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**Original Research**

# Performance of DNA-based biomarkers for classification of adrenocortical carcinoma: a prognostic study

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## Abstract

**Objective:** Adrenocortical carcinoma (ACC) is a rare aggressive malignancy with heterogeneous clinical outcomes. Recent studies proposed a combination of clinical/histopathological parameters (S-GRAS score) or molecular biomarkers (BMs) to improve prognostication. We performed a comparative analysis of DNA-based BMs by evaluating their added prognostic value to the S-GRAS score.

**Design and methods:** A total of 194 formalin-fixed, paraffin-embedded (FFPE) ACC samples were analysed, including a retrospective training cohort ($n = 107$) and a prospective validation cohort ($n = 87$). Targeted DNA sequencing and pyrosequencing were used to detect somatic single-nucleotide variations in ACC-specific genes and methylation in the promoter region of paired box 5 (*PAX5*). The European Network for the Study of Adrenocortical Tumors (ENSAT) tumour stage, age, symptoms at presentation, resection status, and Ki-67 were combined to calculate S-GRAS. Endpoints were overall (OS), progression-free (PFS), and disease-free survival (DFS). Prognostic role was evaluated by multivariable survival analysis and their performance compared by Harrell's concordance index (C index).

**Results:** In training cohort, an independent prognostic role was confirmed at multivariate analysis for two DNA-based BMs: alterations in Wnt/β-catenin and Rb/p53 pathways and hypermethylated *PAX5* (both $P < .05$ for PFS and DFS, hazard ratio [HR] 1.47-2.33). These were combined to S-GRAS to obtain a combined (COMBI) score. At comparative analysis, the best discriminative prognostic model was COMBI score in both cohorts for all endpoints, followed by S-GRAS score (C index for OS 0.724 and 0.765, PFS 0.717 and 0.670, and DFS 0.699 and 0.644, respectively).

**Conclusions:** Targeted DNA-based BM evaluated on routinely available FFPE samples improves prognostication of ACC beyond routinely available clinical and histopathological parameters. This approach may help to better individualise patient's management.

**Keywords:** adrenal cancer, molecular oncology, prognosis, personalised medicine

### Significance

Prognostication of adrenocortical carcinoma—an aggressive malignancy with heterogeneous outcomes—currently relies on clinical/histopathological parameters, but better prognostic markers are needed. Here, we demonstrate that combining the S-GRAS score with two DNA-based biomarkers provides the best discriminatory performance to stratify patients at lower or higher risk of early disease recurrence as well as different progression-free and overall survival. Whereas molecular prognostic markers from snap-frozen tissue samples are challenging to translate to routine clinical care, the here proposed DNA-based biomarkers can be evaluated by targeted sequencing on typically accessible histopathology material. This approach combined with the S-GRAS score, which is also calculated from routinely available clinical/histopathological parameters, could be straightforwardly implemented in clinical practice to improve individualised patient management.

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## Introduction

Adrenocortical carcinoma (ACC) is a rare tumour with a generally poor, but heterogeneous, prognosis (5-year survival rate ranges from 13% to 80%). Tumour stage according to the European Network for the Study of Adrenocortical Tumors (ENSAT) classification, the resection (R) status of the primary tumour, and the Ki67 index are considered the most relevant prognostic factors,[1-5] but they have a limited performance.[2,6] In a recent large collaborative ENSAT study, we demonstrated that the S-GRAS score, a combination of clinical (age, symptoms at diagnosis, and ENSAT tumour stage) and histopathological parameters (R status and Ki67 index), is the most powerful prognostic factor related to survival in patients with ACC.[6] Interestingly, the superiority of S-GRAS score over its individual components remained when comparing patients treated or not with adjuvant mitotane.[6]

Previous pan-genomic studies have identified molecular patterns associated with clinical outcomes in ACC, such as gene expression profile (ie, BUB1B-PINK1 levels), chromosomal alterations, and CpG island methylation patterns.[7,8] More recently, the use of more feasible targeted molecular analysis confirmed that specific DNA-based alterations or RNA-based biomarkers (BM) may play an important prognostic role in ACC.[9-11] However, most of the previous studies were performed on snap-frozen tumour material, which is challenging to obtain in clinical practice. To circumvent this issue, we previously demonstrated that targeted DNA sequencing and methylation analysis is readily obtainable from formalin-fixed, paraffin-embedded (FFPE) tissue ACC samples.[12,13] With this approach, we reported that DNA-based BM, such as a higher number of genes affected by single-nucleotide variations (SNV), the presence of somatic alterations in Wnt/β-catenin alone or together with Rb/p53 pathways, and hypermethylation pattern in the promoter region of four preselected genes, are associated with a very poor prognosis.[12] However, only one hypermethylated gene, ie, the paired box 5 (*PAX5*) gene, has been directly compared to that of the most powerful clinical prognostic factor—the S-GRAS score—showing an independent prognostic role.[13]

The aim of the present study was to evaluate the role of multiple DNA-based BM—detected by targeted analysis in FFPE material—in the prognostic classification of ACC by comparing them to the S-GRAS score.

## Material and methods

### Patient cohort and study design

This is a two-centre study designed and conducted in accordance with the Declaration of Helsinki. We followed the recommendations for tumour prognostic marker studies reported in REMARK.[14] The study protocol was approved by both local ethics committees (#88/11 at the University Hospital of Würzburg; HBRC 11/606 and PrimeAct study REC 20/NW/0207 at the University of Birmingham). Written informed consent was obtained from all subjects.

Patients older than 18 years with histologically confirmed ACC and available FFPE tumour material, clinical and histopathological characteristics at the time of diagnosis, and follow-up data to determine disease status and survival were included. A total of 194 patients were recruited to the study, including a retrospective (training) cohort of 107 cases (cohort 1), which was partially published in a previous study,[12] and an independent, prospective (validation) cohort of 87 cases (cohort 2). Disease status and survival information were updated up to August 2022 for both cohorts (ie, five additional years for cohort 1 compared to published data).

### Clinical data collection

Patient's age at diagnosis, symptoms at presentation (related to autonomous steroid secretion or mass effect), initial ENSAT tumour stage, Ki67 proliferation index, R status of primary tumour, follow-up duration, and clinical outcomes were recorded. The S-GRAS score was calculated as previously published:[6] age at diagnosis (<50 years, 0 points; ≥50 years, 1 point); hormone, tumour, or systemic cancer–related symptoms at presentation (no, 0 points; yes, 1 point); ENSAT stage (1 or 2, 0 points; 3, 1 point; 4, 2 points); R of the primary tumour (R0, 0 points; RX, 1 point; R1, 2 points; R2, 3 points); and Ki67 index (0%-9%, 0 points; 10%-19%, 1 point; ≥20%, 2 points), resulting in S-GRAS scores ranging from 0 to 9 (Table S1). Patients were further stratified into four groups as follows:[6] S-GRAS 0-1, S-GRAS 2-3, S-GRAS 4-5, and S-GRAS 6-9. Details about eventual administration of adjuvant treatment with mitotane (initiated and titrated according to local and European guidelines[2]) were also collected from medical records.

The primary endpoints of the study were progression-free survival (PFS) and overall survival (OS), which were available for all cases. Progression-free survival was defined as the time from diagnosis to first radiological evidence of disease progression. Overall survival was defined as the time from primary tumour resection or diagnosis to death. Additionally, we investigated disease-free survival (DFS) in patients who underwent complete tumour resection (R0) and it was defined as the time from complete tumour resection to first radiological evidence of disease relapse. Specifically, radiological evidence of progression or relapse was detected during periodical radiological surveillance performed every 3 months by thorax–abdomen–pelvis computed tomography scan with contrast (TAP CT scan).

### Tissue sample collection and DNA isolation

We included 194 FFPE tumour tissues paired with peripheral blood samples used as a reference to confirm the somatic nature of detected alterations. Among the tumour tissues, 164 were derived from primary ACC (84.5% of total), 16 from local recurrence, and 14 from metastases. For all tissue samples, tumour localisation and cell content were assessed in a representative FFPE slide by haematoxylin–eosin staining before DNA isolation. Tumour cell content reached a high fraction (median 90%, range 60%-95%). Tumour DNA was isolated with the GeneRead DNA FFPE Kit (Qiagen, Hilden, Germany) and germline leukocyte DNA from peripheral blood with the NucleoSpin Blood L Kit (Macherey-Nagel, Bethlehem, PA, USA) according to the manufacturer's instructions and as previously published.[12,13]

### Targeted DNA sequencing and methylation analysis

#### Targeted DNA sequencing analysis

As described in Lippert et al.,[12] tumour and leukocyte DNAs of the retrospective cohort (cohort 1) were enriched with the GeneRead DNAseq Human Comprehensive Cancer Panel V2 and GeneRead DNAseq Panel PCR Kit V2 (both

Qiagen), according to the manufacturer's protocol. The panel includes coding regions of 160 cancer-specific genes (7951 amplicons and 744 835 bases of target regions), many of them known or suspected to be involved in adrenocortical tumourigenesis. The samples of the prospective cohort (cohort 2) were either enriched with a customised QIAseq™ Targeted DNA Panel (Qiagen) ($n = 58$) or a Cell3™ Target Custom Next-Generation Sequencing (NGS) Panel (Nonacus) ($n = 29$) according to the manufacturer's protocols.[7,8,12] The Qiagen panel was designed to include coding regions of genes affected by recurrent somatic variants at previous targeted sequencing ($n = 100$).[12] The Nonacus panel was designed to include coding regions of a restricted number of 33 ACC-specific genes considered potentially useful for prognostic classification and/or described as drug targets. Details regarding the gene panels are reported in Table S2. Next-generation sequencing was performed on a MiSeq or a NextSeq500 with MiSeq Reagent Kit V2 or NextSeq Mid Output Reagent Kit V2 (both Qiagen), respectively, and 150 bp paired end reads (Illumina Inc). Raw data were aligned and analysed with GensearchNGS (Phenosystems S.A.). For the detection of SNVs or small insertions and deletions (small Indels) in tumour samples, the called variants were filtered as follows: coverage >100, exon distance <21, frequency of appearance >0.1, minor allele frequency (MAF; ie, the frequency at which the second most common allele occurs in a given population) <0.02, and variant balance (indicating how symmetric the variant is seen in forward and reverse strands; ideally, the value is 1) >0. Variants that were also detected in the matched blood samples were excluded. The complete lists of detected somatic variants for the retrospective and the prospective cohorts are shown in Tables S3 and S4, respectively.

The presence of alterations in the Wnt/β-catenin pathway was defined by variants in *CTNNB1*, *ZNFR3*, and/or *APC* genes, while the Rb/p53 pathway was considered altered by the presence of variants in *TP53*, *RB1*, and/or *CDK4* genes.

#### Targeted DNA methylation analysis

Bisulphite pyrosequencing or targeted bisulphite sequencing (TBS) were used for quantitative methylation analysis of the tumour suppressor gene *PAX5* as part of two previous studies from our group.[12,13] The target region was selected to be located within the CpG islands in the promoter region of the gene. Preparation of DNA samples (ie, bisulphite conversion and amplification of target regions) was conducted as previously described.[12,13] Polymerase chain reaction amplicons were then either used for the bisulphite pyrosequencing[12] or for TBS.[13] The methylation level of each CpG was calculated from the ratio of methylated cytosine to total coverage at the appropriate position. The methylation status of a sample for the promoter region of *PAX5* was determined by averaging the methylation levels of the corresponding CpGs. *PAX5* was estimated as hypomethylated with a methylation status of ≤25% and hypermethylated with a methylation status >25%.[10-13]

### Statistical analysis

Descriptive statistics were produced to compare the two cohorts, which did not differ in terms of most relevant characteristics (Table 1). Data are shown as mean ± standard deviation (SD) or median and range, as appropriate. Non-parametric Mann–Whitney *U* test and Fisher or $\chi^2$ tests were used to compare baseline continuous and dichotomic data, respectively. Univariate and multivariate survival analysis was performed in the training cohort ($n = 107$) to identify independent significant DNA-based prognostic BMs. Specifically, Cox survival models were fitted for each of the three survival outcomes (OS, PFS, and DFS) including individual, previously proposed DNA-based BMs, such as the presence of more than one SNV-affected genes, alterations in Wnt/β-catenin and Rb/p53 pathways, and hypermethylated *PAX5*,[12,13] and S-GRAS score grouping[6] separately as an independent variable.

Hazard ratio, 95% CI, and *P* values were reported for each of the three outcomes. To evaluate the additional prognostic value of the DNA-based molecular score as compared to S-GRAS score alone, we merged these two prognostic factors into a combined (COMBI) score (details in Table S1). The discriminative performance of the DNA-based BMs, the S-GRAS grouping, and the combination of them on survival models was compared using Harrell's concordance index (C index) in the training and validation cohorts separately and in the entire cohort.[15] Harrell's C index is a goodness-of-fit measure, with desirable values ranging from 0.50 to 1 and values above 0.70 corresponding to a good model. The two cohorts have been merged together for Kaplan–Meier plots to display the unadjusted survival curves for survival outcomes.

Statistical analysis was performed using R (version: 4.2.1, packages dynpred and survival). A $P$ value $< .05$ was considered statistically significant.

## Results

### Patient characteristics

The baseline demographic, clinical, biochemical, and histopathological characteristics of the 194 patients with ACC (81 males and 113 females; median age 50 years, range 18-87) included in the study are shown in Table 1. The retrospective and prospective cohorts had similar sex, age, symptoms at diagnosis, baseline ENSAT tumour stage, and duration of follow-up (median 49 *vs* 42 months, $P = .09$). They slightly differed in R status and Ki67 index (and frequency of adjuvant treatment with mitotane), but, importantly, this did not translate into a significantly different S-GRAS score.

Overall, 64% of patients presented with symptoms at diagnosis, while 36% were discovered incidentally. A total of 5% patients had ENSAT tumour stage 1, 47% stage 2, 30% stage 3, and 17% stage 4. The R status was R0 in most of the cases (74%), uncertain (RX) in 10%, and R1 or R2 in 8% of cases each. The mean initial Ki67 index was 20.9 ± 18.4% (median 15, range 1-90), being equal to or above 20% in 49% of cases. Finally, the S-GRAS score was 0-1 in 16% (group 0), 2-3 in 39% (group 1), 4-5 in 29% (group 2), and 6 or above in the remaining 15% of cases (group 3).

### Prognostic role of clinical and histopathological parameters and individual DNA-based biomarkers

We first investigated the retrospective training cohort to identify most promising prognostic factors ($n = 107$). The details for the univariable survival analysis are shown in Table 2. Regarding clinical and pathological parameters, at univariable analysis, ENSAT tumour stage, R status, and Ki67 index as well as the S-GRAS score were all significant prognostic markers for the three endpoints ($P < .001$ for OS, PFS, and

**Table 1.** Demographic, clinical, histopathological, and molecular characteristics of each cohort and entire cohort of patients with adrenocortical carcinoma.

| | Entire cohort ($n = 194$) | Retrospective (training) cohort (cohort 1) ($n = 107$) | Prospective (validation) cohort (cohort 2) ($n = 87$) | *P* value |
|---|---|---|---|---|
| Sex—M (%) | 81 (42%) | 46 (43%) | 35 (40%) | .77[c] |
| Age—years (mean ± SD) | 51.2 ± 15.0 | 50.4 ± 14.9 | 52.3 ± 15.1 | .4[b] |
| <50a | 92 | 55 | 37 | .25[c] |
| ≥50a | 102 | 52 | 50 | |
| Symptoms—yes (*n*, %) | 117 (60%) | 66 (62%) | 51 (59%) | .16[c] |
| Unknown | 12 (6.2%) | 12 (11.2%) | — | |
| ENSAT tumour stage | | | | |
| ENSAT stage 1 (*n*, %) | 10 (5.2%) | 4 (3.7%) | 6 (6.9%) | .30[c] |
| ENSAT stage 2 (*n*, %) | 92 (47.4%) | 51 (47.7%) | 41 (47.1%) | |
| ENSAT stage 3 (*n*, %) | 58 (29.9%) | 29 (27.1%) | 29 (33.3%) | |
| ENSAT stage 4 (*n*, %) | 33 (17%) | 23 (21.5%) | 11 (12.6%) | |
| Resection status | | | | |
| R0 (*n*, %) | 141 (72.7%) | 74 (69.2%) | 67 (77%) | .019[c] |
| RX (*n*, %) | 19 (9.8%) | 16 (15%) | 3 (3.5%) | |
| R1 (*n*, %) | 15 (7.7%) | 5 (4.6%) | 10 (11.5%) | |
| R2 (*n*, %) | 15 (7.7%) | 9 (8.4%) | 6 (6.9%) | |
| Unknown (*n*, %) | 4 (2.1%) | 3 (2.8%) | 1 (1.2%) | |
| Ki67 index—% (mean ± SD) | 20.9 ± 18.4 | 18.6 ± 18.1 | 24.1 ± 18.4 | .0469[b] |
| Ki67 ≤ 9 (*n*, %) | 49 (25.3%) | 31 (29%) | 18 (20.7%) | .015[c] |
| Ki67 10-19 (*n*, %) | 36 (18.6%) | 33 (30.8%) | 13 (14.9%) | |
| Ki67 ≥ 20 (*n*, %) | 91 (46.9%) | 43 (40.2%) | 48 (55.2%) | |
| Unknown (*n*, %) | 6 (3.1%) | 0 | 6 (6.9%) | |
| S-GRAS score (0-9) | 3.5 ± 2.0 | 3.4 ± 2.0 | 3.5 ± 1.9 | .77[b] |
| S-GRAS score 0-1 (*n*, %) | 31 (15%) | 19 (17.8%) | 12 (13.8%) | .70[c] |
| S-GRAS score 2-3 (*n*, %) | 76 (39.2%) | 43 (40.2%) | 33 (37.9%) | |
| S-GRAS score 4-5 (*n*, %) | 57 (29.4%) | 28 (26.2%) | 29 (33.3%) | |
| S-GRAS score 6-9 (*n*, %) | 30 (15.5%) | 17 (15.9%) | 13 (14.9%) | |
| Duration of follow-up—months (mean ± SD, median) | 61.2 ± 56.4 (48.5) | 66.5 ± 57.6 (49.0) | 55.1 ± 56.9 (47.0) | .092 |
| Adjuvant mitotane treatment | | | | |
| Yes (*n*, %) | 86 (49.7%) | 40 (42.6%) | 46 (56.8%) | .048[c] |
| No (*n*, %) | 87 (50.3%) | 54 (57.4%) | 33 (40.7%) | |
| Not applicable or not known (*n*, %) | 18 | 12 | 6 | |
| Adjuvant etoposide–cisplatin | | | | |
| Yes (*n*, %) | 3 (1.5%) | 1 (1%) | 2 (2.5%) | |
| DNA-based biomarkers | | | | |
| Number of genes affected by SNV | | | | |
| 0-1 affected genes—*n* (%) | 56 (52.3%) | 65 (74.7%) | 121 (62.9%) | .0027[c] |
| >1 affected genes—*n* (%) | 51 (47.7%) | 22 (25.3%) | 73 (37.1%) | |
| Pathways affected by SNV[a] | | | | |
| 0—*n* (%) | 72 (67.3%) | 67 (77%) | 139 (71.7%) | .098[c] |
| 1—*n* (%) | 25 (23.4%) | 18 (20.7%) | 43 (22.2%) | |
| 2—*n* (%) | 10 (9.4%) | 2 (2.3%) | 12 (6.2%) | |
| *PAX5* methylation (mean ± SD) | 18.2 ± 20.0 | 20.8 ± 18.8 | 19.4 ± 19.5 | .36[b] |
| Methylation ≤ 25%—*n* (%) | 85 (79.4%) | 57 (65.5%) | 142 (73.2%) | .050[c] |
| Methylation > 25%—*n* (%) | 22 (20.6%) | 30 (34.5%) | 52 (26.8%) | |
| DNA-based molecular score (0-3) | | | | |
| 0—*n* (%) | 105 (54.1%) | 60 (56.1%) | 45 (51.7%) | .43[c] |
| 1—*n* (%) | 63 (32.5%) | 30 (28.0%) | 33 (37.9%) | |
| 2—*n* (%) | 22 (11.3%) | 14 (13%) | 8 (9.2%) | |
| 3—*n* (%) | 4 (2.0%) | 3 (2.9%) | 1 (1.1%) | |

S-GRAS score calculated as previously published.[6]
Abbreviations: M, males; R0, complete resection; R1, microscopically incomplete resection; R2, macroscopically incomplete resection; RX, uncertain resection status; SD, standard deviation; SNV, single-nucleotide variant.
[a]Pathways affected by SNV: 0, no alterations in either Wnt/β-catenin or p53; 1, alterations in Wnt/β-catenin only (but not in p53); and 2, alterations in Wnt/β-catenin and p53. [b]Statistical analysis by non-parametric Mann–Whitney *U* test. [c]Statistical analysis by Fisher exact or $\chi^2$ square test (as appropriate).

DFS with HR ranging from 1.59 to 2.84; Table 2). Looking at the variable model performance, the S-GRAS score showed the highest Harrell's C index compared to the individual clinical and histopathological parameters (0.683 for OS, 0.694 for PFS, and 0.655 for DFS; Table 2 and Figure 1).

We then investigated the prognostic role of three previously proposed DNA-based BM, ie, number of genes affected by SNV (none or 1 = 0, 75% of total, *vs* more than one = 1, 25%), presence of alterations in Wnt/β-catenin and/or Rb/p53 pathways (none = 0, 77%; *vs* Wnt/β-catenin alone = 1, 21%; *vs* Wnt/β-catenin and Rb/p53 pathways = 2, 2%), and hypermethylation of *PAX5* (no = 0, 66%, *vs* yes = 1, 34%) (Table 1). At univariable analysis, also all three BM were significantly associated with shorter OS, PFS, and DFS, with HR ranging from 1.70 (OS for SNV-affected genes) to max 2.39 (OS for hypermethylated *PAX5*) (Table 2). Of note, the use

**Table 2.** Univariable survival analysis of single prognostic variables in the retrospective (training) cohort of 107 patients with adrenocortical carcinoma.

| | *P* value | HR (95% CI) | C index |
|---|---|---|---|
| **Overall survival** | | | |
| ENSAT tumour stage (1-2, 3, 4) | .000616 | 1.654 (1.24-2.206) | 0.623 |
| Resection status (0, X, 1, 2) | $3.1e^{-05}$ | 1.589 (1.278-1.975) | 0.637 |
| Ki67 index (0-9, 10-19, 20, or above) | $1.66e^{-05}$ | 1.964 (1.445-2.671) | 0.657 |
| S-GRAS score (0-1, 2-3, 4-5, 6, or above) | $1.97e^{-06}$ | 1.865 (1.443-2.411) | 0.683 |
| SNV-affected genes (0-1, >1) | .0242 | 1.703 (1.072-2.705) | 0.571 |
| Affected pathways[a] | .00083 | 1.719 (1.251-2.362) | 0.604 |
| Hypermethylated *PAX5* (no, yes) | .00101 | 2.395 (1.423-4.03) | 0.562 |
| DNA-based molecular score (0, 1, 2-3) | $1.02e^{-05}$ | 1.863 (1.413-2.456) | 0.645 |
| COMBI score (A, B, C, D, E) | $7.6e^{-09}$ | 1.925 (1.542-2.404) | 0.724 |
| **Progression-free survival** | | | |
| ENSAT tumour stage (1-2, 3, 4) | $1.3e^{-05}$ | 1.833 (1.396-2.408) | 0.632 |
| Resection status (0, X, 1, 2) | $9.6e^{-06}$ | 1.612 (1.305-1.991) | 0.61 |
| Ki67 index (0-9, 10-19, 20, or above) | $2.88e^{-07}$ | 2.103 (1.583-2.794) | 0.659 |
| S-GRAS score (0-1, 2-3, 4-5, 6, or above) | $1.1e^{-09}$ | 2.041 (1.622-2.567) | 0.694 |
| SNV-affected genes (0-1, >1) | .000851 | 2.05 (1.345-3.127) | 0.598 |
| Affected pathways[a] | .000124 | 1.922 (1.377-2.683) | 0.59 |
| Hypermethylated *PAX5* (no, yes) | .00426 | 2.06 (1.255-3.382) | 0.549 |
| DNA-based molecular score (0, 1, 2-3) | $1.25e^{-05}$ | 1.9 (1.425-2.534) | 0.614 |
| COMBI score (A, B, C, D, E) | $1.12e^{-11}$ | 2.184 (1.743-2.736) | 0.717 |
| **Disease-free survival** | | | |
| ENSAT tumour stage (1-2, 3, 4) | .00536 | 1.785 (1.187-2.685) | 0.594 |
| Resection status (0, X, 1, 2) | | | 0.5 |
| Ki67 index (0-9, 10-19, 20, or above) | $2.63e^{-05}$ | 2.117 (1.492-3.004) | 0.65 |
| S-GRAS score (0-1, 2-3, 4-5, 6, or above) | $2.08e^{-06}$ | 2.842 (1.846-4.374) | 0.655 |
| SNV-affected genes (0-1, >1) | .0245 | 1.825 (1.08-3.084) | 0.572 |
| Affected pathways[a] | .00833 | 1.935 (1.185-3.16) | 0.559 |
| Hypermethylated *PAX5* (no, yes) | .00674 | 2.29 (1.258-4.171) | 0.555 |
| DNA-based molecular score (0, 1, 2-3) | .00138 | 1.832 (1.264-2.655) | 0.592 |
| COMBI score (A, B, C, D, E) | $2.5e^{-08}$ | 2.435 (1.781-3.329) | 0.699 |

Abbreviations: C index, Harrell's C index; CI, confidence interval; DFS, disease-free survival; HR, hazard ratio; NA, not applicable; OS, overall survival; PFS, progression-free survival; SNV, single-nucleotide variants.
[a]Affected pathways: 0, no alterations in either Wnt/β-catenin or p53; 1, alterations in Wnt/β-catenin only (but not in p53); and 2, alterations in Wnt/β-catenin and p53.

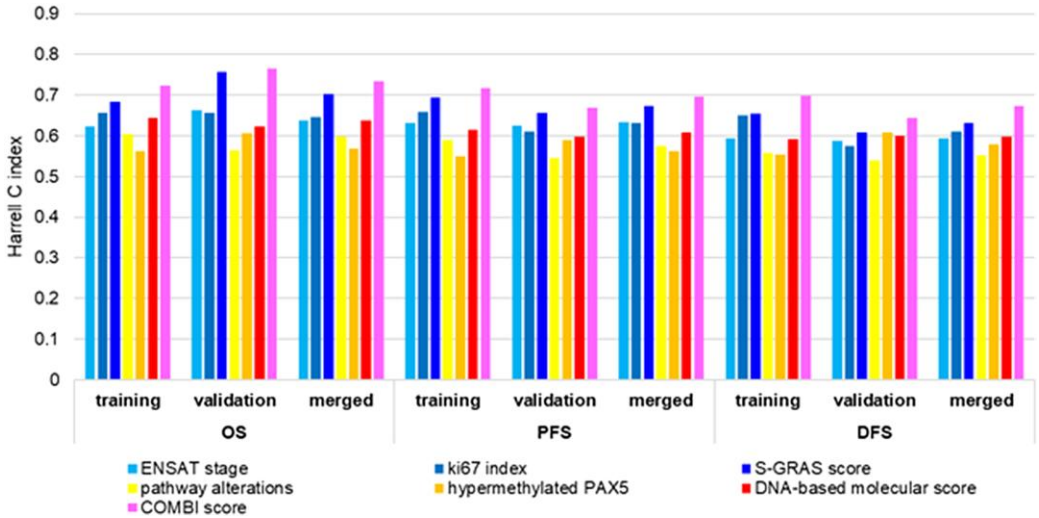


**Figure 1.** Comparative analysis by Harrell's C index calculated for overall survival (OS), progression-free survival (PFS), and disease-free survival in the training cohort ($n = 107$), validation cohort ($n = 87$), and in the entire cohort ($n = 141$) for ENSAT tumour stage, Ki67 index, hypermethylation in PAX5, number of genes affected by SNV (none, 0, *vs* 1 or more, 1), presence of alterations in Wnt/β-catenin and/or Rb/p53 pathways (none, 0; *vs* Wnt/β-catenin alone, 1; *vs* Wnt/β-catenin and Rb/p53 pathways, 2), DNA-based molecular score, S-GRAS score, and COMBI score. Of note, COMBI score confirmed to be the best performing prognostic marker in all three cohorts for all three endpoints.

of adjuvant treatment with mitotane did not show any relationship with any of the endpoints (OS: HR 0.895 [0.538-1.489], $P = .67$; PFS: 1.15 [0.728-1.818], $P = .55$; and DFS: HR 1.28 [0.7521-2.178], $P = .36$).

At multivariable Cox regression analysis including the DNA-based parameters and the S-GRAS score grouping, only the presence of alterations in Wnt/β-catenin and/or Rb/p53 pathways and hypermethylated *PAX5* remained significant

**Table 3.** Multivariable survival analysis including the three individual DNA-based biomarkers and S-GRAS score grouping in the retrospective (training) cohort of 107 patients with adrenocortical carcinoma.

| | Variable | HR (95% CI) | *P* value |
|---|---|---|---|
| OS | Number of genes affected by SNV | 1.078 (0.6376-1.822) | .77954 |
| | Pathways affected by SNV[a] | 1.314 (0.9110-1.894) | .14407 |
| | *PAX5* methylation | 2.149 (1.2260-3.768) | .00756 |
| | S-GRAS score | 1.844 (1.4089-2.414) | $8.35e^{-06}$ |
| PFS | Number of genes affected by SNV | 1.394 (0.8783-2.213) | .15869 |
| | Pathways affected by SNV[a] | 1.471 (1.0357-2.090) | .03111 |
| | *PAX5* methylation | 2.334 (1.3904-3.920) | .00134 |
| | S-GRAS score | 2.011 (1.5709-2.575) | $2.98e^{-08}$ |
| DFS | Number of genes affected by SNV | 1.252 (0.7032-2.231) | .4447 |
| | Pathways affected by SNV[a] | 1.774 (1.0530-2.990) | .0313 |
| | *PAX5* methylation | 1.954 (1.0202-3.741) | .0434 |
| | S-GRAS score | 3.052 (1.8931-4.921) | $4.67e^{-06}$ |

Abbreviations: CI, confidence interval; DFS, disease-free survival; HR, hazard ratio; OS, overall survival; PFS, progression-free survival; SNV, single-nucleotide variants.
[a]Affected pathways: 0, no alterations in either Wnt/β-catenin or p53; 1, alterations in Wnt/β-catenin only (but not in p53); and 2, alterations in Wnt/β-catenin and p53.

independent prognostic factors besides S-GRAS for PFS (HR 1.47, 95% CI 1.03-2.09, and HR 2.33, 95% CI 1.39-3.92, respectively), and DFS (HR 1.77, 95% CI 1.05-2.99, and HR 1.95, 95% CI 1.02-3.74, respectively) (Table 3). Only hypermethylated *PAX5* confirmed to be an independent prognostic factor for OS (HR 2.15, 95% CI 1.23-3.77). The number of SNV-affected genes did not appear to be an independent prognostic factor when adjusted for S-GRAS score for any of the endpoints (Table 3).

### Added prognostic value of combined DNA-based biomarkers and S-GRAS score

According to the findings obtained in the training cohort, we built up a DNA-based molecular score merging the scoring obtained from the presence of alterations in Wnt/β-catenin and/or Rb/p53 pathways and hypermethylated *PAX5*, ranging from a minimum of 0 to a maximum of 3. The score system is detailed in the Table S1. Overall, the majority of the patients was classified in the DNA-based molecular score group 0 and distribution of patients in each group of DNA-based molecular score was similar between the training and validation cohorts (group 0 56.1% *vs* 51.7%, group 1 28.0 *vs* 37.9%, and group 2 15.9% *vs* 20.7%; Table 1). Of note, our DNA-based molecular score was strongly associated with all three endpoints at univariable analysis (training cohort: HR ranging from 1.83 to 1.90, all $P < .005$, details in Table 2). Moreover, the C index showed that the DNA-based molecular score has a better discriminatory performance than the single DNA-based parameters (0.645 for OS, 0.614 for PFS, and 0.592 for DFS; Table 2 and Figure 1).

We then evaluated the potential additional prognostic value of DNA-based molecular score as compared to S-GRAS score alone. To this aim, we merged them into a COMBI score (ranging from a minimum of 0 to a maximum of 6) (details in Table S3). Patients were then grouped into four categories according to the COMBI score as follows: score 0, group A; score 1, group B; score 2, group C; score 3-4, group D; and score 5-6, group E. In the training cohort, the COMBI score had a strong prognostic role for OS, PFS, and DFS at univariate analysis (all $P < .0001$, HR ranging from 1.92 to 2.43; Table 2). Even more importantly, the COMBI score showed the best prognostic performance compared to all other individual parameters and scores, with a C index of 0.724 for OS, 0.717 for PFS, and 0.699 for DFS, respectively, followed by the S-GRAS score that represented the second-best discriminative model (Table 2 and Figure 1).

### Validation of added prognostic value of COMBI score

We then intended to validate the prognostic role of COMBI score by using comparative analysis in our independent, prospective (validation) cohort ($n = 87$). Here, we could further demonstrate the superiority of COMBI score compared to all other factors, showing the highest Harrell's C index values for all three endpoints (ie, 0.765 for OS, 0.670 for PFS, and 0.644 for DFS). These findings are graphically shown in Figure 1 including a comprehensive comparison with all the individual clinical/histopathological parameters and DNA-based BM, as well as the S-GRAS and the DNA-based molecular scores. Our findings clearly show that COMBI score has a superior discriminative power to predict clinical outcome in ACC than all other evaluated BMs.

The Kaplan–Meier curves for OS, PFS, and DFS for S-GRAS score, DNA-based molecular score, and COMBI score are shown for the entire cohort ($n = 194$) in Figure 2 (A-C, D-F, and G-I, respectively).

## Discussion

In the present study, we compared for the first time the prognostic role of promising DNA-based BM to the most acknowledged prognostic factors for ACC, ie, ENSAT tumour stage, Ki67 index, and most importantly the recently established S-GRAS score.[6] We investigated a large cohort of 194 patients with ACC, including a training and a validation cohort, and used methods easily applicable in the clinical practice—starting from FFPE material and straightforward targeted molecular analysis[9,10]—already validated on FFPE.[12,13] Hereby, we could confirm that in the training cohort, three previously proposed prognostic DNA-based BM, ie, the number of genes affected by SNV,[12] the presence of alterations in Wnt/β-catenin and/or Rb/p53 pathways,[7,9,12] and hypermethylation of *PAX5*,[7,9,12,13] play a significant role for prognostication of patients with ACC at univariable survival analysis. However, only the presence of alterations in Wnt/β-catenin and/or Rb/p53 pathways and hypermethylated *PAX5* proved to be independent prognostic factors for OS, PFS, and DFS at multivariable regression analysis including the S-GRAS score grouping. This is of clinical relevance since the S-GRAS score is the strongest prognostic factor for the prognostic stratification of ACC, as shown in a large international study coordinated by our group.[6] Of note, the crucial role of the S-GRAS score has been further confirmed in multiple independent studies on both adult and paediatric patients with ACC.[16,17] Accordingly, looking at the variable model performance, the S-GRAS score resulted to be more strongly associated with the clinical outcome than the individually clinical/histopathological parameters, ie, ENSAT tumour stage, R status, and Ki67 index, in both training and validation cohorts.

From a biological perspective, PAX5 is a well-known tumour suppressor gene, which is down-regulated in multiple tumours

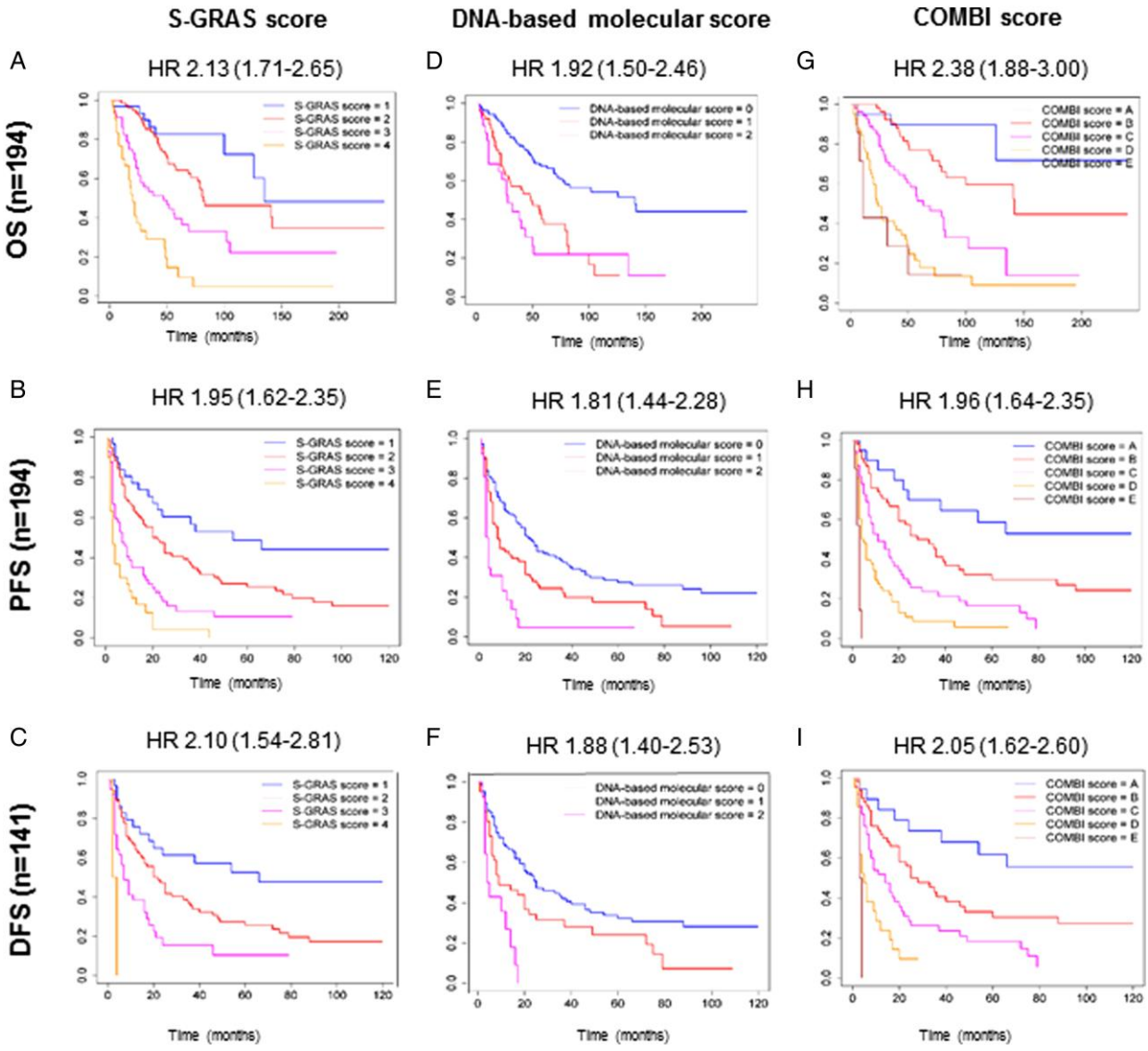


**Figure 2.** Kaplan–Meier survival curves for overall survival (OS, $n = 194$), progression-free survival (PFS, $n = 194$), and disease-free survival (DFS, $n = 141$) for S-GRAS (A-C), DNA-based molecular score (D-F), and COMBI score (G-I). The S-GRAS score and grouping is calculated and defined as previously published.[6] The combined DNA-based molecular score range is calculated as follows: alterations in Wnt/β-catenin and/or Rb/p53 pathways (none, 0; *vs* Wnt/β-catenin alone, 1; *vs* Wnt/β-catenin and Rb/p53 pathways, 2) plus hypermethylation of *PAX5* (no, 0, *vs* yes, 1) and groups for survival curves defined as follows: group 0, 0; group 1, 1; and group 2, above 1. The COMBI score is calculated as follows: DNA-based molecular score (0-1-2-3) plus S-GRAS score (0-1-2-3) for a minimum of 0 and a maximum of 6 points. The COMBI score grouping is defined as follows: 0, A; 1, B; 2, C; 3-4, C; and 5-6, D. Statistical analysis by log rank test showed significant *P* values for all three scores and all three endpoints. Hazard ratios (HR) and 95% confidence intervals (95% CI) are shown in the graphs.

through its promoter methylation. Hypermethylation in *PAX5* has been also associated with worst outcome and proposed as prognostic marker in other cancer types.[18-20] Moreover, its involvement in tumourigenesis has been associated with the inhibition of the Wnt/β-catenin pathway,[21] which is frequently up-regulated in ACC. Therefore, its potential involvement in ACC development and aggressiveness would be not surprising, even if it has not been directly investigated. On the other side, the contribution of genetic alterations in the Wnt/β-catenin and/or Rb/p53 pathways in the pathogenesis ACC is well known and has been clearly demonstrated.[22,23]

According to the results of the multivariable survival analysis, we then proposed a DNA-based molecular score including the scoring obtained from the presence of alterations in Wnt/β-catenin and/or Rb/p53 pathways and hypermethylated *PAX5*. This score was strongly associated with clinical outcomes for all three endpoints and, importantly, yielded better discriminatory performance than the single DNA-based parameters. We then evaluated the potential additional prognostic value of DNA-based molecular as compared to the S-GRAS score alone. To this end, we merged these two prognostic markers into a COMBI score which showed a strong prognostic role for OS, PFS, and DFS. We then demonstrated by comparative analysis that COMBI score had the best discriminative performance in both training and validation cohorts (see Figure 1). Therefore, we can conclude that the straightforward assessment of few molecular alterations could significantly improve the prognostic classification and disease recurrence risk stratification in patients with ACC. This could have relevant clinical impact for personalised management

helping to guide clinician's choices, for instance, avoiding the prescription of mitotane adjuvant treatment and/or prolonging the frequency of radiological follow-up outcome in patients with best predicted clinical outcome (eg, COMBI score A) or considering more aggressive adjuvant treatment in patients with worst anticipated survival (eg, COMBI score D-E).

Previous studies on snap-frozen tissue material already proposed the use of targeted DNA-based sequencing to assess the molecular classification of ACC and its role for prognostic classification.[9] However, here, we propose for the first time a simplified targeted analysis based on SNV in only six genes (ie, *CTNNB1*, *ZNRF3*, *APC*, *TP53*, *RB1*, and *CDK4*) and hypermethylation in only one gene (*PAX5*) starting from routinely available FFPE samples. The use of FFPE material rendered the analysis of chromosomal alterations (previously proposed as useful prognostic BM) unreliable. Nevertheless, this did not decrease the discriminatory power of our DNA-based molecular score. Moreover, previous studies using frozen material showed that RNA-based markers such as BUB1-PINK1 expression may be more efficient than DNA-based markers to identify better prognosis ACC.[9] We also evaluated mRNA expression of BUB1B and PINK1 by quantitative real-time RT-PCR in our initial cohort of 107 FFPE samples[12] and observed that high BUB1B-PINK1 differential expression was indeed associated with a shorter PFS. However, considering that good quality RNA was only obtained in 32.5% of cases, this approach was not ideal. Moreover, the use of 3′-end RNA-sequencing recently emerged as an interesting technology to investigate transcriptome profile in FFPE tissue material. In fact, in a recent collaborative study, we could demonstrate that this approach represents a convenient solution for determining adrenocortical tumour molecular class from FFPE samples,[24] potentially facilitating routine use and large retrospective studies. However, 3′-end RNA-sequencing is not yet widely available and cannot be proposed for implementation in clinical practice.

Major strengths of our study include the large and well-characterised patient cohort, as well as the homogeneous and well-established methods for data extraction, sequencing, and data analysis, which allowed robust conclusions in this rare cancer. However, the study has some limitations. First, subdividing participants down by S-GRAS group and the presence of DNA-based BMs may result in a smaller sample size, making it difficult to determine if BM importance varies based on S-GRAS score. Future larger studies should consider the possibility of these interaction effects. Second, the impact of eventual adjuvant treatments or additional therapeutic interventions (ie, cytotoxic drugs) was not taken into account for OS and PFS. Nonetheless, this is not relevant for DFS, which showed a pattern similar to the other two endpoints.

In conclusion, we demonstrated that targeted DNA-based BM evaluated on routinely available FFPE samples improve prognostication of ACC beyond the S-GRAS score alone. This approach could be easily applicable in clinical practice in most clinical institutions and may guide tailored patient management, sparing unnecessary adjuvant treatment or frequent radiological surveillance. However, further prospective collaborative studies would be required before implementing a routine use of COMBI score.

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## Supplementary material

Supplementary material is available at *European Journal of Endocrinology* online.

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