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Germline NGS targeted analysis in adult patients with sporadic adrenocortical carcinoma

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ABSTRACT

Background: Adrenocortical carcinoma (ACC) is a rare cancer that arises sporadically or due to hereditary syndromes. Data on germline variants (GVs) in sporadic ACC are limited. Our aim was to characterize GVs of genes potentially related to adrenal diseases in 150 adult patients with sporadic ACC.
Methods: This was a retrospective analysis of stage I-IV ACC patients with sporadic ACC from two reference centers for ACC in Italy. Patients were included in the analysis if they had confirmed diagnosis of ACC, a frozen peripheral blood sample and complete clinical and follow-up data. Next generation sequencing technology was used to analyze the prevalence of GVs in a custom panel of 17 genes belonging to either cancer-predisposition genes or adrenocortical-differentiation genes categories.
Results: We identified 18 GVs based on their frequency, enrichment and predicted functional characteristics. We found six pathogenic (P) or likely pathogenic (LP) variants in *ARMC5, CTNNB1, MSH2, PDE11A* and TP53 genes; and twelve variants lacking evidence of pathogenicity. New unique P/LP variants were identified in *TP53* (p.G105D) and, for the first time, in *ARMC5* (p.P731R). The presence of P/LP GVs was associated with reduced survival outcomes and had a significant and independent impact on both progression-free survival and overall survival.
Conclusions: GVs were present in 6.7 % of patients with sporadic ACC, and we identified novel variants of *ARMC5* and *TP53*. These findings may improve understanding of ACC pathogenesis and enable genetic counseling of patients and their families.

1. Introduction

Adrenocortical carcinoma (ACC) is a rare and aggressive neoplasm that arises either sporadically or in the context of hereditary cancer syndromes [1–3]. Studies in southern Brazil, where the incidence of ACC is exceedingly high, have linked ACC development to germline *TP53* mutations frequently found in the local population [4,5]. Because of the rarity of ACC, population-based registries of patients with hereditary ACC living in countries other than Brazil are lacking, and current knowledge regarding the heritable fraction of ACC mainly comes from linkage studies of families with hereditary cancer syndromes (Li-Fraumeni syndrome [LFS], Beckwith-Wiedemann syndrome [BWS], Lynch

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syndrome, Multiple Endocrine Neoplasia Type 1 [MEN1], and Carney Complex) [1,6,7]. Germline variants (GVs) of specific cancer-associated genes have rarely been assessed in adult ACC patients [1].

The Cancer Genome Atlas (TCGA) project analyzed germline alterations related to ACC in two key studies: a pan-cancer study and a specific ACC study [8,9]. Apart from TCGA analysis, most studies have focused only on a limited number of genes [10]. From the analysis of the core dataset of 91 ACC cases in TCGA pan-cancer study [8], a low rate of GVs was found, which places adult sporadic ACC in the lowest quartile among the 33 cancers screened. In the TCGA-ACC study, nine GVs were found among 177 genes potentially linked to ACC [9].

Given the sparse evidence available on GVs in sporadic ACC, the present study aimed to evaluate the frequency and clinical implications of GVs in a targeted group of genes potentially related to adrenal diseases in 150 adult patients with ACC. To the best of our knowledge, this study represents the largest multigene germline analysis of adult patients with ACC.

2. Methods

2.1. Study overview

This study was conducted at two reference centers in Italy (San Luigi Hospital, Orbassano, and A.S.S.T. Spedali Civili Hospital, Brescia). Next-Generation Sequencing (NGS) and bioinformatics analyses were conducted at the Molecular Oncology Laboratory, Edo, and Elvo Tempia Foundation (Biella). All subjects included in the study provided written informed consent. The study was approved by the Institutional Review Boards of each institution and was conducted in accordance with the Declaration of Helsinki, Good Clinical Practice (GCP), and in compliance with local regulations.

2.2. Patients

A retrospective cohort of patients with ACC was obtained from two institutional review board-approved biological sample repositories, established independently at both centers. Each repository included a collection of peripheral blood samples with comprehensive clinical annotation. Family and clinical histories were obtained through medical documentation and patient interviews with expert medical personnel.

Patients with ACC consecutively referred to our centers between January 1998 and March 2019 were included if they met the following inclusion criteria: age $\geq$ 18 years, pathologically confirmed diagnosis according to the Weiss criteria [11,12], availability of a peripheral whole blood sample, and complete follow-up information. For oncocytic ACC, Lin-Weiss-Bisceglia classification was used according to the WHO classification, 5th edition [13]. The entire study cohort included 150 patients with presumably sporadic ACC, 32 patients with benign adrenocortical adenoma, and seven healthy controls. None of our patients had a family history of ACC, or was known to harbor a genomic alteration that would increase their risk for ACC, or had clinical characteristics suggestive of genetic syndromes associated with ACC.

2.3. NGS custom panel design

NGS custom panel was designed to cover the coding sequence and flanking region (20 bp) of the following 17 candidate genes: *AIP*, *APC*, *ARMC5*, *ARNT*, *BRCA1*, *BRCA2*, *CTNNB1*, *IGF2*, *MEN1*, *MSH2*, *MSH6*, *PDE8B*, *PDE11A*, *PRKACA*, *PRKACB*, *PRKAR1A*, and *TP53*. The panel genes were selected from the recommended list of 56 genes of the American College of Medical Genetics and Genomics (ACMG) for genomic reporting [14] based on literature evidence of their possible role in the pathogenesis of adrenal tumors [1,15–28] (Appendix Table S1). Specifically, six genes (*APC, BRCA1, BRCA2, MSH2, MSH6*, and *TP53*) are known to be cancer-predisposing genes according to the ACMG criteria, and 11 genes (*AIP, ARNT, ARMC5, CTNNB1, IGF2, MEN1, PDE8, PDE11A, PRKACA, PRKACB*, and *PRKAR1A*) are involved in pathways linked to adrenal tumorigenesis.

2.4. NGS analysis and bioinformatic interpretation

Germline DNA was isolated from leukocytes in peripheral whole blood samples using standard techniques. NGS analysis was performed using Ion Torrent technologies (Thermo Fisher Scientific) as previously described [29] (see Appendix for details).

A semi-automated bioinformatics pipeline was used, which involved manual inspection of the data quality and contributions from molecular biologists, bioinformaticians, and clinicians. Variant prioritization was calculated after the filtering steps. Polymorphisms in intronic regions or those classified in the ClinVar database [30] as benign or likely benign were excluded. To predict the impact of each amino acid substitution on the structure and function of a protein, each mutation was studied using three in silico tools: Polyphen-2, SIFT, and Grantham [31–33]. In addition, one molecular and one clinical geneticist independently evaluated all variants according to the ACMG rules [32] using literature, public databases, and variant-specific databases (IARC TP53, LOVD, and HGMD). Variants interpreted as pathogenic (P), likely pathogenic (LP), or increased risk alleles were considered as potentially pathogenic [34]. Selected variants were confirmed by Sanger Sequencing in leukocytes and archival FFPE tumor samples, if available (see Appendix for details).

2.5. Statistical analysis

Descriptive statistics were used to analyze the clinical indicators. Associations between variables were assessed using appropriate statistical tests. No imputation was performed for missing data.

Overall survival (OS) was calculated from the diagnosis of ACC to death or the date of the last follow-up. Disease-free survival (DFS) was defined as the time from radical surgery to the first radiological evidence of ACC relapse or date of the last follow-up. Progression-free survival (PFS) was calculated from diagnosis to the first evidence of progressive disease (PD) or death in metastatic patients, and from disease relapse to progression, death, or last follow-up in non-metastatic patients.

Survival curves were generated using the Kaplan–Meier method and compared using the log-rank test. Known clinical variables with a potential prognostic value for each survival endpoint (enter level $p \leq 0.05$, univariate analysis) were included in the multivariate Cox models. Race was not controlled because 98 % of the patients were European non-Finnish. The results were reported as hazard ratios (HR) with 95 % confidence intervals (95 %CI). Cohen's d value was calculated to measure the effect of germline variant size on survival endpoints. For all tests, statistical significance was set at $P < 0.05$. All analyses were performed using SPSS v.23.0 (IBM-SPSS Statistics, USA) and R Core Team (2020) version 4.0.2.

3. Results

3.1. Patient characteristics

Our cohort of 150 patients had a median age at diagnosis of 47 years (range 18–82) and male-to-female ratio of 1:1.83. The patient characteristics are described in Appendix Table S2.

A personal history of cancer other than ACC was found in 20 patients (13.4 %), and 44 patients (29.4 %) had a family history of cancer. At diagnosis, the majority of ACCs were ENSAT stages I-II (58.7 %). Excess hormones were detected in 55.3 % of cases, and the majority (93.4 %) of patients underwent upfront surgery (see Appendix for details).

3.2. Characterization of germline variants

Of the 150 patients, 21 (14 %) had 18 unique germline variants (GVs) in the panel of analyzed genes (Figure 1). These unique variants were

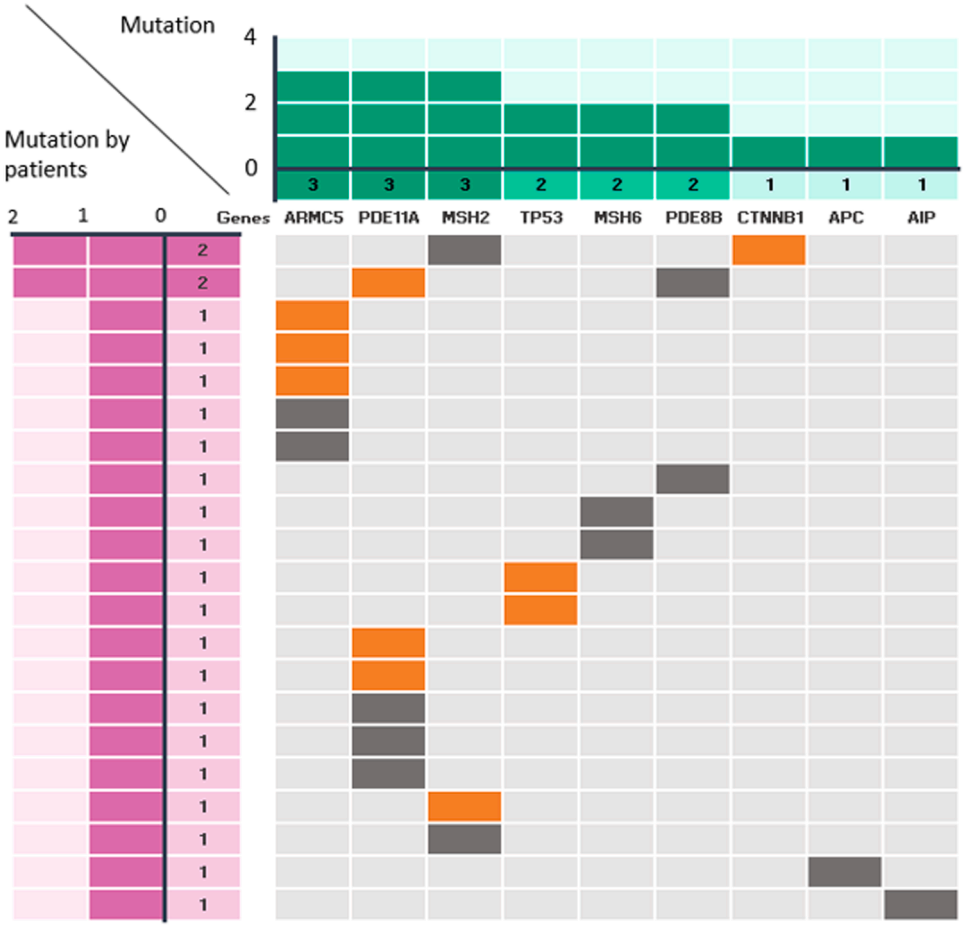

Fig. 1. Landscape of germline variants (GVs) in 150 patients with adrenocortical carcinoma. In green, the number of GVs that were unique for each gene. In purple, the number of GVs per patient. In orange, the distribution of potentially pathogenic (P/LP) GVs by gene and patient; in dark grey the distribution of non-P/LP GVs by gene and patient.

identified in nine different genes: *APC* (n = 1), *ARMC5* (n = 3), *MSH2* (n = 3), *PDE11A* (n = 3), *TP53* (n = 2), *MSH6* (n = 2), *PDE8B* (n = 2), AIP (n = 1), and CTNNB1 (n = 1) (Table 1). The 18 GVs found in our patients were classified using ACMG classification as follows: a) 6 pathogenic (P) or likely pathogenic (LP) variants in *ARMC5, CTNNB1, MSH2*, *PDE11A*, and *TP53* (n = 2) genes; b) 12 variants in *AIP, APC, ARMC5, MSH2, MSH6, PDE11A* and *PDE8B* lacking a clear evidence of pathogenicity. Overall, 10 of the 150 (6.7 %) patients had potentially pathogenic variants.

Specifically, 15 missense mutations and three in-frame deletions were identified (Appendix Table S3). Supplemental Fig. S1 shows the corresponding human proteins with their functional domains and alterations. Most variants described in this series were present in a single patient at a higher frequency than those reported for GnomAD. The variants p.P731R in *ARMC5*, p.R307X and p.I552T in *PDE11A* were particularly enriched, as they were found in more than one patient (Appendix Table S4). Two patients had concurrent mutations involving *MSH2* and *CTNNB1*, *PDE11A* and PDE8B genes, respectively (Appendix Table S5). The *TP53* p.G105D variant was found in a female patient whose sister was later diagnosed with ACC and was confirmed to carry the *TP53* p.G105D variant. Sanger sequencing of the specific regions of interest was performed on archival FFPE tumor samples of patient #113, and the *TP53* p.R110P mutation was detected in the absence of the gene-negative allele (LOH). In patient #117, Sanger sequencing showed the *ARMC5* p.P731R variant with a frequency comparable to germline expression (heterozygous). No other pathogenic variant was found by analyzing the entire coding sequence of *ARMC5*.

With our experimental approach and bioinformatics pipeline, no deleterious variants were found in 32 subjects with adrenal adenoma and seven healthy controls.

3.3. Histological analysis of patients with germline variants

Table 2 summarizes the results of the histological analysis of 10 of 21 patients carrying potentially pathogenic GVs. Predominant oncocytic features were observed in the *MSH2* mutated patient and immunohistochemistry for mismatch repair proteins showed an altered pattern of MSH6 protein. One oncocytic and one conventional ACC showing an altered p53 pattern on immunohistochemistry with protein overexpression were present in patients with *TP53* germline variants. *PDE11A* and *ARMC5* mutated patients had ACC with a minor component of the oncocytic or myxoid subtype. One *PDE11A*-associated case showed a well-circumscribed lesion surrounded by a thin capsule, with a Weiss score of 5 and unequivocal signs of vascular invasion (Supplemental Fig. S2). One *ARMC5*-associated case showed the combined features of ACC and macronodular cortical nodular disease (Supplemental Figure A3).

3.4. Correlation between germline genotype and clinical phenotype

Table 2 reports the clinical characteristics of patients carrying GVs. Women represented 80.0 % of the 10 carriers of potentially pathogenic variants, and their median age at diagnosis was 58.5 years (range, 29–72 years). Demographic and clinical characteristics did not significantly differ between patients who were or were not carrying GVs (Table 3).

Despite being diagnosed with apparently sporadic ACC, 40.0 % of

Table 1
Unique germline variants identified in 150 patients with adrenocortical carcinoma.

Germline Variants Identified	ExAC# frequency	dbSNP	ClinVarID	ACMG Classification	Patient ID
APC (NM_000038.6)					
c.3410A>G p.(D1137G)	NA	rs1765418674	836538	VUS	#022
ARMC5 (NM_001105247.2)					
c .26C>T p.(T9M)	NA	rs1166729776	NA	VUS	#057
c.66_68del p.(A23del)	NA	rs778338263	NA	VUS	#103
c .2192C>G p.(P731R)	1.91e^{-03}	rs200951744	1303338	**LP**	#089 #107 #117
MSH2 (NM_000251.3)					
c .136C>G p.(H46D)	NA	rs1553348821	1058599	VUS	#131
c.1786_1788del p.(N596del)	1.50e^{-05}	rs63749831	1757	**P**	#112
c .1804C>G p.(L602V)	1.50e^{-05}	rs748797209	219668	VUS	#032
PDE11A (NM_016953.4)					
c .919C>T p.(R307X)	4.45e^{-03}	rs76308115	5286	**LP**	#011 #063 #109
c .1655T>C p.(I552T)	1.46e^{-03}	rs138427178	725066	VUS	#148, #077
c. 2531G>C p.(R844P)	1.50e^{-05}	NA	NA	VUS	#088
TP53 (NM_000546.6)					
c .314G>A p.(G105D)	NA	rs587781504	141114	**LP**	#102
c .329G>C p.(R110P)	NA	rs11540654	233627	**P**	#113
MSH6 (NM_000179.3)					
c .3788G>A p.(R1263H)	7.50e^{-05}	rs147852216	127593	VUS	#042
c .3800T> C p.(M1267T)	1.60e^{-04}	rs148445930	142672	VUS	#134
PDE8B (NM_003719.5)					
c .1183C>T p.(R395C)	4.50e^{-05}	rs778969486	NA	VUS	#049
c .1831T>G p.(S611A)	1.50e^{-05}	rs201596222	906328	VUS	#109
AIP (NM_003977.4)					
c .161G>A p.(R54Q)	3.01e^{-05}	rs762938281	819687	VUS	#017
CTNNB1 (*NM_001904.4*)					
c.2262_2300del p.(D755_P767del)	NA	NA	NA	**LP**	#032

Legend of abbreviations in alphabetical order. LP, likely pathogenic; P, pathogenic; VUS, variant of uncertain significance; # European (non-Finnish), ExAC v1.0.

patients with GVs had a family history of cancer (with a median of three relatives) compared to 28.6 % of patients who did not. Moreover, one patient with the *TP53* p.G105D variant had a previously unknown family history of ACC. The frequency of a personal history of cancer did not differ between patients who carried GVs and those who did not (Table 3).

3.5. Survival outcomes and prognostic factors

The database was closed for the final analysis on October 20, 2022. At that time, 94 of the 127 (74.0 %) operated patients had disease relapse and 100 % (23/23) of the metastatic patients had disease progression. Overall, 82 patients (54.6 %) were alive and 50/150 (33.3 %) were alive and free from progression. The median OS in the entire series was 142 months (range, 1–297 months) and the 5 year-OS was 65 %. The median DFS and PFS were 31 months (range, 1–225+ months) and 27 months (range, 1–275+ months), respectively (Fig. 2).

Univariate and multivariate analyses of clinical characteristics showed that ENSAT stage and resection of the primary tumor were prognostic factors for both OS and PFS, whereas age and cortisol excess significantly affected OS. Adjuvant treatment and a familial history of cancer were prognostic factors for DFS (Appendix Table S6). In univariate analysis, potentially pathogenic GVs were significant predictors of PFS (HR 2.39, 95 %CI, 1.09–5.27; p = 0.029) and OS (HR 2.18, 95 % CI, 1.01–4.80; p = 0.046) (Appendix Table S7-S8). The multivariate Cox model retained the prognostic significance of potentially pathogenic GVs for both endpoints (PFS, HR 2.41, 95 %CI, 1.05–5.53; p = 0.037; OS, HR 2.43, 95 %CI, 1.01–5.84; p = 0.046) (Appendix Table S7-S8). Among patients with metastatic disease, carriers of potentially pathogenic GVs had a median PFS of 9 months versus 27 months in gene-negative patients (p = 0.023). The corresponding median OSs were 39 and 142 months (p = 0.046), respectively (Fig. 2).

4. Discussion

We investigated the presence of GVs in 17 selected genes (NGS custom panel) in 150 adult patients with sporadic ACC. We investigated known cancer-predisposing genes (*APC, BRCA1, BRCA2, MEN1, MSH2, MSH6,* and *TP53*) linked to ACC, mostly in the context of hereditary syndromes [1] because limited knowledge is available on whether they are associated with sporadic ACC [35]. We also included genes (*AIP, ARMC5, ARNT, CTNNB1, IGF2, PDE8B, PDE11A, PRKACA, PRKACB, PRKAR1A*) involved in several endocrine diseases characterized by disruption of specific adrenal cortex pathways, or affected by somatic mutations in ACC [9,36–38].

By applying this “plausible association” approach, we found that 21 (14 %) of 150 adult patients with apparently sporadic ACC were carriers of at least one GV, and 10 (6.7 %) carried potentially pathogenic variants. This frequency of GV carriers is similar to that reported in an ACC-specific TCGA study [9].

The prevalence of GVs in adult patients was lower than that previously reported in pediatric patients [39], thus supporting the hypothesis that the prevalence of germline variants is inversely correlated with age [40]. In our series, 40 % of patients with potential pathogenic GVs had a family history of cancer, an intriguingly high figure that may suggest a genetic predisposition in some of these cases. Our results underscore the need to offer patients with presumed sporadic ACC genetic counseling to identify an underlying hereditary syndrome [1].

Most of the variants described in our series were present in individual patients, and some variants had a frequency that was much higher than that reported in the GnomAD database. This enrichment suggests a new, yet not described, role for these variants in the pathogenesis of ACC.

However, some patients are carriers of multiple variants. In fact, we identified the co-presence of variants in *MSH2* and *CTNN1B*, *PDE11A* and *PDE8B*. Patients who carried multiple GVs did not appear to have a worse prognostic profile than those who did not, and their baseline characteristics did not differ significantly from those of the others.

We identified one potentially pathogenic variant in *MSH2*. This finding supports the view that ACC may be considered a part of Lynch syndrome [7,21] and highlights the role of the GVs of genes involved in DNA damage repair in the pathogenesis of ACC. However, we have to

Table 2
Clinical characteristics of patients carrying potentially pathogenic (P/LP) variants.

Patient ID	Gender	Age at diagnosis (years)	OS (months)	Live status	Personal history of cancer other than ACC	Family history of cancer	ACC stage	Secretion of ACC	Gene/Variant	ACMG classification	Complementary IHC	Histological variant	Weiss score	Helsinki score	Ki-67 (%)
#089	F	60	44	DOD	NO	NO	II	Cosecretion of cortisol and other steroids	*ARMC5* p. P731R	LP	Not executable	Focal oncocytic (20 %)	6	18	10
#107	M	57	55	ANED	NO	NSCLC, CRC, BLCA, PCa	II	Cosecretion of cortisol and other steroids	*ARMC5* p. P731R	LP	NA	NA	NA	NA	NA
#117	M	64	74	DOD	NO	NO	I	Cosecretion of cortisol and other steroids	*ARMC5* p. P731R	LP	No evaluable target	Focal myxoid (10 %)	4	23	20
#032	F	72	36	DOD	NO	NO	I	Cosecretion of cortisol and other steroids	CTNN1B p. D755_767del	LP	NA	NA	NA	NA	NA
#112	F	65	2	DOD	NO	CRC	IV	No secretion	*MSH2* p. N596del	P	MSH6 negative (altered pattern)	Oncocytic variant	NA (biopsy)	NA (biopsy)	20
#011	F	29	76	ANED	NO	NO	I	No secretion	*PDE11A* p. R307X	LP	Not executable	Focal oncocytic (30 %)	5	3	3
#063	F	36	27	DOD	NO	CRC, NSCLC	III	No secretion	*PDE11A* p. R307X	LP	Not executable	Myxoid(40 %)	9	78	70
#109	F	42	92	ANED	NO	NO	II	No secretion	*PDE11A* p. R307X	LP	No evaluable target	Focal oncocytic (30 %)	5	NA	NA
#102	F	35	39	DOD	NO	ACC, NSCLC, LGG, others	II	Cosecretion of cortisol and other steroids	*TP53* p.G105D	LP	Not executable	Oncocytic variant	NA	23	18
#113	F	61	34	DOD	BRCA	NO	III	No secretion	*TP53* p.R110P	P	p53 overexpressed (altered pattern)	Conventional	7	48	40

Legend of abbreviations in alphabetical order. ACC, adrenocortical carcinoma; ANED, alive with no evident disease; BLCA, bladder carcinoma; BRCA, breast carcinoma; CRC, colorectal cancer; DOD, dead of disease; LGG, low-grade glioma; LP, likely pathogenic; NA: not available; NSCLC, non-small cell lung cancer; P, Pathogenic; PCa, prostate cancer.

Table 3
Comparison of patients with or without potentially pathogenic (P/LP) germline variants (GVs).

Variable		Patients with potentially pathogenic GVs (n = 10)	Patients without potentially pathogenic GVs (n = 140)	p-value
Age, (years)	Median	58.5	47.0	0.268
	Range, (IQR)	29-72 (28.5)	18-82 (21.7)	
Sex, n (%)	M	2 (20)	51 (36)	0.479
	F	8 (80)	89 (64)	
ENSAT stage, n (%)	Stage I-II	7 (70)	81 (58)	0.748
	Stage III	2 (20)	37 (26)	
	Stage IV	1 (10)	22 (16)	
Clinical hypersecretion, n (%)	No	5 (50)	62 (44)	0.982
	Yes	5 (50)	78 (56)	
Type of hypersecretion, n (%)	Hypercortisolism	4 (40)	50 (36)	0.747
	Other steroids	6 (60)	90 (64)	
Proliferation index (KI67 %)	Median	20[a]	20[b]	0.778
	Range, (IQR)	4-35 (12.5)	2-75 (20)	
Tumor size, (cm)	Median	7.5	10[c]	0.254
	Range, (IQR)	4-21 (10)	2-25 (3.5)	
Weiss score	Median	7.5[d]	6[e]	0.084
	Range, (IQR)	4-9 (3)	2-9 (6.3)	
Surgery of primary tumor, n (%)	No	1 (10)	9 (6)	0.510
	Yes	9 (90)	131 (94)	
Adjuvant treatment, n (%)	No	3 (30)	46 (33)	1.000
	Yes	7 (70)	94 (67)	
Personal history of cancer other than ACC, n (%)	No	9 (90)	121 (86)	1.000
	Yes	1 (10)	19 (14)	
Family history of cancer, n (%)	No	6 (60)	100 (71)	0.480
	Yes	4 (40)	40 (29)	

Mann Whitney was used for continuous variables, Fisher exact test for ordinary variables.
Legend: [a] available on 7 patients; [b] available on 129 patients; [c] available on 129 patients; [d] available on 6 patients; [e] available on 113 patients.

acknowledge that our gene panel did not cover the whole genomic spectrum of Lynch syndrome. Moreover, a partially discordant result was observed between genomic profiling and immunohistochemistry for MMR proteins, since MSH2 protein expression was preserved. However, MSH2 protein retained expression has been described in patients harboring germline *MSH2* variants [41], possibly as the consequence of an alteration of the protein function but not of the expression of the protein domain that acts as epitope for the antibody. Indeed, the loss of MSH6 protein in this patient further support a damage of the MSH2/MSH6 complex and the pathogenicity of the MSH2 variant detected.

Of the two GVs detected in *TP53*, the p.G105D variant has been observed at low frequencies in large population studies [42]. This variant produces an in-frame deletion at the end of exon 4, as observed in patients with breast cancer [43]. We found this variant in a 35-year-old female and subsequently in her sister, who was found to have androgen-secreting ACC at the age of two years. The fact that the p.G105D variant was found in two patients from the same family suggests a pathogenic role for this variant, which fits well with its localization in a highly conserved protein domain. According to the Chompret criteria that have been recently proposed to identify affected families beyond the classical criteria of Li-Fraumeni syndrome [44], this is a Li-Fraumeni family that was previously unknown and recognized through the study.

The p.R110P variant has previously been reported in two males from separate families with Li-Fraumeni syndrome, one with gastric cancer at 32 years of age and the other with two primary sarcomas at 37 and 44 years of age [45,46] as well as in an individual with soft tissue sarcomas [47]. Several functional studies have demonstrated that this alteration is deficient in transcriptional activation, DNA binding, apoptosis induction, and cell growth suppression [48,49]. One study suggested the assembly of mutant p53 into large aggregates, resulting in impaired nuclear import [50]. We identified this variant in a 61-year-old female with ACC and breast cancer. Immunohistochemical analysis confirmed cytoplasmic overexpression of *TP53* protein in the tumor, and Sanger sequencing showed the presence of LOH for the variant in the *TP53* allele. Neither of these *TP53* variants has previously been reported in patients with ACC, and our findings suggest that they may play a role in ACC development.

ARMC5 is a tumor suppressor gene responsible for the familial form of primary bilateral macronodular adrenal hyperplasia (PBMAH). The presence of inactivating *ARMC5* mutations is associated with a severe form of ACTH-independent Cushing syndrome as well as an overall increase in adrenal mass [23,51,52]. For these reasons, it has been suggested (but never confirmed) that the GVs of *ARMC5* represent a genetic risk factor for ACC [38,53].

We detected one variant of *ARMC5* in three patients (0.2 %), which has already been reported in PBMAH [23]. We identified the p.P731R variant in three patients whose clinical characteristics included older age, large tumors, and cortisol excess. Such findings reflect those observed in patients with PBMAH [23,38]. In contrast to the findings in PBMAH [38], we were unable to demonstrate a secondary alteration of the ARMC5 gene in one patient with available tumor material. To the best of our knowledge, this is the first report of GVs in the *ARMC5* gene in patients with ACC. Interestingly, one patient carrying the ARMC5 variant displayed pathological features of both ACC and PBMAH. This finding leads to the hypothesis that progression from benign to malignant cortical proliferation, from PBMAH to ACC, is possible.

Recent evidence supports the notion that germline mutations may contribute to tumor progression [54,55]. In our series, potentially pathogenic GVs were associated with reduced survival outcomes and had a significant and independent impact on both PFS and OS.

This study had some limitations. First, we focused on a set of genes that did not encompass the full genetic variability of ACC: due to shortage of funding, we did not perform whole exome sequencing. Therefore, the frequency of pathogenic GVs may have been underestimated in this study. Second, we were unable to perform a systematic parallel analysis of somatic DNA since most patients were referred to us after being operated in other centers. Thus, we could not thoroughly investigate the double-hit events and clearly ascertain the specific pathogenicity of individual variants.

In conclusion, we have characterized the largest series to date of adult patients with sporadic ACC for GVs using an NGS target gene panel. Our results showed a 6.7 % prevalence of potentially pathogenic variants, which were mainly found in genes involved in DNA damage repair. In addition, we reported, for what we believe is the first time, the presence of GVs of *ARMC5* in patients with ACC, and we found two novel pathogenic variants of *TP53*. The present study thus extends the knowledge on the germline component in this rare cancer and highlights the role of genetic counseling for patients with apparently sporadic ACC and their families.

CRediT authorship contribution statement

Deborah Cosentini: Writing – review & editing, Visualization, Validation, Data curation, Investigation. **Soraya Puglisi:** Writing – review & editing, Visualization, Validation, Investigation, Data curation. **Marta Laganà:** Writing – review & editing, Visualization, Validation, Investigation, Data curation. **Paola Perotti:** Writing – review & editing, Visualization, Validation, Project administration. **Laura Saba:** Writing – review & editing, Visualization, Validation, Investigation, Data curation. **Elisa Rossini:** Writing – review & editing, Visualization, Validation, Investigation, Data curation. **Sandra Sigala:** Writing – review &

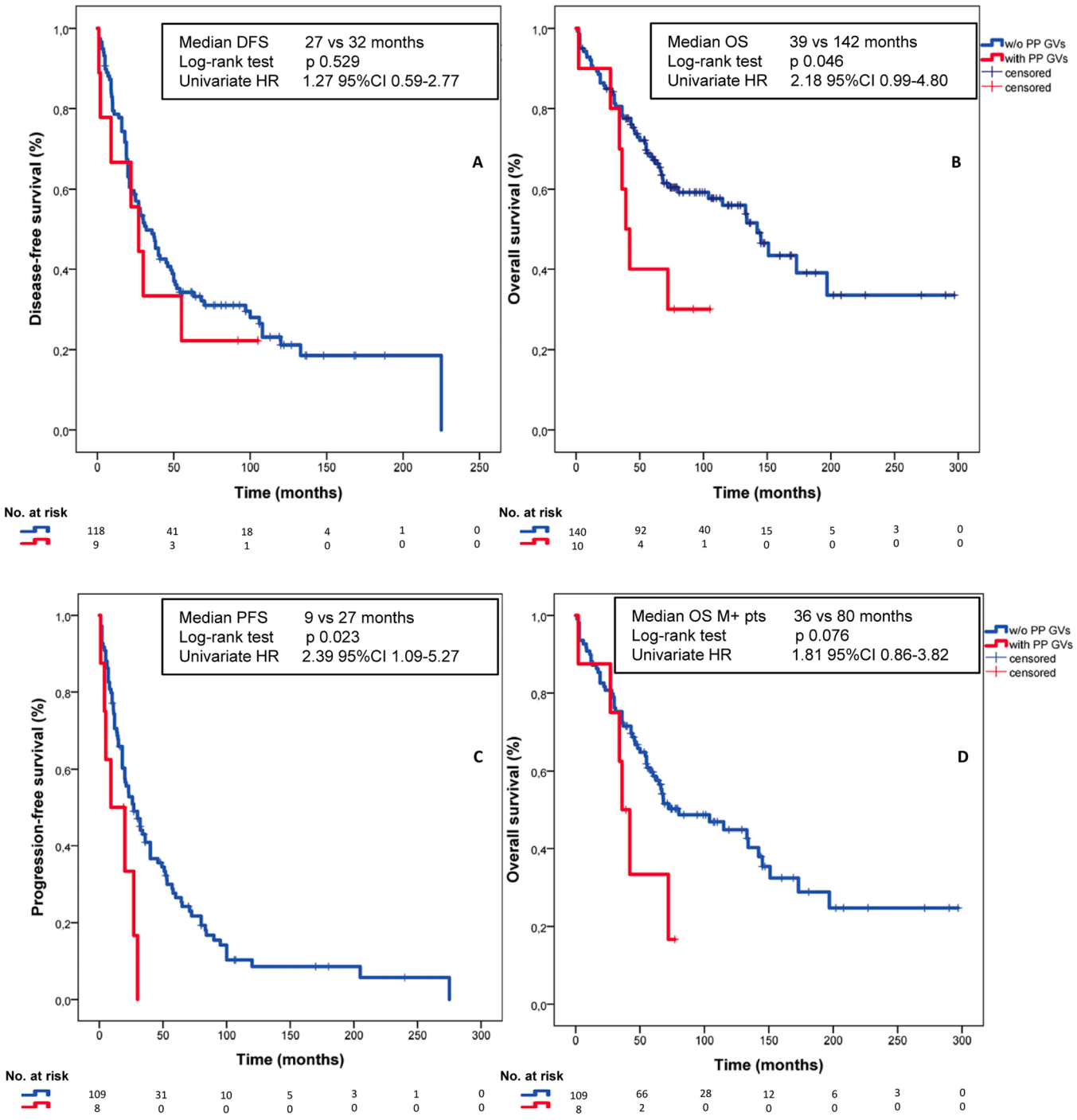

Fig. 2. Survival curves stratified by the presence (red line) or absence (blue line) of potentially pathogenic (P/LP) germline variants (GVs). A: Disease-free-survival in operated patients; B: Overall survival in the whole series; C: Progression-free survival in relapsed/metastatic patients; D: Overall survival in relapsed/metastatic patients.

editing, Visualization, Validation, Supervision. **Maria Scatolini:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Formal analysis, Conceptualization. **Salvatore Grisanti:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Formal analysis, Conceptualization. **Marco Volante:** Writing – review & editing, Visualization, Validation, Supervision. **Alfredo Berruti:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Conceptualization. **Pasquale Tomaiuolo:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Investigation. **Flavia Palermo:** Validation, Visualization, Writing – review & editing. **Massimo Terzolo:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Funding acquisition, Conceptualization. **Enrico Grosso:** Writing – review & editing, Visualization, Validation, Software, Investigation. **Vittoria Basile:** Writing – review & editing, Visualization, Validation, Investigation, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

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Conflict of interest

The authors declare no potential conflicts of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejca.2024.114088.

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