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REVIEW ARTICLE

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The role of molecular profiling in adrenocortical carcinoma

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Abstract

Adrenocortical carcinoma (ACC) is a rare, aggressive cancer with still partially unknown pathogenesis, heterogenous clinical behaviour and no effective treatment for advanced stages. Therefore, there is an urgent clinical unmet need for better prognostication strategies, innovative therapies and significant improvement of the management of the individual patients. In this review, we summarize available studies on molecular prognostic markers and markers predictive of response to standard therapies as well as newly proposed drug targets in sporadic ACC. We include in vitro studies and available clinical trials, focusing on alterations at the DNA, RNA and epigenetic levels. We also discuss the potential of biomarkers to be implemented in a clinical routine workflow for improved ACC patient care.

KEYWORDS

adrenocortical cancer, biomarkers, precision medicine, prognosis, targeted treatment

1 | INTRODUCTION

Personalized or stratified medicine are keywords that give rise to hopes, especially in relation to cancers. The aim of personalized medicine is to treat each patient in the best possible way based on his or her individual characteristics. A prerequisite for this approach is detailed knowledge about pathomechanisms leading to disease. Advances made in high-throughput techniques enable comprehensive molecular characterisation of tumour entities at genomic, transcriptomic and epigenomic levels, contributing significantly to the detection of diagnostic, prognostic and predictive biomarkers. While stratified medicine is already part of a clinical routine in common tumour types it is still a challenge for patients with rare cancers like adrenocortical carcinomas (ACCs).

ACC is a rare malignancy of the adrenal cortex with an incidence of 0.5–2.0 individuals per million per year.[1–3] Prognosis is generally poor but heterogeneous with a 5-year survival rate ranging from 13% to 80%,[2,4] especially depending on tumour stage at diagnosis. However, there are over 10% of long-term survivors with an initial metastatic disease and >20% of patients with low tumour stages that die within the first 3 years.[4] For improved prognostication, current guidelines recommend considering tumour stage according to the European Network for the Study of Adrenocortical Tumours (ENSAT) classification,

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resection status, Ki67 index (or mitotic count), steroid oversecretion and patient's general condition.[3,5]

Also in ACCs, genome-wide and targeted studies identified molecular markers associated with a clinical outcome that may explain less and more aggressive subtypes within all tumour stages.[6–9] These findings might help to improve prognostic stratification in ACC and, therefore, improve decisions regarding therapy, but have not been implemented in clinical routine care so far.

A very limited number of therapeutic alternatives is available for ACC. The only curative treatment option for patients with ACC is complete surgical resection, but many patients experience recurrence including distant metastases.[3,5] The only drug formally approved for ACC is mitotane. In advanced ACC the combination of etoposide, doxorubicin, cisplatin plus mitotane (EDP-M) is the first-line standard chemotherapy treatment.[10] Unfortunately, response rates are low for both therapies and treatment may be limited by severe adverse reactions (reviewed by Else et al.).[2] The efficacy of multiple-targeted therapies has been tested in previous preclinical studies or relatively small case series, but the results were mostly disappointing (reviewed by Mohan et al.[11] and Altieri et al.).[12]

In this review, we summarize studies on molecular prognostic and predictive markers and new proposed therapies in sporadic ACC. We focus on markers identified at the DNA, RNA and epigenetic level using targeted or pan-genomic studies and discuss their potential to be implemented in a clinical routine workflow for improved patient care (illustrated in Figure 1). The main findings from the most relevant studies are also summarized in Tables 1 and 2. Here, we concentrate on somatic alterations, while for germline genetic testing and ACC-related hereditary syndromes, we rely on available literature.[13–15]

2 | MOLECULAR PROGNOSTIC MARKERS IN ACC

2.1 | Alterations at DNA level

2.1.1 | Copy number alterations

Already publications from the 1980s described losses of heterozygosity (LOH) at loci on Chromosome 11, 13q and 17p to be highly specific for malignant adrenocortical tumours (ACT) in comparison to benign adrenocortical lesions.[16,17] Some of these alterations have also been reported to play a role as prognostic markers. For instance, LOH at 17p13—the location of*TP53*—and 11p15—the location of *insulin-like growth factor 2* (*IGF2*)—have been proposed as strong predictors of shorter disease-free survival (DFS) in patients with localized tumour and complete resection.[18] Comparative genomic hybridisation (CGH) analyses—which can be used for a genome-wide survey of copy number alterations (CNAs)—consistently showed that fewer alterations are found in genomes of adenomas (ACA) than in carcinomas.[19–21] Aberrations in ACCs can be detected in specific regions distributed over the entire genome, for example, amplification at Chromosomes 5, 12 and 19, and deletions at parts of Chromosomes 13 and 22 among

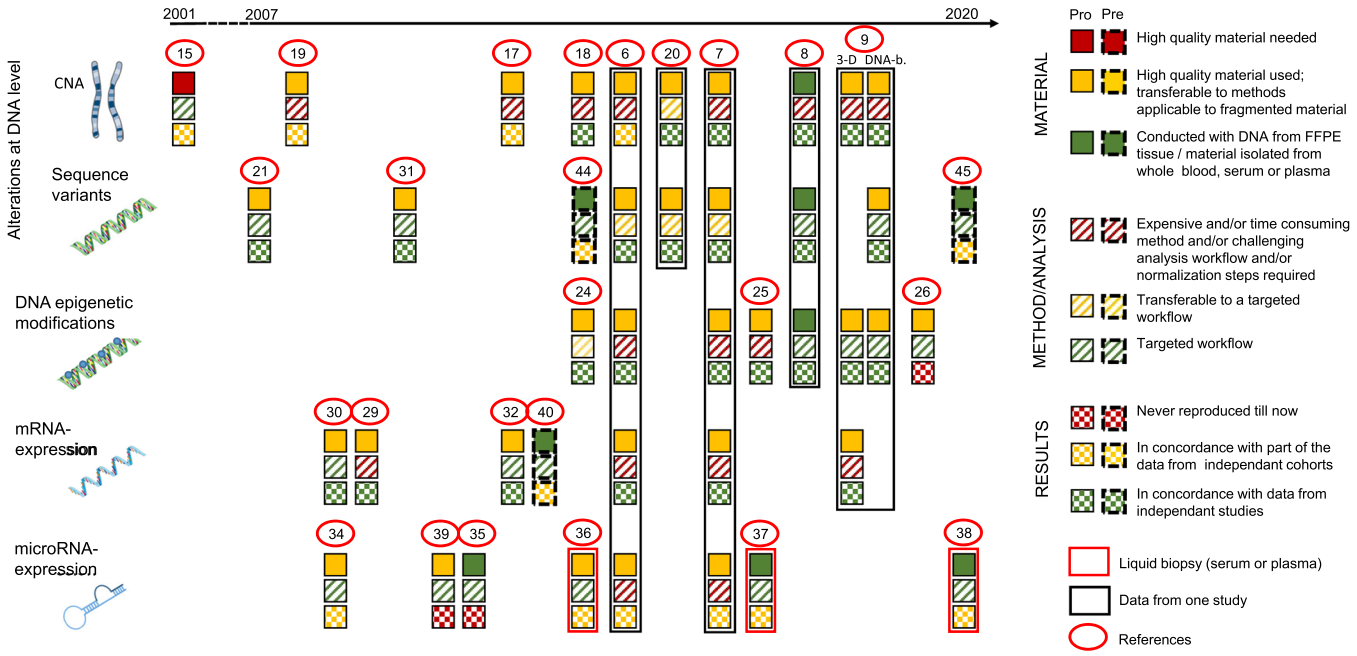

FIGURE 1 Prognostic (Pro) and predictive (Pre) biomarkers for patients with adrenocortical carcinoma. Research results were assessed according to their applicability in clinical routine care in terms of required material, the complexity of methods and analysis, costs and reliability. Studies were sorted according to the date of publication (2001–2020). 3D, 3D-targeted classifier; CNA, copy number alteration; DNA-b, DNA-based-targeted classifier; FFPE, formalin-fixed paraffin-embedded; mRNA, messenger RNA; Pre, predictive; Pro, prognostic [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Summary of available publications about molecular prognostic biomarkers and markers predictive of response to standard therapies for adrenocortical carcinoma

References	Main findings	Cohort	Material & method	Perspective for personalized medicine and implementation in clinical routine care
Alterations at DNA level—copy number alterations				
Gicquel et al.[18]	17p13 LOH as a predictor of relapse and shorter DFS	96 ACTs	DNA and RNA from ff tissue Southern blotting Dot-blot analysis	*No promising molecular marker*, as: data generation feasible with low-quality (FFPE) material, but more reliable with high-quality material- complex analysis workflow mostly based on genome-wide data
Stephan et al.[22]	Accumulation of gains and losses (in specific regions linked to OS)	25 ACCs	DNA from ff tissue High-resolution CGH	
Barreau et al.[20]	Specific chromosomal alterations associated with different OS	21 ACCs	DNA from ff tissue CGH array Hierarchical clustering	
Ronchi et al.[21]	Characteristic CNAs associated with different OS	22 ACCs	DNA from ff tissue SNP array and CGH array Hierarchical clustering	
Juhlin et al.[23]	Trend to decreased OS in patients with *ZNRF3* deletions	41 ACCs	DNA from ff and FFPE tissue WES	
Lippert et al.[8]	Specific CN patterns associated with different PFS	107 ACCs	DNA from FFPE tissue Targeted NGS (160 genes)	
Alterations at DNA level—sequence variants				
Libé et al.[24]	*TP53* mutations associated with shorter DFS	36 ACTs	DNA and RNA from ff tissue Sanger sequencing	*Promising molecular prognostic marker*, as:- data generation feasible with low-quality (FFPE) material —analysis via targeted workflows—stable markers that were reproduced several times
Ragazzon et al.[34]	*TP53* and *CTNNB1* variants exclusively found in tumours associated with dismal DFS and OS	51 ACCs	DNA from ff tissue Sanger sequencing	
Juhlin et al.[23]	Trend to decreased OS in patients with *TP53* mutations	41 ACCs	DNA from ff and FFPE tissue WES	
Lippert et al.[8]	Variants in the ß-catenin pathway and the number of protein-altering mutations associated with poor survival. Additional variants in p53/Rb1 pathway associated with worst PFS	107 ACCs	DNA from FFPE tissue Targeted NGS (160 genes) Sanger sequencing (ZNRF3)	

(Continues)

TABLE 1 (Continued)

References	Main findings	Cohort	Material & method	Perspective for personalized medicine and implementation in clinical routine care
D'Avolio et al.[52]	*CYP2B6* polymorphism as a predictor of mitotane plasma concentrations	56 ACCs	DNA from whole blood TaqMan allelic discrimination kit	*Promising molecular predictive marker*, as: data generation feasible with low-quality (FFPE) material—analysis via targeted workflows
Altieri et al.[53]	*CYP2W1* and *CYP2B6* polymorphisms for prediction of response to adjuvant mitotane treatment	182 ACCs	DNA from whole blood Sanger sequencing	
DNA epigenetic modifications				
Barreau et al.[27]	Three subgroups with different methylation profiles (non-CIMP, CIMP-low, CIMP-high) correlated to OS	51 ACCs	DNA from ff tissue Infinium HumanMethylation27 Beadchip Targeted analysis (MS-MLPA)	*Promising molecular marker*, as: data generation feasible with low-quality (FFPE) material—analysis via targeted workflows—reproducible markers
Jouinot et al.[28]	Mean DNA methylation status of four genes (*GSTP1*, *PAX5*, *PAX6* and *PYCARD*) as an independent prognostic factor for DFS and OS	253 ACCs (50 t. cohort; 203 v. cohort)	DNA from ff tissue Targeted analysis (MS-MLPA)	
Lippert et al.[8]	Mean DNA methylation of four genes (*GSTP1*, *PAX5*, *PAX6* and *PYCARD*) confirmed as an independent prognostic factor for PFS	107 ACCs	DNA from FFPE tissue Targeted analysis (pyrosequencing)	
Mohan et al.[29]	Hypermethylation of *G0S2* associated with poor DFS and OS	80 t-n p ACCs 12 n-n/np ACCs	DNA from ff tissue Targeted analysis (bisulfite sequencing and ms restriction digest/qPCR)	
Gene expression changes				
de Reyniès et al.[33]	Gene expression profiles differentiate aggressive and nonaggressive ACCs (DFS and OS) (C1A and C1B). Targeted approach for prognostication with gene expression data (*BUB1B-PINK*)	153 ACTs	RNA from ff tissue Microarray and qRT-PCR	*No promising molecular marker*, as: data generation requires high-quality material (RNA cannot be reliably isolated from FFPE tissue so far)
Giordano et al.[32]	Two groups with distinct gene expression associated with different OS and different mitotic count	33 ACCs	RNA from ff tissue High density oligonucleotide arrays RNA from ff tissueqRT-PCR	
Fragoso et al.[35]	Validation of the prognostic value of the combined expression of BUB1B, DLGAP5 and PINK1 genes (DFS and OS)	20 pACCs14 ACCs	RNA from ff tissue qRT-PCR	

TABLE 1 (Continued)

References	Main findings	Cohort	Material & method	Perspective for personalized medicine and implementation in clinical routine care
Volante et al.[48]	*RRM1* gene expression is associated with mitotane sensitivity	92 ACCs	RNA from FFPE tissues and cell lines qRT-PCR	*No promising molecular marker*, as: data generation requires high-quality material (RNA cannot be reliably isolated from FFPE tissue so far)
miRNA expression				
Soon et al.[37]	Downregulation of miR-195 and upregulation of miR-483-5p associated with poorer OS	22 ACCs	microRNA from ff tissue Exiqon miRCURY LNA microarray qRT-PCR	*Promising molecular marker*, as: data generation feasible with low-quality (FFPE) material—minimally invasive examinations (can be measured in serum or plasma)—analysis via targeted workflow
Özata et al.[42]	High expression of miR-503, miR-1202 and miR-1275 associated with poor OS	22 ACCs	microRNA from ff tissue Human Agilent's miRNA microarray system qRT-PCR	
Schmitz et al.[38]	miR-335 and miR-675 expression levels may identify aggressive ACC	4 ACCs3	microRNA from FFPE tissue TaqMan MicroRNA Array v2.0 (667 miRNAs)	
		mACCs	Validation with qRT-PCR	
Chabre et al.[39]	Circulating miR-483-5p and miR-195 levels linked to RFS and OS	aACCs	microRNA from ff tissue and serum miRNA assays	
		naACCs	miRXplore Microarrays TaqMan	
Salvianti et al.[40]	Pre- and postsurgery miR-483 and miR-483-5p levels are higher in advanced ACC. Circulating miR-483-5p levels correlated with RFS and OS	27 ACCs	microRNAs from plasma Targeted analysis (qRT-PCR of miR-483 and miR-483-5p)	
Oreglia et al.[41]	High early post-surgery serum miR-483-5p associated with shorter RFS and OS	26 ACCs	microRNAs from serum Targeted analysis (qRT-PCR miRNA-483-5p)	

Abbreviations: aACC, aggressive ACCs; ACC, adrenocortical carcinoma; ACT, adrenocortical tumour; CGH, comparative genomic hybridisation; CIMP, CpG island methylation phenotype; CN, copy number; CNA, copy number alteration; DFS, disease-free survival; DNA, deoxyribonucleic acid; EFS, event-free survival; ff, fresh frozen; FFPE, formalin-fixed and paraffin-embedded; LOH, loss of heterozygosity; mACC, metastatic adrenocortical carcinoma; mRNA, messenger RNA; miRNA, microRNA; ms, methylation-sensitive; MS-MLPA, methylation-sensitive multiplex ligation-dependent probe amplification; naACC, nonaggressive ACC; NGS, next-generation sequencing; n-n, nonnaïve; np, nonprimary; OS, overall survival; p, primary; PFS, progression-free survival; PCR, polymerase chain reaction; q, quantitative; RT, real time; RFS, recurrence-free survival; RNA, ribonucleic acid; SNP, single-nucleotide polymorphism; t., training; t-n, treatment-naïve; v., validation; WES, whole-exome sequencing.

TABLE 2 Summary of main findings in available pan-genomic/comprehensive studies

References	Main findings	Cohort	Material & method	Perspective for personalized medicine and implementation in clinical routine care
Assié et al.[6]	mRNA expression status (C1A and C1B), DNA methylation status (CIMP-high, CIMP-low, non-CIMP), miRNA clusters (Mi1-3), variants in driver genes (*CTNNB1*, *TP53* and *ZNRF3*) and mutation rate associated with OS	130 ACCs	DNA and RNA from ff tissue WESSNP arrays Methylation assays Gene expression arrays miRNA expression profiling	*No promising approach*, *as*:- data generation requires high-quality material (RNA cannot be reliably isolated from FFPE tissue so far)—analysis workflow too expensive and complex
Zheng et al.[7]	Three clusters of cluster groups; built by methylation status (CIMP-low/-intermediate/-high), mRNA expression profile (steroid or proliferation phenotype high and/or low or C1A or C1B), microRNA expression profile (miRNA 1–6) and chromosomal pattern (quiet, chromosomal or noisy) and associated with EFS	91 ACCs	DNA from ff tissue WESmRNA and miRNA sequencing DNA-methylation array Reverse-phase protein arrays SNP array	
Lippert et al.[8]	COMBI-score built by molecular markers (number of sequence variants (more than one), affected pathways (alterations in Wnt/ß-catenin and p53 pathways) and methylation pattern) and clinical/histopathological parameters for prediction of PFS	107 ACCs	DNA from FFPE fissue Targeted NGS (160 genes) Sanger sequencing Targeted methylation analysis (pyrosequencing)	*Promising molecular marker*, as: data generation feasible with low-quality (FFPE) material (Lippert et al.)—analysis via targeted workflow
Assié et al.[9]	3D-targeted classifier (targeted gene expression, targeted methylation and targeted measures of chromosome alterations) or DNA-based-targeted classifier (targeted methylation, targeted chromosome alteration profile and mutational status) combined with tumour stage and proliferation index correlated to OS and DFS	224 ACCs (v. cohort)	DNA from ff tissue Targeted NGS (18 genes) SNP array Targeted gene expression profiling (qRT-PCR) MS-MLPA	

Abbreviations: ACC, adrenocortical carcinoma; CIMP, CpG island methylation phenotype; DFS, disease-free survival; DNA, deoxyribonucleic acid; EFS, event-free survival; ff, fresh frozen; FFPE, formalin-fixed and paraffin-embedded; mRNA, messenger RNA; miRNA, microRNA; MS-MLPA, methylation-sensitive multiplex ligation-dependent probe amplification; NGS, next-generation sequencing; OS, overall survival; PFS, progression-free survival; PCR, polymerase chain reaction; q, quantitative; RT, real time; RNA, ribonucleic acid; SNP, single-nucleotide polymorphism; v., validation; WES, whole-exome sequencing.

others.[20,22] The accumulation of those changes is correlated with survival, that is, patients with minimal aberrations have better survival rates in comparison to patients with accumulated aberrations.[20,22]

We performed also unsupervised genomic clustering to define genetic patterns associated with prognosis in ACC.[7,21] In a single centre study, we described two clusters with distinct outcome.[21] One group is characterized by large amplifications or deletions (i.e., at Chromosome 5, 7, 12 and 19 and Chromosome 1, 2, 13, 17 and 22, respectively) and the other group showed an extremely variable pattern of genetic alterations. In The Cancer Genome Atlas (TCGA) study, three different groups were defined, which were termed chromosomal, noisy and quiet.[7] Tumours with a chromosomal pattern are characterized by a high frequency of whole-chromosome arm gains and losses, tumours with a noisy pattern by a significantly higher number of chromosomal breaks and frequent loss of 1p and tumours with a quiet pattern exhibit only a few large CNAs. A significant decrease in survival was observed in the noisy group compared to the quiet and chromosomal group.

2.1.2 | Sequence variants

In addition to larger chromosomal changes, several specific genes and pathways were identified by comprehensive and targeted studies to be

altered in sporadic ACCs. Especially by whole-exome sequencing (WES) the catalogue of genes involved in the tumourigenesis of ACCs was expanded.[6,7,23] Among those, several genes that are part of the p53/Rb1 or Wnt/ß-catenin pathway (i.e., *CDK4*, *CDKN2A*, *MDM2*, *RB1* and *TP53 and APC*, *CTNNB1* and *ZNRF3*, respectively) are described to be related to poor survival in ACCs. Libé et al.[24] were the first to study the ACC-phenotype with somatic mutations in *TP53* in 36 patients with a 17p13 LOH. *TP53* somatic mutations were found in 33% of the cohort—especially in hot-spot regions of Exons 5–8—and associated with shorter recurrence-free survival.[24] In a landmark paper by Assie et al.[6] using WES, variants in driver genes, such as *CTNNB1*, *TP53* and *ZNRF3* were more frequent in the cluster with shorter overall survival (OS). Similarly, in the TCGA study, variants in the *CTNNB1* gene were mostly detected in patients in "cluster of cluster" group II and III with shorter event-free survival.[7] Finally, Juhlin et al.[23] noted a trend towards decreased OS for patients with *ZNRF3* deletions and *TP53* mutations. We also recently showed in a study on targeted next-generation sequencing that patients with somatic variants in genes of the Wnt/ß-catenin pathway had shorter progression-free survival (PFS) than patients with no somatic mutations or only variants in the genes of the p53/Rb1 pathway, while those with somatic variants in genes of both the Wnt/ß-catenin and the p53/Rb1 pathway were in the group with even worst prognosis.[8] Beneath specific gene alterations, the number of mutations per sample is described to be associated with the worst 5-year OS or shorter PFS by calculating the tumour mutational burden (TMB) with WES data[6,7] or by considering the absolute number of protein-altering variants found with a targeted sequencing workflow, respectively.[8]

2.2 | Alterations at DNA epigenetic level

Methylation differences and their role in tumourigenesis in ACTs were first reported in context with the imprinted 11p15 locus.[25] The first genome-wide methylation analysis in ACTs was conducted in 2012.[26] By comparing the genome-wide methylation status of normal, benign, primary malignant and metastatic malignant adrenocortical tissue, differentially methylated sites were detected and used to distinguish different types of samples.[26]

In contrast, Barreau et al.[27] investigated CpG sites located within proximal promoter regions of genes and confirmed whole-genome methylation differences between ACAs and carcinomas. By unsupervised hierarchical clustering of ACCs, they also identified three subgroups that differ in their methylation status, defined as CIMP (CpG island methylation phenotype)-high, CIMP-low and non-CIMP, which are correlated with OS. This data were also reproduced in comprehensive genomic studies in ACCs.[6,7] Interestingly, the CIMP status identified by unsupervised clustering could be validated by methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA).[27]

More recently, Jouinot et al.[28] used MS-MLPA for setting up a simplified and optimized tool for measuring methylation in ACCs. In a training cohort, methylation array data were compared to MS-MLPA data. From the 27 analysed probes, the four tumour suppressor genes *GSTP1*, *PYCARD*, *PAX6* and *PAX5* positively correlated with CpG island methylation. According to the mean methylation status, the cohort was subdivided into a hypo- and hypermethylated group with a methylation level of 25% as the best cut-off and hypermethylation being associated with shorter DFS and OS.[28] As MS-MLPA data analysis necessitates complicated normalisation procedures, we recently confirmed the correlation between hypermethylation status of these genes and survival with pyrosequencing, which provides absolute methylation values and can be performed with DNA isolated from formalin-fixed and paraffin-embedded (FFPE) samples.[8] By reanalysing the methylation data from the ACC–TCGA study, Mohan et al.[29] were able to reduce the complex genome-wide CpG island hypermethylation signature to a single, binary molecular marker. They identified hypermethylated G0S2 as a marker for rapidly recurrent ACCs. The authors describe their targeted bisulfite sequencing approach as inexpensive, straightforward and compatible with a timeline feasible for clinical decision-making. These results need to be validated in an independent cohort.

2.3 | Alterations at RNA level

2.3.1 | mRNA expression

As already shown for the other prognostic markers, the first studies on gene expression profiling in adrenal tumours focused on the differentiation of benign from malignant tumours.[30,31] Larger, unsupervised transcriptome-based tumour classification studies reported then the existence of distinct groups of ACCs with diverse clinical outcome[32,33] results that were confirmed by several other groups.[6,7,34]

ACTs were subdivided into a group of malignant (C1) and a group of benign (C2) tumours. While in the C1 group genes playing a role in the M phase of the cell cycle and/or in DNA replication were differently expressed, in the C2 group altered expression mostly affected genes involved in inflammatory processes and immune response. The malignant C1 group was further subdivided into a more aggressive C1A group enriched in transcription and mitotic cell cycle genes and a good prognosis C1B group enriched in cell metabolism, intracellular transport, apoptosis and cell differentiation genes.[27,33]

Starting from microarray data, de Reyniès et al.[33] were able to define a two-gene malignancy signature. The combination of budding uninhibited by benzimidazoles 1 homologue beta (*BUB1B*) and PTEN-induced putative kinase 1 (*PINK1*), both involved in the cell cycle regulation, provided the best prediction rule of OS. In the meantime, the correlation of *BUB1B*–*PINK1* expression and survival has been confirmed several times.[8,9,35]

2.3.2 | microRNA expression

The role of microRNAs (miRNA or miR) in human cancers was first discovered in association with B-cell chronic lymphocytic

leukaemia cells.[36] It took seven years until the first data on microRNAs in ACCs were published. Microarray profiling revealed 23 microRNAs differentially expressed between ACAs and ACCs and two microRNAs whose down- (miR195) or upregulation (miR483-5p) was significantly associated with poorer disease-specific survival.[37] The microRNA that was repeatedly correlated with a more aggressive phenotype in subsequent studies was miR-483-5p, which is transcribed from an intronic sequence ofthe *IGF2* gene. Not only microRNAs isolated from tissue—even from FFPE tissue[38]—were investigated but also circulating microRNAs isolated from serum or plasma. Chabre et al.[39] found a positive correlation between circulating miR-483-5p levels and tumour size and an association between high presurgical circulating miR-483-5p levels and worse prognosis. High pre- and post-operative plasma levels from miR-483 and its mature variant miR-483-5p were significantly associated with the ENSAT stage and worst clinical outcome.[40] Furthermore higher miR-483-5p concentrations 3 months after surgery, were linked to a more than fourfold risk of progression and were predictive of poor OS.[41] Further microRNAs—that is, miR-195[39] or miR-503, miR-1202 and miR-1275, miR-195[42]—have been associated with poor survival of ACC patients, but have not been reproduced in other studies (excluding once miR-195). Comprehensive characterisation of ACCs in both the ENSAT cohort and the TCGA cohort revealed three and six stable patterns of miRNAs, respectively, associated with prognosis.[6,7]

2.4 | Intratumour heterogeneity

Intratumor heterogeneity in relation to sequence variants, CNAs and epigenetic modifications is well known from other tumour entities (reviewed in detail by McGranahan and Swanton[43] and Mazor et al.).[44] In ACC only a few and small studies addressed this topic. Vatrano et al.,[45] Gara et al.[46] and Jouinot et al.[47] describe a high degree of genetic heterogeneity in relation to sequence variants in primary tumours versus recurrent and/or metatstatic lesions analysed with targeted or WES approach. These changes, on the one hand, affect well-known ACC driver genes correlated with prognosis and, on the other, potential molecular drug targets. In contrast, epigenetic modifications—that is, DNA methylation alterations—were shown to be rather stable as are chromosome alteration profiles[47] and might therefore be most suitable for prognostic assessment.

2.5 | Implementation of DNA- and RNA-based prognostic markers in clinical practice

Comprehensive pan-genomic studies have shown that molecular markers—each presented separately in the paragraphs before—cluster in certain groups defining patients with good, intermediate and poor prognosis[6,7] (summarized in Table 2 and illustrated in Figure 2). However, the methods used to generate this 'omics' data are still too expensive and analysis workflow too complex to be implemented in a routine workflow.

In fact, the ideal marker to implement in a routine setup should be simple, reliable, standardized and cost-effective to study. It would therefore be a marker that can be analysed using material from FFPE tissue, which is generally of poorer quality than material from fresh-frozen tissue but is routinely available. Using a targeted approach is cheaper and requires less bioinformatics input than genomic sequencing approaches. The studies reported in the previous sections are summarized in Figure 1 and Table 1 to give an overview of markers with regard to applicability in a routine workflow using the following criteria: (1) quality of the material needed for the analysis; (2) workload, costs and complexity of the tests regarding laboratory work and data analysis; (3) reproducibility of the results in independent cohorts. According to those criteria, the best implementable in a clinical routine workflow would be the analysis of DNA sequence variants and DNA epigenetic modifications as they can be conducted with DNA isolated from FFPE tissue and targeted studies have been published for both showing similar results. As a further step towards personalized medicine in ACCs, we and the group of Assié recently proposed the combination of molecular markers—assessed in targeted workflows—with clinical markers to a so-called COMBI-score or 3D targeted or DNA-based targeted classifier, respectively.[8,9] According to these studies, the combination of molecular and clinical and histopathological markers provides the most accurate prognostication for ACC patients.

In any case, before implementing the analysis of molecular markers into a clinical routine workflow, a large, international prospective study is urgently needed.

So far, most markers are tested on DNA or RNA isolated from tissue, which requires biopsy or resection. Besides the fact that these methods are invasive, neither the examination of biopsy material nor a section of the resected tumour can fully capture the mutation spectrum. In contrast, liquid biopsy, that is a blood-based analysis, is a minimally invasive method for examining molecular changes that can be traced back to the tumour, as a predictive and diagnostic tool, as well as for monitoring of disease progress or response to therapy. Therefore, the studies analysing circulating microRNAs (or cell-free DNA) seem to offer a promising, powerful, sensitive and noninvasive approach for individualized care of ACC patients.[39–41]

3 | PREDICTORS AND TARGETS OF THERAPEUTICAL APPROACHES IN ACC

3.1 | Predictive markers of response to current therapies

As described above, mitotane and EDP-M are currently the most recommended systemic therapies for recurrent or advanced ACC[3] despite their low efficacy rates. The relationship between the response to current therapies and diverse molecular alterations has been evaluated in previous studies.

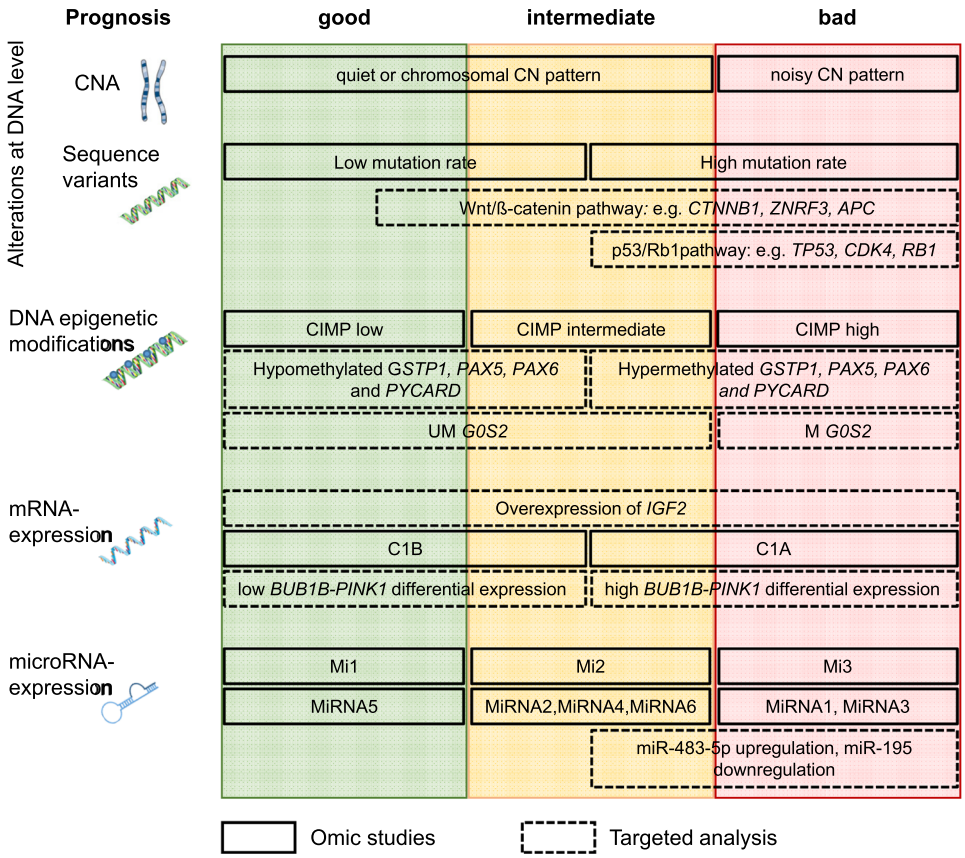

FIGURE 2 Comprehensive and targeted studies reveal that ACCs can be subdivided into subgroups with distinct clinical outcomes according to molecular alterations. ACC, adrenocortical carcinoma; CN, copy number; CNA, copy number alteration; CIMP, CpG island methylation phenotype; M, methylated; Mi, microRNA; miR, microRNA; mRNA, messenger RNA; UM, unmethylated [Color figure can be viewed at wileyonlinelibrary.com]

3.1.1 | Alterations associated with mitotane treatment

Volante et al.[48] analysed the predictive role of the gene expression level of ribonucleotide reductase large subunit 1 (*RRM1*) and excision repair cross complementation group 1 (*ERCC1*) for clinical outcome and response to mitotane treatment in ACC patients was based on their prognostic relevance in other cancer types[49] and on the sequential use of platinum- and gemcitabine-based therapy in ACC.[50] The study revealed that only the gene expression level of *RRM1* is predictive of response to mitotane treatment in an adjuvant setting as low *RRM1* gene expression and adjuvant mitotane treatment was associated with improved DFS. An effect that was not seen in patients with high *RRM1* expression. Together with in vitro experiments, which also displayed that *RRM1* expression is functionally associated with mitotane sensitivity, it was assumed that the determination of *RRM1* expression has potential clinical utility to select patients for adjuvant mitotane therapy.

Other studies focused on factors influencing mitotane plasma level.[51] For instance, at DNA level, it was shown that polymorphisms in genes coding for members of the CYP superfamily may affect the response to mitotane plasma levels in ACC patients. D'Avolio et al.[52] demonstrated that the presence of *CYP2B6* single-nucleotide polymorphism (SNP) enabled prediction of reaching therapeutic mitotane plasma levels during adjuvant mitotane treatment. In fact, a multivariate logistic regression analysis showed that the *CYP2B6* rs3745274GT/TT genotype was a predictor of mitotane concentrations of at least 14 μg/ml after 3 months of treatment. Moreover, our group recently coordinated a multicentric study on behalf of ENSAT on the relationship between the presence of *CYP2W1* and *CYP2B6* SNPs and both plasma mitotane levels and response to treatment in a large cohort of 182 patients with ACC.[53] Of note, we could demonstrate that the presence of *CYP2W1**6 SNP (rs3808348) was associated with a reduced probability to reach mitotane therapeutic range and lower response rates, whereas *CYP2B6**6 (rs3745274) correlated with higher mitotane levels. Moreover, a higher rate of patients with the profile *CYP2W1**6WT + CYP2B6*6 (60.6%) achieved mitotane therapeutic range, suggesting that this combination may predict the individual response to mitotane in patients with advanced ACC. However, these findings need to be validated in a prospective study before being implemented in clinical practice.

3.1.2 | No reliable predictors of cytotoxic therapies in ACC

Few molecular predictors of response to cytotoxic chemotherapies have been proposed in ACC. For instance, ERCC1 protein expression was suggested as a predictor of response for platin-based therapy,[54] but subsequent studies could not confirm these results.[55] In addition, protein expression of human equilibrative nucleoside transporter type 1 (hENT1) and RRM1 was presumably associated with resistance to gemcitabine, but in a large series, we could not establish this association.[56]

Moreover, at the moment, there are no promising DNA- or RNA-related biomarkers associated with the response to standard chemotherapies in ACC.

3.2 | New therapeutic approaches in ACCs

There is an urgent need for alternative therapies for aggressive ACCs when standard treatments fail. Novel therapies are on one hand based on the idea to attack cancer cells with the help of the patient's own immune system—immunotherapies—and on the other hand to inhibit a specific molecular pathway deregulated in a specific disease leaving other cells unharmed—targeted therapy. In other tumour entities, new effective therapeutic strategies were identified by molecular studies. Regarding ACC, while the efficacy of a few therapies has been tested in clinical trials, other drug targets have only been studied in vitro or in *animal models*. Overall, no breakthrough by any new therapy has been identified until now.

3.2.1 | Potential predictors for immunotherapy in ACC

Immunotherapy has revolutionized the therapeutical concepts in many cancers. However, the results of initial studies of different immune checkpoint inhibitors in ACC were heterogeneous. Up to now, four small phase 2 trials with a total of 115 patients have been published and demonstrated an objective response in only 15 patients[57–60] (for details see review).[12] Therefore, predictors of response would be highly desirable. TMB, microsatellite instability, tumour infiltrating lymphocytes or expression of PD1/PD-L1 have been established as predictive biomarkers in some, but not all tumour entities. Unfortunately, none of these markers could be proven to be helpful in ACC (most likely due to the limited samples size of the studies so far).

3.2.2 | Potential molecular drug targets

Clinical studies

As already mentioned, few clinical trials from Phase 1 to 3 have been conducted in patients with advanced ACC in recent years, targeting pathways known to be deregulated. Inhibitors of receptor tyrosine kinases or mammalian target of rapamycin pathway were tested so far and have been recently reviewed by Altieri et al.[12] Most promising but also most disappointing was the use of therapeutics targeting the IGF pathway, which is deregulated in over 90% of ACCs. Encouraging in vitro and in vivo studies as well as early-phase clinical trials were the rationale for testing linsitinib, a dual inhibitor of IGF receptor 1 and insulin receptor, in a randomized placebo-controlled phase III trial.[61] Overall, no effect of linsitinib on PFS and OS could be demonstrated; however, in the intervention arm, four patients experienced objective response or stable disease for more than 12 months (including one patient with a still ongoing complete response). However, these responses could not be associated with a specific molecular profile. Similar results with small proportions of ACC patients showing a response to certain targeted therapy have been also seen in other studies (summarized by Altieri et al.).[12] Of note, these findings might suggest that subgroups of patients might benefit from a specific treatment due to their tumour molecular pattern and should be verified in further studies.

Preclinical studies

As diverse as the changes that can be found in ACCs at different levels, so are the therapeutic approaches that are investigated in preclinical studies. Based on molecular data, a first study investigating the presence of potentially targetable genetic events in 40 patients with advanced ACC stages was published in 2013 by De Martino et al.[62] They used targeted sequencing and CGH array analysis and identified 40% of ACC tumours with alterations in the G1 cell cycle progression pathway and therefore proposed drugs targeting the cell cycle as the most relevant potential new therapeutic strategy for patients with advanced ACC. From genes involved in cell cycle regulation, we recently identified *CDK4* as the most promising drug target in our cohort with 43% of the tumours having a *CDK4* copy number gain.[8] We and others demonstrated already in in vitro studies the effect of CDK inhibitors in different ACC cell lines using, for example, the CDK4/6 inhibitors palbociclib and ribociclib.[63–65] Palbociclib was particularly effective when tested in combination with dual IGF1R/IR inhibitor linsitinib.[65]

Preclinical data from Nilubol et al.[66] suggest the evaluation of the combination therapy with flavopiridol and carfilzomib, a CDK-inhibitor and a proteasome inhibitor, respectively. These compounds were selected based on results from quantitative high-throughput screening and resulted in an antiproliferative effect and an increase in cell death in vitro and in inhibited tumour growth in mice with the human ACC xenograft model.

An obviously interesting target in ACC is the Wnt/ß-catenin signalling pathway. As described above, there are alterations in different genes of the pathway leading to activation (e.g., *ZNRF3* homozygous deletion or loss-of-function mutations and constitutive activating *CTNNB1* mutations in Exon 3). In vitro experiments using the NCI-H295R cell line, which harbours a *CTNNB1* p.Ser45Pro mutation in Exon 3, showed inhibited proliferation or increased

apoptosis, decreased cell viability and impairment of adrenal steroidogenesis by the use of PKF115–584[67] or PNU-74654,[68] respectively, both acting as antagonists of the formation of T-cell factor/ß-catenin complex. However, as ß-catenin is a critical regulator of development and homoeostasis of numerous tissues, many inhibitors of ß-catenin-dependent transcription cause on-target toxicity in Wnt-dependent tissues.[11] Of note, so far, there are no approved anticancer drugs targeting CTNNB1 or ZNRF3 genetic alterations.

Antiproliferative effects in ACC cell lines are also described for demethylating agents, but those are rarely discussed as potential targeted therapies. Indeed, the demethylating agents 5-azacitidine (5-Aza-CR) and 5-aza-2′-deoxycytidine (5-Aza-CdR = decitabine) are FDA-approved for the treatment of myelodysplastic syndromes and chronic myelomonocytic leukaemia.[69,70] The effect of decitabine on the proliferation of NCI-H295R cells had already been studied.[71–73] Further studies showed an increased expression of hypermethylated genes after treatment with demethylating agents.[74] However, further research is required to determine the role of epigenetically targeted drugs in the treatment of ACC. Although in other types of cancer epigenetic therapies are an emerging option for overcoming drug resistance, it still needs to be investigated in ACC.[75]

Finally, also microRNAs could be used for targeted therapies. As they can function as oncomiRs, which are generally overexpressed in tumours, or as oncocosuppressor miRs, whose expression is downregulated, there are different strategies for therapeutic applications: first, through antisense-mediated inhibition of overexpressed miRNAs; second, through replacement of under-expressed miRNAs with either miRNA mimetics or viral vector-encoded miRNAs; and third, by modulating miRNA expression to augment a patient's response to existing treatment modalities.[76] So far, only Özata et al.[42] tested the effect of altered microRNA expression in cell culture. They used NCI-H295R cells to inhibit miR-483-3p or miR-483-5p, known to be overexpressed in ACCs, and to overexpress miR-195 or miR-497, known to be downregulated in ACCs, and saw reduced cell proliferation.

4 | CONCLUSION

ACC is a rare, aggressive cancer with still partially unknown pathogenesis, heterogeneous clinical behaviour and no effective treatment for advanced stages. An individualized management approach could be therefore extremely relevant for these patients.

Tumour molecular profiling was important to better elucidate pathogenic pathways and identify some prognostic features. However, so far there is still no role of molecular analysis in clinical routine care of ACC. Nevertheless, we are convinced that there are methods that hold the potential to be implemented in a clinical routine workflow in the near future. For instance, we expect that easily available clinical and histopathological characteristics combined with molecular profiles obtained from FFPE tumour material will guide clinicians (and patients) for treatment decisions in the near future.[8,9,77] Such 'scores' will be used to identify patients at high or low risk of disease recurrence or progress and therefore help to judge for or against adjuvant therapy.

Moreover, liquid biopsy-related approaches might represent promising tools in the field of prognostication and surveillance for ACC patients.

While it is relatively straightforward to study predictive markers forecasting the response to standard therapies, especially when they are SNPs that can be studied with DNA isolated from whole blood (evaluation implemented in Figure 1 and Table 1), it is proving very difficult to find new therapies for ACC patients. Most studies ended disappointingly for the majority of the patients. Hence, there is still a need for further preclinical studies to identify better potential drug targets and large clinical trials to test the efficacy of available/proposed targeted therapies in relation to the molecular profile (e.g., with newly available ACC cell lines[78–80]). On the other hand, in several countries, tumour sequencing programs, especially for rare diseases, have been implemented and might lead to new insights on 'druggable targets' in single patients.

Finally, the last 10–20 years have seen several international and interdisciplinary networks (e.g., ENSAT, A5 etc.) and we are optimistic that these collaborative efforts will finally facilitate rational treatment progress even for ACC.

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CONFLICT OF INTERESTS

The author declares that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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