Future directions in the diagnosis and medical treatment of adrenocortical carcinoma

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Abstract

Adrenocortical carcinoma (ACC) is a rare disease with a poor prognosis. Discrimination between ACCs and adrenocortical adenomas (ACAs) remains challenging, with the current gold standard being the Weiss score, consisting of several histopathological characteristics. However, new markers like Ki67, a marker for proliferation, and the staining of reticulins are promising not only as it comes to identifying malignancy but also as prognostic markers in patients with ACC. Currently, surgery is still the only curative treatment for ACC. Mitotane, an adrenolytic drug, is used in the adjuvant setting and in case of metastatic or advanced disease. Patients with progressive disease are frequently treated with mitotane, alone or in combination with etoposide, doxorubicine and cisplatin. Radiotherapy is indicated in selected cases. The low response rates and high toxicity of the systemic therapies emphasize the need for markers that enable the identification of responders and non-responders. Consequently, research is focusing on predictive factors varying from the expression of DNA repair genes to clinical patient characteristics. Subgroups of ACC with different prognosis have been identified based on transcriptome characteristics. As a conclusion from large molecular studies, ACCs appear to harbor many abnormalities compared to ACAs. Altered pathways driving ACC pathogenesis include the IGF, TP53 and the Wnt signaling pathway, allowing these as new potential targets for medical therapy. However, despite efforts in preclinical and clinical studies investigating efficacy of targeting these pathways, most novel therapies appear to be effective in only a subset of patients with ACC. New treatment concepts are therefore urgently needed.

Key Words

► adrenocortical cancer
► diagnosis
► treatment
► prognostic markers

Introduction

Adrenocortical carcinoma (ACC) is an aggressive but rare malignancy with an incidence of 0.5 to 2 cases per million per year (Kebebew et al. 2006, Golden et al. 2009, Fassnacht et al. 2013, Kerkhofs et al. 2013b). Five-year survival rates vary from 16 to 40% and are largely dependent on the ACC stage at diagnosis (Fassnacht et al. 2009, 2010). Most ACCs occur sporadically, but ACCs can also be associated with various genetic syndromes, e.g. Li Fraumeni syndrome.
(Kleihues et al. 1997, Birch et al. 2001, Gonzalez et al. 2009), Beckwith–Wiedemann syndrome (BWS) (Wiedemann 1983, Steenman et al. 2000, Lapunzina 2005), multiple endocrine neoplasia type 1 (MEN1) (Waldmann et al. 2007, Gatta-Cherifi et al. 2012) and Lynch syndrome (Medina-Aranal et al. 2011, Karamurzin et al. 2012, Raymond et al. 2013). To a lesser extent, ACC can be associated with familial adenomatous polyposis (Gaujoux et al. 2010), neurofibromatosis type 1 (Wagner et al. 2005) and Werner syndrome (Takazawa et al. 2004). Despite much effort to improve care for patients with ACC, diagnosis and treatment still have limited opportunities. A better understanding of the pathogenesis and the identification of potential new therapeutic targets could lead to a more personalized approach in patients with ACC. Furthermore, it should be emphasized that ACC patients should only be referred to specialized centers that have extensive experience in the management of this rare cancer (Lacroix 2010). In this review, we provide an overview of the current diagnostic opportunities and challenges in ACC, and focus on the therapeutic strategies and targets for therapy. We describe the current standard care as well as perspectives for future directions based on findings from basic science and clinical research.

**Diagnosis of ACCs**

**Current tools to diagnose ACCs**

**Imaging** A thorough preoperative diagnostic work up is essential in patients with an (incidentally discovered) adrenal mass to differentiate between ACC and adrenocortical adenoma (ACA) (Lacroix 2010). Initial assessment of malignancy risk is predominantly performed by the evaluation of radiological characteristics on (contrast-enhanced) CT or MRI (Nieman 2010). Most patients with ACC present with large tumors, measuring more than 6 cm in diameter, but with local disease (Schulick & Brennan 1999, Icard et al. 2001, Boland et al. 2008, Johnson et al. 2009).

Other CT characteristics that (to a certain extent) discriminate between ACCs and ACAs include lack of a well-defined margin, increased heterogeneity, central low attenuation, calcifications, and extension into the inferior vena cava (Nieman 2010, Zhang et al. 2012). On contrast enhanced CT a high contrast washout and O10 Hounsfield Units (HU) are characteristic for malignancy. Size is thought to be the most important predictor for malignancy, with an increase from 52 to 80% specificity for malignancy for tumors larger than 4–6 cm respectively (Sturgeon et al. 2006). In the largest series on adrenal imaging so far, Petersenn et al. (2015) suggest that a threshold of 13 HU instead of 10 HU should be used to more adequately diagnose ACC. If the characteristics on unenhanced CT followed by contrast-enhanced examinations do not show a classic ACC appearance, MRI can provide additional information regarding the diagnosis (Ilias et al. 2007). Although these findings together will not always indicate a clear diagnosis, the previously mentioned characteristics on a CT scan are currently used to guide the decision on adrenalectomy. Adrenalectomy is generally performed in case of lesions larger than 4 cm (Petersenn et al. 2015).

In 2011, a systematic review included 21 studies which investigated the value of 18F-fluorodeoxyglucose positron emission tomography (18F-FDG PET) to differentiate benign from malignant adrenal tumors (Boland et al. 2011). In 1217 patients, a mean sensitivity of 97% and a specificity of 91% was found. No differences were found between 18F-FDG PET and 18F-FDG PET/CT. After this systematic review, several other studies were performed confirming the high sensitivity and negative predictive value for diagnosing ACCs. Also, it is reported that 18F-FDG PET and CT can be complementary as it comes to initial diagnosis of ACC and recurrence detection (Leboulleux et al. 2006, Nunes et al. 2010, Gust et al. 2012).

Important considerations that should be taken into account with the 18F-FDG PET/CT scans are the increased uptake seen in case of an adrenal metastasis or in several benign conditions. Furthermore, 18F-FDG PET/CT is considered less sensitive and specific for characterizing smaller lesions (<1 cm) and 18F-FDG uptake can also be increased in the contralateral adrenal after adrenalectomy following mitotane treatment (Leboulleux et al. 2011). Recently, a retrospective study (n = 106) showed that only for a minority (~5%) of patients undergoing 18F-FDG PET/CT, the scan would have changed the clinical management at initial staging (Takeuchi et al. 2014). In case of chemotherapy, PET/CT could predict response earlier than the detection of anatomic changes on CT (Takeuchi et al. 2014). Up to this moment, there are equivocal findings as it comes to 18F-FDG PET/CT measurements as a prognostic marker, probably because of the low number of patients included in the studies.

**Staging** The ENSAT-staging, a reclassification of the Union for International Cancer Control staging system, is the system currently used for staging of adrenal tumors (Table 1; Fassnacht et al. 2009, Lughezzani et al. 2010). The staging is based on the evaluation of a total of 1065 patients with ACC. Recently, Asare et al. (2014) reported
Biochemistry  Patients with ACC often present with symptoms due to hormonal overproduction (40–60% of cases, of which 50–80% are due to hypercortisolism). Patients without hormone overproduction present with nonspecific symptoms due to local tumor growth or spread of tumor to surrounding or distant tissues (Allolio & Fassnacht 2006, Fassnacht & Alloio 2009). Biochemical evaluation, which is in part guided by hormone-related clinical symptoms of patients, is performed by measurement of steroid hormones potentially produced by the tumor. For several reasons it is important to perform biochemical evaluation prior to surgery (Nieman 2010): i) it can further add to judge the risk of malignancy, since this risk increases in case of androgen or estrogen production; ii) in case of glucocorticoid excess cortisol lowering- or antagonizing therapy can be indicated; iii) patients with cortisol producing ACCs need hydrocortisone replacement post-surgery; iv) hormonal parameters can be used as tumor markers; v) pre-surgical testing for pheochromocytoma-related hormones can avoid complications during surgery (Song et al. 2011).

Pathology  The Weiss score (WS) is currently the most widely used classification system for the pathological assessment of adrenocortical tumors (Weiss 1984, Lau & Weiss 2009). It consists of nine morphological parameters and since 1989 a threshold for malignancy of at least three criteria present in the tumor (Weiss et al. 1989). Different more simplified algorithms have been proposed with only the most reliable parameters included (Aubert et al. 2002). Pennanen et al. (2015) recently developed the Helsinki score, which consists of the sum of 3 × mitotic rate + 5 × presence of necrosis + maximum proliferation index. This scoring system was able to diagnose metastatic ACC with 100% sensitivity and 99.4% specificity, whereas the revised WS of Aubert et al. had a sensitivity of 100% and specificity of 96.9%. The WS lacks reproducibility and is difficult to apply in ACC variants and pediatric adrenocortical tumors. The reliability of the WS is challenged in borderline cases, where a WS of 2 can be suggestive for an ACC (Tissier et al. 2012, Papotti et al. 2014). To prevent overdiagnosis in oncocytic variants with the classic WS, an alternative diagnostic system was proposed (Bisceglia et al. 2004) and also validated to correctly predict malignancy in this ACC variant (Wong et al. 2011). ACCs can also be classified as myxoid, sarcomatoid or mixed variants. Because of the remaining difficulties with the WS and the Lin–Weiss–Bisceglia system, and because only a definite diagnosis can be made in the presence of metastasis, pathologists have put effort in developing new techniques to refine the diagnostic assessment of adrenal tumors.

Ki67, a marker for proliferation, has raised attention for its use in the differential diagnosis of adrenal tumors (Table 2). The monoclonal antibody MIB1, which reacts with Ki67, is used for immunohistochemistry (Cattoretti et al. 1992). Ki67 evaluation seems to be reproducible, with intra- and inter-observer differences of 3.7 and 4.2% respectively (Morimoto et al. 2008). The general agreement is that ACCs have a Ki67 labeling index of ≥ 5%. In a large study (n = 319, validation cohort n = 250; all patients after complete resection of the tumor) evaluating the prognostic value of histopathological, clinical and immunohistochemical markers, Ki67 alone most powerfully predicted recurrence-free and overall survival (Fig. 1, Beuschlein et al. 2015). In addition, the authors recommend that based on their results Ki67 should be introduced in the routine pathology for adrenocortical tumors.

Volante et al. (2009) demonstrated that disruption of reticular networks, defined as the loss of continuity of reticular fibres or basal membrane network as highlighted by histochemical staining, was present in all ACCs included in their study (n = 92; Table 2). By adding at least one of the following three parameters – necrosis, high mitotic rate or vascular invasion – this reticulin algorithm identified malignancy with a sensitivity and specificity of 100% (Volante et al. 2009). A study aiming to validate especially the first part of the algorithm, the presence of reticulin fibre disruptive changes, in 178 adrenocortical tumors, showed that a specific training increased the interobserver reproducibility to 86% (Duregon et al. 2013a). Specifically for cortical tumor variants like oncocytic and myxoid subtypes this algorithm might be
Future directions in diagnosing ACC

Because both imaging and histopathological criteria cannot completely predict biological behavior of adrenal tumors, research now focuses on new imaging techniques and genomic or molecular markers for discrimination between ACCs and ACAs.

Imaging  Several studies have focused on the diagnostic value of positron emission tomography (PET) using $^{11}$C-labeled metomidate (MTO) for lesions in the adrenal cortex. Metomidate binds with high specificity and affinity to CYP11B enzymes, which are expressed in the adrenal cortex. Two studies compared MTO-PET with FDG PET in adrenocortical tumors (Minn et al. 2004, Zettinig et al. 2004). In a total of 37 patients, MTO-PET appeared to only differentiate lesions of adrenal from those of non-adrenal origin, while FDG PET was able to identify malignancy of the adrenal tumor. Another study that investigated the correlation between MTO-PET scan results, histopathology, and hormonal secretion of the adrenals, found that MTO-PET could diagnose adrenocortical origin of the lesion with a sensitivity of 89% and specificity of 96% ($n = 75$) (Hennings et al. 2006).

Table 2  Overview of the diagnostic value of the IGF2 gene, the proliferation marker Ki67, and the staining of reticulins to discriminate ACCs from ACAs. Only studies which analyzed the discriminative value of the molecular markers were included in this overview. Total sensitivity and specificity represents a weighted mean.

<table>
<thead>
<tr>
<th>Study</th>
<th>ACC (n)</th>
<th>ACA (n)</th>
<th>Sens (%)</th>
<th>Spec (%)</th>
<th>Cutoff</th>
<th>Reference diagnosis</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IGF2</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Gicquel et al. (1994)</td>
<td>6</td>
<td>17</td>
<td>83</td>
<td>88</td>
<td>$IGF2$ mRNA $&gt; 100$ times that in normal adrenals</td>
<td>Clinical data, CT and pathology</td>
<td></td>
</tr>
<tr>
<td>Gicquel et al. (1997)</td>
<td>18</td>
<td>35</td>
<td>61</td>
<td>91</td>
<td>Presence of 11p13-15 LOH</td>
<td>Histological features</td>
<td></td>
</tr>
<tr>
<td>Gicquel et al. (1997)</td>
<td>29</td>
<td>35</td>
<td>86</td>
<td>100</td>
<td>$IGF2$ mRNA $&gt; 10$-582 times that in normal adrenals</td>
<td>Histological features</td>
<td></td>
</tr>
<tr>
<td>Erickson et al. (2001)</td>
<td>67</td>
<td>64</td>
<td>93</td>
<td>45</td>
<td>Positive IGF2 IHC</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>Schmitt et al. (2006)</td>
<td>22</td>
<td>22</td>
<td>76</td>
<td>100</td>
<td>Positive IGF2 IHC</td>
<td>WS, Hough and van Slooten</td>
<td></td>
</tr>
<tr>
<td>Soon et al. (2009a)</td>
<td>25</td>
<td>25</td>
<td>64</td>
<td>72</td>
<td>Positive IGF2 IHC</td>
<td>WS</td>
<td></td>
</tr>
<tr>
<td>Wang et al. (2014)</td>
<td>25</td>
<td>25</td>
<td>64</td>
<td>72</td>
<td>Positive IGF2 IHC</td>
<td>WS and clinical and biochemical data</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>185</td>
<td>239</td>
<td>81</td>
<td>80</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ki67</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Iino et al. (1997)</td>
<td>17</td>
<td>28</td>
<td>65</td>
<td>100</td>
<td>$LI &gt; 2.5$</td>
<td>NR</td>
<td></td>
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<tr>
<td>Vargas et al. (1997)</td>
<td>20</td>
<td>20</td>
<td>95</td>
<td>100</td>
<td>$TPF &gt; 80$</td>
<td>WS</td>
<td></td>
</tr>
<tr>
<td>Wachenfeld et al. (2001)</td>
<td>8</td>
<td>26</td>
<td>75</td>
<td>81</td>
<td>$LI &gt; 5$</td>
<td>WS and clinical data</td>
<td></td>
</tr>
<tr>
<td>Terzolo et al. (2001)</td>
<td>11</td>
<td>26</td>
<td>100</td>
<td>100</td>
<td>$TPF &gt; 70–90$</td>
<td>WS</td>
<td></td>
</tr>
<tr>
<td>Schmitt et al. (2006)</td>
<td>16</td>
<td>22</td>
<td>88</td>
<td>95</td>
<td>$LI &gt; 5$</td>
<td>WS, Hough and van Slooten</td>
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<td>Soon et al. (2009a)</td>
<td>25</td>
<td>25</td>
<td>64</td>
<td>96</td>
<td>$LI &gt; 5$</td>
<td>WS</td>
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<td>Wang et al. (2014)</td>
<td>25</td>
<td>25</td>
<td>64</td>
<td>96</td>
<td>$LI &gt; 5$</td>
<td>WS and clinical and biochemical data</td>
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<tr>
<td><strong>Total</strong></td>
<td>120</td>
<td>188</td>
<td>78</td>
<td>96</td>
<td></td>
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<tr>
<td><strong>Reticulin staining</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Volante et al. (2009)</td>
<td>92</td>
<td>47</td>
<td>100</td>
<td>100</td>
<td>$RA$</td>
<td>WS</td>
<td>Lin–Weiss–Bisceglia system only OACTs included</td>
</tr>
<tr>
<td>Duregon et al. (2011)</td>
<td>6</td>
<td>1</td>
<td>83</td>
<td>100</td>
<td>$RA$</td>
<td>WS</td>
<td></td>
</tr>
<tr>
<td>Duregon et al. (2013a)</td>
<td>184</td>
<td>61</td>
<td>97</td>
<td>100</td>
<td>$RA$</td>
<td>WS</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>282</td>
<td>109</td>
<td>98</td>
<td>100</td>
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</tr>
</tbody>
</table>

Sens, sensitivity; spec, specificity; NR, not reported; LOH, loss of heterozygosity; IHC, immunohistochemistry; LI, labeling index, defined as the number of Ki67/MIB1-positive cells per 100 tumor cells; TPF, tumor proliferating fraction, expressed as the number of Ki67/MIB1-positive nuclei per 1000 tumor cells; RA, reticulin algorithm, defined as the presence of disruption of reticular networks with at least one of the following parameters – necrosis, high mitotic rate or vascular invasion; OACT, oncocytic adrenocortical tumors.
Molecular markers

Differential gene expression Several studies have shown that ACCs and ACAs have different gene expression profiles, which can be used to discriminate the two entities. IGF2 is the most widely known overexpressed gene in ACCs. Besides the microarray studies, several studies have shown overexpression of IGF2 with qPCR and immunohistochemistry. However, IGF2 alone appears not to be sufficient to accurately discriminate ACCs from ACAs (Table 2). By comparing microarray data of 33 ACAs and 24 ACCs, de Fraipont et al. (2005) identified two clusters of genes whose combined levels of expression could correctly discriminate ACCs from ACAs. Overall, 75% of ACCs expressed high levels of the IGF2 cluster, containing eight genes, whereas 93% of ACAs highly expressed fourteen genes representing the steroidogenesis cluster. After this finding, several other studies also reported differential expression levels in ACCs compared to ACAs, as well as a more heterogeneous transcriptional profile in ACCs versus ACAs (Giordano et al. 2003, 2009, Velazquez-Fernandez

[123]IMTO in adrenocortical tissue (Hahner et al. 2008). IMTO binds to both 11β-hydroxylase and aldosterone synthase and is able to identify the adrenocortical origin of the lesion, but not malignancy of the lesion. IMTO does show uptake in metastasis of ACC (Hahner et al. 2013, Kreissl et al. 2013). In two studies, sensitivity and specificity for characterization of adrenal lesions was 89% and 85%, and 38% and 100% respectively (Hahner et al. 2013, Kreissl et al. 2013).

Early results suggest that proton MR spectroscopy may be useful in discriminating pheochromocytomas and adrenal adenomas from adrenal metastases and ACC. Faria et al. (2007) found that a choline:creatine ratio greater than 1.20 yielded a sensitivity of 92% and a specificity of 96%. Furthermore, choline:lipid ratios greater than 0.38 could differentiate adenomas and pheochromocytomas from carcinomas and metastases with a sensitivity of 92% and a specificity of 90% (Faria et al. 2007). Further studies are needed to validate this approach.
et al. 2005, Slater et al. 2006). Soon et al. (2009a) more specifically selected two factors, IGF2 and Ki-67, which in combination resulted in a high diagnostic accuracy for ACCs (96% sensitivity, 100% specificity). Several other factors were also differentially expressed in ACCs compared to ACAs, like MAD2L1, CCNB1, ABLIM1, NAV3, SEPT4, and RPRM (Soon et al. 2009a). Another microarray study showed 614 significant differentially expressed genes (Tombol et al. 2009), of which several were previously described to be similarly differentially expressed between ACCs and ACAs (Giordano et al. 2009, Soon et al. 2009a). The most differentially expressed genes in this series were TOP2A and IGF2, CCNB2, CDC2, CDC25C, and CDKN1C (Tombol et al. 2009). In another series by Laurell et al. (2009) comparing 11 ACCs and 17 ACAs, ALDH1A1, IGF2, USP4 and UFD1L were the four most differentially expressed genes. The gene expression profiles were subjected to hierarchical clustering, resulting in two subclusters of patients with short survival (<9 months) and long survival (>67 months), suggesting that gene expression profiles can be used to predict survival (Laurell et al. 2009). Another gene of interest in adrenal tumors, the steroidogenic factor 1 (SF1) gene, has been shown to have a role in adrenocortical cell proliferation (Doghman et al. 2007). It also appeared to identify the adrenocortical derivation of the tumor with high diagnostic accuracy and also has a high prognostic value (Sbiera et al. 2010, Sangoi et al. 2011, Duregon et al. 2013b). Besides the fact that overexpression of SF1 is associated with a poor prognosis, its oncogenic effect is also emphasized by its chromosomal location (9q34), which is frequently gained in ACC (see the ‘Chromosomal aberrations’ section).

These findings together highlight that expression profiles provide more insights into the pathogenesis of ACCs and the main pathways involved (Fig. 2). However, the interpretation of these findings is still difficult, since there are considerable differences between the different studies. Whether this is due to the heterogeneity of the series of patients studied, the different analyses methods or both, remains unclear.

**Methylation** The rationale that aberrant methylation patterns in tumor cells can cause altered gene expression

**Figure 2**
Most frequently altered pathways in adrenocortical carcinomas (ACC) compared to adrenocortical adenomas (ACA), with molecular aberrations involving the cell cycle-, the IGF/mTOR-, and the Wnt/β-catenin pathway.
resulting in tumorigenesis is now another focus in ACC research (Das & Singal 2004). To date, research has focused on candidate gene approaches as well as genome-wide methylation level analysis. Insights and interest in the imprinted IGF2 gene comes from an association of ACC with the BWS (Wiedemann 1983). In these patients, genes regulated by the 11p15 chromosomal region – IGF2, H19, and CDKN1C – show altered expression (Demars et al. 2011). In sporadic ACC, DNA methylation of the H19 promoter has been shown to be correlated with H19 and IGF2 expression (Fig. 2; Gao et al. 2002). TPS3 methylation, in contrast to some other types of cancer, is not present in ACC as a mechanism of tumor suppressor gene inactivation (Sidhu et al. 2005). A genome-wide approach to study methylation status was first performed by Rechache et al. (2012). Global hypomethylation was found in primary (n=8) and metastatic (n=12) ACC samples compared to normal adrenals (n=19) and ACAs (n=48). Fifty-two genes were down-regulated and hypermethylated in primary adrenocortical tumor samples, suggesting methylation as a potential regulator of expression in ACC (Rechache et al. 2012). Fonseca et al. (2012) analyzed 27 578 CpG sites in 6 normal adrenals, 27 ACAs and 15 ACCs. Two hundred and twelve CpG islands in promoter regions of genes involved in cell cycle regulation, apoptosis, and transcriptional regulation, were significantly hypermethylated in ACCs compared to ACAs and normal adrenal tissues. Of six selected genes, mRNA expression levels were concordantly significantly reduced in ACCs compared to ACAs and normal adrenal tissue (Fonseca et al. 2012). Along with this finding, Barreau et al. (2013) also confirmed ACC-specific hypermethylation in promoter regions in a series of 51 ACCs and 84 ACAs. In addition, Barreau et al. (2013) also correlated the methylation levels with prognostic features in patients with ACC (see the ‘Prognostic and predictive markers’ section).

In conclusion, DNA methylation patterns appear to identify subgroups of adrenal tumors with benign or malignant behavior. The main challenge is to use these global methylation studies not only for a better understanding of ACC pathogenesis, but also to identify specific abnormalities that can be informative for the individual patient.

miRNAs Several studies have focused on the relevance of microRNAs (miRNAs), short noncoding sequences regulating gene expression post-transcriptionally (Malumbres 2013), in the pathogenesis and diagnosis of adrenocortical tumors. miR-483-5p and miR-483-3p are the most consequentially overexpressed miRNAs in ACCs compared to ACAs, whereas miR-195 is often found to be underexpressed (Soon et al. 2009b, Patterson et al. 2011, Ozata et al. 2011, Chabre et al. 2013). The hypothesized mechanism of pathogenesis of these specific miRNAs in ACC are mainly based on in vitro results and studies in other types of tumors (Igaz et al. 2015). Overexpression of miR-483-5p, miR-503, miR-1202, and miR-1275, and underexpression of miR-195 were associated with poor survival in ACC (Soon et al. 2009b, Ozata et al. 2011). Different combinations of several miRNAs (miR-483-5p, miR-195, miR-503, miR-511, miR-335, miR-675, miR-139-3p) could identify malignancy of adrenal tumors (Soon et al. 2009b, Tombol et al. 2009, Patterson et al. 2011, Schmitz et al. 2011). Other studies, such as Caramuta et al. (2013), have shown overexpression of miRNA-processing enzymes, i.e. DICER, TARBP2 and DROSHA, at protein level, of which TARBP2 also strongly discriminated carcinomas from adenomas (Caramuta et al. 2013). To date, three studies expanded on using serum miRNAs, of which miR-483 harbors the highest potential for use as a noninvasive biomarker (Chabre et al. 2013, Patel et al. 2013, Szabo et al. 2014). Although it would be very valuable to attain a noninvasive biomarker for the follow-up of patients with ACC, these findings still have to be validated.

Genetics

Chromosomal aberrations Comparative genomic hybridization (CGH) studies can identify structural chromosomal alterations within ACCs. Studies have shown that ACCs harbor mainly monoclonal cells, whereas benign tumors can be monoclonal as well as polyclonal (Beuschlein et al. 1994, Gicquel et al. 1994). This suggests the presence of a genetic alteration resulting in a growth advantage in ACCs. Studies of adrenocortical tumors have shown a complex pattern of chromosomal alterations in ACCs, while ACAs present few regions of chromosomal gains and losses (Kjellman et al. 1996, Zhao et al. 1999, Dohna et al. 2000, Sidhu et al. 2002, Gruschwitz et al. 2010, Barreau et al. 2012). It is thought that oncogenes and tumor suppressor genes are located in regions of gains and losses respectively. CGH studies have identified frequent allelic losses in ACCs in the TPS3 region 17p13 (85%), the MEN1 locus 11q13 (92%), and the Carney Complex region 2p16 (90%) (Kjellman et al. 1996, Gicquel et al. 2001). Some studies support the concept of a progression model, whereas genetic aberrations were correlated with tumor size (Kjellman et al. 1996, Zhao et al. 1999, 2002,
Sidhu et al. (2002). Sidhu et al. (2002) showed that ACCs ($n=13$) harbored the most frequent gains on chromosome 5, 12, 19 and 4. Losses were most commonly seen on chromosome 1p, 17p, 22, 2q and 11q. A cut-off of 4 or more CGH alterations in one tumor was strongly suggestive for malignancy of the adrenocortical tumor (Sidhu et al. 2002). Stephan et al. (2008) reported that some of the alterations found (amplifications in 6q, 7q and 12q, and losses in chromosomes 3, 8, 10p, 16q, and 19q) were associated with decreased overall survival. Barreau et al. (2012) found frequent gains of the 9q34 region in adenomas, which includes the steroidogenic factor 1 (SF1) gene. Gain of region 9q34 is also frequently found in pediatric ACC (Figueiredo et al. 1999, James et al. 1999, Pianovski et al. 2006), in which it has also been suggested to be involved in tumorigenesis based on mRNA over-expression and strong SF1 staining (Figueiredo et al. 2000, Almeida et al. 2010). Barreau et al. (2012), who used a higher-resolution CGH array, also developed a diagnostic tool to identify malignancy of adrenal tumors with a sensitivity of 100% and a specificity of 83% by combining DNA copy number estimates at six loci (5q, 7p, 11p, 13q, 22q, and 17p). This tool was validated in an independent cohort of 79 tumors. Cluster analysis based on gains and losses in DNA could also identify two groups of ACC with different survival rates (Barreau et al. 2012). Partly in concordance, in a study by Ronchi et al. (2013) chromosomes 1, 5, 7, and 12 were selected to separate ACCs ($n=22$) from ACAs ($n=24$), which appeared more evident when considering only chromosome 5. More recently, frequent recurrent copy number variations were identified at 5p15 and deletions at 22q12.1 (Juhlin et al. 2015). Regions contain TERT, encoding telomerase reverse transcriptase, and the ZNRF3 gene, which is recently reported to act as a tumor suppressor gene respectively (Hao et al. 2012).

These studies together show the diversity and heterogeneity of chromosomal gains and losses in ACC (Fig. 2). It is thus not surprising that so far no specific pattern among different tumors has been characterized. The utility of chromosomal aberrations in diagnosing malignancy of adrenocortical tumors remains to be elucidated and needs to be further investigated in larger more specific studies focusing on the most promising regions.

**Mutations** The association of TP53 gene mutations with ACC has been discovered in patients with the Li–Fraumeni syndrome (Birch et al. 2001), who appeared to have TP53 germline mutations and presented with ACCs (Malkin et al. 1990). Another line of indirect evidence of TP53 involvement in adrenocortical tumorigenesis is the frequent loss of chromosomal locus 17p (see the ‘Chromosomal aberrations’ section) (Libe et al. 2007). TP53 mutations occur in 25 to 35% of sporadic ACC in adults and are thought to be associated with a shorter disease-free survival (Reincke et al. 1994, Libe et al. 2007, Wasserman et al. 2012). Furthermore, the prevalence of TP53 mutations is higher in pediatric ACC (Wagner et al. 1994, Varley et al. 1999). Other studies have confirmed the relatively high frequencies of TP53 mutations in ACC, ranging from 15 to 19.5% (De Martino et al. 2013, Assie et al. 2014, Juhlin et al. 2015).

The second most frequently mutated driver gene in ACC is CTNNB1 ($\beta$-catenin). Mutations in CTNNB1 lead to activation of the WNT signaling pathway and these mutations have been shown to be a common event in both ACCs and ACAs (varying from 20 to 30% of samples; Tissier et al. 2005, Gaujoux et al. 2008, Masi et al. 2009). Upregulation of $\beta$-catenin in adrenocortical tumors was also confirmed with immunohistochemistry (Tissier et al. 2005). More recently, the high frequency of CTNNB1 mutations in ACC was confirmed by several studies, which reported somatic mutation frequencies of 10–16% (De Martino et al. 2013, Assie et al. 2014, Juhlin et al. 2015). Notably, TP53 and CTNNB1 mutations are mutually exclusive.

Recently, Assie et al. (2014) identified ZNRF3 as a new tumor suppressor gene driving ACC pathogenesis, with inactivation of ZNRF in 21% of ACCs. Inactivation was caused by a homozygous deletion in 75% of the mutated cases, whereas the other 25% were caused by missense and nonsense mutations. The frequency of ZNRF3 mutations was even higher than TP53 mutations (16%) in this study (Assie et al. 2014). In addition, mutations in ZNF3 and CTNNB1 appeared to be mutually exclusive. A second recent study confirmed this mutually exclusive behavior, although the frequency of ZNF3 mutations was lower (10%) compared to the former study (Juhlin et al. 2015).

Other genes that are relatively frequently mutated in ACC include ATM ($\sim 13\%$), CDKN2A ($\sim 11\%$), RB1 ($\sim 4$ to $7\%$), MEN1 ($\sim 7\%$), KREMMEN1 ($\sim 7\%$), DAXX ($\sim 6\%$), TERT ($\sim 6\%$), MED12 ($\sim 5\%$) and JAK3 ($\sim 4\%$), which almost always co-occurs with mutations in TP53, CTNNB1, or ZNF3 (De Martino et al. 2013, Assie et al. 2014, Ragazzon et al. 2014, Juhlin et al. 2015). Three additional studies screened for EGFR mutations in ACC and reported different frequencies, i.e. 0, 11 and 0% (Ameur et al. 2009, Kotoul et al. 2009, Adam et al. 2010).

Four studies have screened ACCs simultaneously for mutations and copy number alterations using (targeted)
next generation sequencing and CGH. In the first study, in which a large number of structural DNA changes in ACC were analyzed, TP53 was found to be mutated in 15% of cases, ATM in 12.5% of cases and CTNNB1 in 10% (De Martino et al. 2013). Most frequent copy number alterations were amplification of the CDK4 gene, and deletion of the CDKN2A and CDKN2B genes. Interestingly, these genes are known actors of the RB/E2F pathway. Overall, 19/40 ACCs (47.5%) had at least one molecular abnormality (De Martino et al. 2013). In a second study, Ross et al. (2014) recently performed a comprehensive genomic profiling of 29 ACC samples and found at least one alteration (a mutation, amplification, deletion, or truncation) in 22 cases (76%). Genomic alterations in NFI (14%), CDKN2A (14%), ATM (10%), CCND2 (7%), CDK4 (7%) and DNMT3A (7%) were considered as the most common and potentially clinically relevant at the same time (Ross et al. 2014). The third study showed, considering the different omics classifications, a strong correlation between clustering of patients with different prognosis based on transcriptome clusters, DNA methylation and miRNA expression (Assie et al. 2014). The fourth study investigated recurrent copy number variations using the coverage of paired exome sequencing results (patient’s tumor vs normal), and reported somatic amplification of the TERT gene and deletion of ZNR F3 and KRE MEN1 genes (Juhlin et al. 2015).

Based on the two most recent studies that used different genomic approaches, we can conclude that the Wnt signaling pathway is most frequently altered in ACCs (Assie et al. 2014, Juhlin et al. 2015). Figure 2 gives an overview of the most frequently altered pathways in ACCs compared to ACAs. However, because of the lack of a discriminative value and the relative rarity of genetic abnormalities in ACCs, mutation studies are not primarily used to diagnose ACCs, but specifically to identify potential novel targets for therapy (see the ‘Future directions and pathway driven therapies’ section).

Urine metabolomics Urine metabolomics might offer an alternative diagnostic tool for malignancy of adrenal tumors and is based on excessive amounts of adrenal steroids secreted by ACCs. It has been shown to be relevant as a diagnostic tool and as a tumor marker during follow-up (Grondal et al. 1990). More recently, in a series of 102 patients with ACAs and 45 with ACCs, urinary steroid profiling differentiated ACCs from ACAs with a sensitivity and specificity of 90% (Arlt et al. 2011). Kerkhofs et al. (2015) showed that tetrahydro-11-deoxycortisol (THS) at a cut-off value of 2.35 μmol/24 h differentiated ACC (n = 27) from other adrenal disorders (n = 125) with a sensitivity of 100% and specificity of 99%.

Treatment of ACC

Current therapeutic strategies

Surgery Complete R0 resection of ACC is currently the keystone and only curative treatment modality for patients with ACC. However, even after complete resection the recurrence rates are high (30–50%; Fassnacht et al. 2010, 2011, Lafemina & Brennan 2012) and often occur with metastases (Bellantone et al. 1997, Schulick & Brennan 1999, Icard et al. 2001, Terzolo & Berruti 2008). Resection status is one of the most important prognostic factors. To reduce the amount of recurrences, it is recommended to perform adrenalectomies only in specialized centers performing at least 20 adrenalectomies per year (Kerkhofs et al. 2013a, Ronchi et al. 2014a). A recent systematic review reported that open adrenalectomy with lymph node dissection should be regarded as standard treatment for ACC (Bellantone et al. 2015). However, for patients with stage I-II ACCs with a diameter <8 to 10 cm, laparoscopic resection may be performed if oncological standards are respected. In addition, for patients in ENSAT stage IV or patients with hormone excess, debulking surgery can be helpful. Though, clinical effects and effects on response to systemic therapy after surgery are still unclear (Ronchi et al. 2014a). However, Livhits et al. (2014) showed that even in patients with metastatic disease, surgery was associated with improved survival.

Adjuvant treatment Mitotane, a synthetic derivative of the insecticide dichlorodiphenyltrichloroethane, is an adrenolytic drug. Mitotane is thought to act primarily by disruption of mitochondria and thereby activate an apoptotic process (Poli et al. 2013). Sbiera et al. (2015) recently identified endoplasmic reticulum stress as a key molecular pathway activated by mitotane. Sterol-O-Acyl-Transferase 1 (SOAT1) was identified as a key molecular target, which expression was also correlated with response to mitotane. This adrenal specific drug is difficult to manage clinically and mitotane use is often accompanied by severe adverse effects, sometimes leading to drug withdrawal (Allolio & Fassnacht 2006). Side effects mainly consist of gastrointestinal (nausea and diarrhea), neurological (confusion and sleepiness), metabolic, and endocrine effects. The target plasma concentration of mitotane is 14 to 20 mg/l and monitoring is of great importance.
Several studies have shown that patients with advanced ACC who reached this target concentration had less recurrences and showed a prolonged recurrence-free survival (Fig. 3; Terzolo & Berruti 2008, Hermens et al. 2011, Terzolo et al. 2013). Kerkhofs et al. (2013c), who investigated the optimal dosing strategy, showed that 50% (10/20) of patients from the high dose starting regimen and 33% (4/12) of patients from the low-dose regimen reached the therapeutic level within 3 months. No significant differences were observed in frequency and severity of adverse events. Mitotane is known to induce CYP3A4 activity, which indicates relevant drug interactions with mitotane (Kroiss et al. 2011). This issue needs to be considered when designing clinical trials in patients with ACC (Kroiss et al. 2011). This CYP3A4 induction can also, together with suppression of 11β-hydroxylase and cholesterol side chain cleavage, lead to hypocortisolism (Touitou et al. 1978, Ghataore et al. 2012). The adrenolytic effect of mitotane on the healthy contralateral adrenal, as well as enhanced production of cortisol-binding globuline in mitotane treated patients, also play a role in the occurrence of hypocortisolism (Nader et al. 2006), which should be prevented by supraphysiological hydrocortisone replacement therapy.

In case of radically resected ACC, the first line adjuvant treatment recommendation is mitotane (Terzolo & Berruti 2008, Terzolo et al. 2012, Fassnacht et al. 2013). Adjuvant treatment is mandatory in patients with high recurrence risk, because the postoperative 5 years disease-free survival is only around 30% (Allolio & Fassnacht 2006). However, studies investigating efficacy of mitotane as adjuvant treatment all have a retrospective design (Table 3). Therefore, this issue is currently addressed in a multicenter phase III trial recruiting patients with low to intermediate risk of recurrence (ADIUVO). In case of locally advanced or metastatic disease, approximately 25–30% of patients with ACC respond (defined according to different response evaluation criteria) to mitotane (Table 4). Combination of mitotane with chemotherapy for advanced ACC is investigated in the first randomized trial in ACC, showing that patients receiving mitotane with etoposide, doxorubicine and cisplatin (EDP) had a longer median progression-free survival compared to patients receiving streptozotocin and mitotane (5.0 vs 2.1 months) (Fassnacht et al. 2012). Based on this trial, mitotane with EDP is preferred above mitotane with etoposide. However, the median overall survival in the mitotane with EDP group was still only 14.8 months, underscoring the limitation of cytotoxic drugs.

**Postoperative radiotherapy** Previously, ACC was considered a radiotherapy resistant disease and studies reported poor and contradictive results of radiotherapy after surgery (Polat et al. 2009, Else et al. 2014).
### Table 3  Efficacy of adjuvant mitotane treatment in patients with adrenocortical carcinomas. Total rates represent weighted means

<table>
<thead>
<tr>
<th>Study</th>
<th>Design</th>
<th>Multi-center</th>
<th>With mitotane</th>
<th>Without mitotane</th>
<th>Follow-up</th>
<th>RR With mitotane</th>
<th>RR Without mitotane</th>
<th>DFS</th>
<th>OS</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bodie et al. (1989)</td>
<td>Retrospective</td>
<td></td>
<td>21</td>
<td>25</td>
<td>5 y</td>
<td>NR</td>
<td>NR</td>
<td>=</td>
<td>=</td>
<td>Comparison between no adjuvant treatment (n=44) and adjuvant treatment (mitotane n=7, radiotherapy n=3)</td>
</tr>
<tr>
<td>Pommier &amp; Brennan (1992)</td>
<td>Retrospective</td>
<td></td>
<td>7</td>
<td>43</td>
<td>2.4 y</td>
<td>7/7</td>
<td>35/43</td>
<td>=</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>Vassilopoulou-Sellin et al.</td>
<td>Retrospective</td>
<td></td>
<td>8</td>
<td>6</td>
<td>Minimal 12 mo</td>
<td>NR</td>
<td>NR</td>
<td>↓</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>Haak et al. (1994)</td>
<td>Retrospective</td>
<td></td>
<td>11</td>
<td>36</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>=</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>Barzon et al. (1997)</td>
<td>Retrospective</td>
<td></td>
<td>7</td>
<td>11</td>
<td>Median 2/7</td>
<td>8/11</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>Terzolo et al. (2007)</td>
<td>Retrospective</td>
<td></td>
<td>x</td>
<td>47</td>
<td>130</td>
<td>23/47</td>
<td>110/130</td>
<td>↑</td>
<td>=</td>
<td>Two control groups were used, 1 from Italy and 1 from Germany</td>
</tr>
<tr>
<td>Bertherat et al. (2007)</td>
<td>Retrospective</td>
<td></td>
<td>86</td>
<td>80</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>=</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>Grubbs et al. (2010)</td>
<td>Retrospective</td>
<td></td>
<td>22</td>
<td>196</td>
<td>Mean 88 mo</td>
<td>12/22</td>
<td>160/190</td>
<td>↑</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>Berruti et al. (2014)</td>
<td>Retrospective</td>
<td></td>
<td>x</td>
<td>251</td>
<td>Median 50 mo</td>
<td>NR</td>
<td>NR</td>
<td>↑</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>Beuschlein et al. (2015)</td>
<td>Retrospective</td>
<td></td>
<td>x</td>
<td>84</td>
<td>235</td>
<td>Median 43.7 mo</td>
<td>NR</td>
<td>=</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>Beuschlein et al. (2015)</td>
<td>Retrospective</td>
<td></td>
<td>x</td>
<td>142</td>
<td>679</td>
<td>Median 69.8 mo</td>
<td>NR</td>
<td>53.0% (44/83)</td>
<td>=</td>
<td>Validation cohort</td>
</tr>
<tr>
<td>Total</td>
<td>Retrospective</td>
<td>x</td>
<td>679</td>
<td>1111</td>
<td>Median 69.8 mo</td>
<td>NR</td>
<td>NR</td>
<td>83.7% (313/374)</td>
<td>=</td>
<td></td>
</tr>
</tbody>
</table>

NR, not reported; RR, recurrence rate; DFS, disease-free survival; OS, overall survival; y, years; mo, months; =, no statistically significant difference between mitotane or no mitotane administration; ↓, decreased survival time; ↑, increased survival time under adjuvant mitotane treatment.
Table 4  Efficacy of mitotane as therapy for advanced/metastatic ACCs

| Study                  | Design                  | n  | Concomitant other therapy | Follow-up    | CR | PR  | SD | PD   | Response duration | Comments                                                                 |
|------------------------|-------------------------|----|**************************|--------------|----|-----|----|------|-------------------|---------------------------------------------------------------------------|
| Venkatesh et al. (1989)| Retrospective           | 64 | NR                       | NR           | 0/64| 21/64| 0/64| 43/64| NR                |                                                                           |
| Williamson et al. (2000)| Prospective             | 16 | NR                       | NR           | 0/16| 2/16 | 2/16| 12/16| NR                |                                                                           |
| Luton et al. (1990)    | Retrospective           | 37 | 29                       | Mean 24.9 mo | 0/37| 8/37 | 2/37| 27/37| 5–56 mo           | Concomitant other therapy: 4 chemotherapy, 11 radiotherapy, 14 amino-glutethimide |
| Decker et al. (1991)   | Prospective             | 36 | 0                        | NR           | 2/36| 6/36 | 0/36| 28/36| Median 8.9 mo     |                                                                           |
| Pommier & Brennan (1992)| Retrospective           | 29 | NR                       | Median 28 mo | 0/29| 7/29 | 0/29| 22/29| NR                |                                                                           |
| Haak et al. (1994)     | Retrospective           | 52 | NR                       | NR           | 8/52| 7/52 | 0/52| 37/52| 2–190 mo          |                                                                           |
| Barzon et al. (1997)   | Retrospective           | 11 | 0                        | NR           | 0/11| 2/11 | 0/11| 9/11 | 12 and 21 mo      |                                                                           |
| Baudin et al. (2001)   | Prospective             | 13 | NR                       | Median 21 mo | 1/13| 3/13 | 0/13| 9/13 | 10–48 mo          |                                                                           |
| Hermsen et al. (2011)  | Retrospective           | 91 | 64                       | NR           | 1/91| 16/91| 25/91| 49/91| NR                | 14/17 patients with CR or PR received concomitant chemotherapy           |
| Total                  |                         | 416|                          |              |    |      |     |      |                  |                                                                           |

All patients had advanced/metastatic disease. Total rates represent weighted means. NR, not reported; n, number of patients participated in study; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; mo, months.
More recently, several studies with a total of 45 patients treated in an adjuvant setting show local control in 56 to 100% of patients (Fassnacht et al. 2006, Hermsen et al. 2010, Sabolch et al. 2011, Habra et al. 2013, Ho et al. 2013). However, no increase of disease-free or overall survival was found. The ultimate protocol should be adapted according to patient and tumor characteristics. In vitro results suggest that the combination of irradiation with simultaneous mitotane synergistically inhibits ACC cell growth (Cerquetti et al. 2008).

Apart from the adjuvant setting, radiotherapy can be indicated: i) when microscopic tumor residues are visible after surgery; ii) when patients are not suitable for surgery (in this case radiotherapy is often in combination with mitotane); and iii) for palliative care. Several studies have shown efficacy of radiotherapy for adequate palliation, but with divergent results and mainly based on case series (Else et al. 2014). The three most recent studies reported 8, 12 and 22 patients treated in palliative setting, respectively, with response rates varying from 77% to 100% (Polat et al. 2009, Hermsen et al. 2010, Ho et al. 2013).

**Treatment of hormone excess** In 40–60% of patients with ACC, the main complaints are due to hormone overproduction (Allolio & Fassnacht 2006, patients with ACC, the main complaints are due to hormone overproduction. In 40–60% of patients with ACC, the main complaints are due to hormone overproduction (Allolio & Fassnacht 2006, Ho et al. 2013). However, no increase of disease-free or overall survival was found. The ultimate protocol should be adapted according to patient and tumor characteristics. In vitro results suggest that the combination of irradiation with simultaneous mitotane synergistically inhibits ACC cell growth (Cerquetti et al. 2008).

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**Treatment of hormone excess** In 40–60% of patients with ACC, the main complaints are due to hormone overproduction (Allolio & Fassnacht 2006, Fassnacht & Allolio 2009). Treatment of these elevated hormone levels is mandatory for either improvement of quality of life, alleviation of symptoms, and in some cases to potentially prolong survival rates. By different mechanisms, mitotane treatment can already result in some control of hormone levels (see the ‘Adjuvant treatment’ section). Adrenal steroidogenesis inhibitors like ketoconazole or metyrapone (alone or in combination; Corcuff et al. 2015) can also be used, or more rarely aminoglutethimide or etomidate (Creemers et al. 2015). Mifepristone, a glucocorticoid receptor antagonist, is another treatment modality for excess cortisol levels (Fleseriu et al. 2012). However, there are still no parameters to monitor and guide treatment with mifepristone.

To control androgen effects in women with androgen-secreting tumors and mineralocorticoid effects in patients with mineralocorticoid-secreting tumors, spironolactone can be administered (Hunter & Carek 2003). Monitoring of the patient parameters is important in all cases, considering the risk on adrenal insufficiency.

**Future directions and pathway driven therapies**

As previously discussed, extensive effort has been made with different genomic approaches, like CGH, gene expression arrays, methylation analysis and whole genome sequencing, to identify driver mutations and altered signaling pathways in ACC. Since ACC is a very heterogeneous disease with multiple genetic hits affecting different signaling pathways, several therapeutic targets have been identified in different pathways, which are described in the following sections.

**IGF-mTOR pathway** Familial forms of ACC have enabled identification of IGF2 overexpression in ACCs. Nonetheless, for a long time there has been debate about the role of IGF2 in progression of ACC and consequently its utility as a therapeutic target. Guillaud-Bataille et al. (2014) confirmed the active role of IGF2 on adrenocortical tumor growth in ACC cells by knockdown of IGF2. In this study, ACCs expressing low levels of IGF2 showed higher levels of other growth factors (e.g. FGF9, PDGFA) compared to ACCs that expressed high levels of IGF2, suggesting alternative growth promoting pathways driving ACC pathogenesis. Abnormal activation of the insulin-like growth factor receptor 1 (IGF1R) has also been observed in ACCs (Weber et al. 1997). Based on these findings, and in vitro and preclinical studies with promising results, targeting the IGF pathway had aroused high expectations (Barlaskar et al. 2009). Linsitinib (OSI-906) was the first IGF1R blocker that reached a phase III trial, but unfortunately did not show an increased overall survival compared to placebo (Fassnacht et al. 2015).

Table 5 shows that various clinical studies mainly show disappointing results. A potential explanation can be found in compensatory activation of other growth promoting pathways. Important future considerations are reconsideration of the dosing strategy and efforts to identify potential responders to IGF targeted therapies. Combination therapy with other targeting drugs could be considered.

The role of the mammalian target of rapamycin (mTOR), a downstream effector of IGF2, has been investigated in adrenal tumors by several studies, and mTOR appeared to be a potential therapeutic target in a subset of patients with ACC (Table 5; De Martino et al. 2014). Doghman et al. (2010) reported for the first time involvement of miRNAs in regulation of mTOR signaling in childhood adrenocortical tumors. Targeting mTOR signaling by everolimus caused tumor cell growth reduction in vitro and in mouse xenografts (Doghman et al. 2010). Preclinical studies support the idea that mTOR inhibitors can upregulate AKT phosphorylation in tumor tissue (Hay & Sonenberg 2004, O’Reilly et al. 2006, Wan et al. 2007, Liu et al. 2009). To address and circumvent the
### Table 5: Overview of clinical studies investigating drugs targeting the IGF-mTOR and VEGF pathway in patients with advanced or metastatic ACC

<table>
<thead>
<tr>
<th>Study</th>
<th>Design</th>
<th>Drug 1</th>
<th>Mechanism of action drug 1</th>
<th>Drug 2</th>
<th>Mechanism of action drug 2</th>
<th>n</th>
<th>Follow-up</th>
<th>PR</th>
<th>SD</th>
<th>PD</th>
<th>Response duration</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IGF-mTOR</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haluska et al. (2010)</td>
<td>Phase I</td>
<td>Figitumumab</td>
<td>IGF1 monoclonal antibody</td>
<td></td>
<td></td>
<td>14</td>
<td>150 days</td>
<td>0/14</td>
<td>8/14</td>
<td>6/14</td>
<td>150 days</td>
<td>6/14 patients received concurrent mitotane</td>
</tr>
<tr>
<td>Gangadhar et al. (2011)</td>
<td>Case series</td>
<td>Sirolimus</td>
<td>mTOR inhibitor</td>
<td>Sunitinib</td>
<td>Multi-TKI</td>
<td>2</td>
<td>NR</td>
<td>1/2</td>
<td>0/2</td>
<td>1/2</td>
<td>44 weeks</td>
<td></td>
</tr>
<tr>
<td>Naing et al. (2011)</td>
<td>Phase I</td>
<td>Cixutumumab</td>
<td>IGF1 monoclonal antibody</td>
<td>Temsirolimus</td>
<td>mTOR inhibitor</td>
<td>10</td>
<td>28 days</td>
<td>0/10</td>
<td>4/10</td>
<td>6/10</td>
<td>28 days</td>
<td></td>
</tr>
<tr>
<td>Fraenkel et al. (2013)</td>
<td>Case series</td>
<td>Everolimus</td>
<td>mTOR inhibitor</td>
<td></td>
<td></td>
<td>4</td>
<td>4 mo</td>
<td>0/4</td>
<td>0/4</td>
<td>4/4</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>Naing et al. (2013)</td>
<td>Expansion of phase I study</td>
<td>Cixutumumab</td>
<td>IGF1 monoclonal antibody</td>
<td>Temsirolimus</td>
<td>mTOR inhibitor</td>
<td>26</td>
<td>6 mo</td>
<td>0/26</td>
<td>11/26</td>
<td>15/26</td>
<td>≥6 mo</td>
<td></td>
</tr>
<tr>
<td>Lerario et al. (2014)</td>
<td>Phase II</td>
<td>Cixutumumab</td>
<td>IGF1R inhibitor</td>
<td>Mitotane</td>
<td>Adrenalytic drug</td>
<td>20</td>
<td>mean 200 weeks</td>
<td>1/20</td>
<td>7/20</td>
<td>12/20</td>
<td>6.2-38 weeks</td>
<td></td>
</tr>
<tr>
<td>Jones et al. (2015)</td>
<td>Phase I</td>
<td>Linisitinib</td>
<td>IGF1R and IR inhibitor</td>
<td></td>
<td></td>
<td>15</td>
<td>2/15</td>
<td>0/15</td>
<td>13/15</td>
<td>199 and 703 days</td>
<td>24 weeks</td>
<td></td>
</tr>
<tr>
<td>Fassnacht et al. (2015)</td>
<td>Phase III</td>
<td>Linisitinib</td>
<td>IGF1R and IR inhibitor</td>
<td></td>
<td></td>
<td>90</td>
<td>24 weeks</td>
<td>3/90</td>
<td>6/90</td>
<td>81/90</td>
<td>None of the patients in the placebo group had SD at 24 weeks</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>181</td>
<td></td>
<td>3.9% (7/181)</td>
<td>19.9% (36/181)</td>
<td>76.2% (138/181)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>VEGF</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hong et al. (2009)</td>
<td>Phase I</td>
<td>Sorafenib</td>
<td>Multi-TKI</td>
<td>Tipifarnib</td>
<td>Farnesyltransferase inhibitor</td>
<td>2</td>
<td>NR</td>
<td>0/2</td>
<td>2/2</td>
<td>0/2</td>
<td>7 and 11 mo</td>
<td></td>
</tr>
<tr>
<td>Wortmann et al. (2010)</td>
<td>Case series</td>
<td>Bevacizumab</td>
<td>VEGF antibody</td>
<td>Capecitabine</td>
<td>Cytotoxic drug</td>
<td>10</td>
<td>25 mo</td>
<td>0/10</td>
<td>0/10</td>
<td>10/10</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>Butler et al. (2010)</td>
<td>Case report</td>
<td>Sorafenib</td>
<td>Multi-TKI</td>
<td>Sunitinib</td>
<td>mTOR inhibitor</td>
<td>1</td>
<td>28 mo</td>
<td>0/1</td>
<td>1/1</td>
<td>1/1</td>
<td>28 mo</td>
<td></td>
</tr>
<tr>
<td>Gangadhar et al. (2011)</td>
<td>Case series</td>
<td>Sorafenib</td>
<td>Multi-TKI</td>
<td>Sunitinib</td>
<td>mTOR inhibitor</td>
<td>2</td>
<td>NR</td>
<td>1/2</td>
<td>0/2</td>
<td>1/2</td>
<td>44 weeks</td>
<td></td>
</tr>
<tr>
<td>Berruti et al. (2012)</td>
<td>Phase II</td>
<td>Sorafenib</td>
<td>Multi-TKI</td>
<td>Paclitaxel</td>
<td>Cytotoxic drug</td>
<td>9</td>
<td>8 weeks</td>
<td>0/9</td>
<td>0/9</td>
<td>0/9</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>Kroiss et al. (2012)</td>
<td>Phase II</td>
<td>Sunitinib</td>
<td>Multi-TKI</td>
<td></td>
<td></td>
<td>35</td>
<td>36 mo</td>
<td>0/35</td>
<td>5/35</td>
<td>30/35</td>
<td>3 mo</td>
<td></td>
</tr>
<tr>
<td>O’Sullivan et al. (2014)</td>
<td>Phase II</td>
<td>Axitinib</td>
<td>VEGFR TKI</td>
<td></td>
<td></td>
<td>13</td>
<td>Median 2.59 y</td>
<td>0/13</td>
<td>8/13</td>
<td>5/13</td>
<td>3 mo</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>72</td>
<td></td>
<td>1.4% (1/72)</td>
<td>22.2% (16/72)</td>
<td>76.4% (55/72)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All patients had advanced/metastatic disease. Combination therapies are indicated by drug 1 and drug 2. Two studies were randomized; Fassnacht et al. randomized into linisitinib or placebo, Lerario et al. randomized into cixutumumab and mitotane or mitotane alone. Sorafenib and sunitinib are inhibitors of several tyrosine kinases, but mainly target the VEGFR. Total rates represent weighted means. NR, not reported; PR, partial response; SD, stable disease; mo, months; y, years; TKI, tyrosine kinase inhibitor.
proliferation by the small-molecule inhibitor PKF115-584 (Doghman et al. 2008). Gaujoux et al. showed that β-catenin silencing caused decreased cell proliferation, alterations in the cell cycle and increased apoptosis in adrenocortical cancer cells in vitro (Gaujoux et al. 2013). Clinical trials with Wnt inhibitors in ACC have not yet been performed.

**Angiogenesis** Angiogenesis is an important feature of tumorigenesis. Expression of vascular endothelial growth factor (VEGF), as well as its receptor (VEGFR), have been shown to be increased in ACC tumor tissue (Zacharieva et al. 2004, de Fraipont et al. 2005, Xu et al. 2011). In other types of cancer encouraging results have been achieved with VEGF inhibitor treatment. Several studies have been undertaken with VEGFR inhibitors in patients with ACC (Table 5). Three phase II studies evaluated sorafenib in combination with paclitaxel, sunitinib or axitinib respectively (Berruti et al. 2012, Kroiss et al. 2012, O’Sullivan et al. 2014). Sorafenib did not show an anti-tumor effect in patients, whereas sunitinib and axitinib showed a partial response in 14 and 62% of the patients respectively (Table 5). The mitotane-induced CYP3A4 increase may limit the therapeutic efficacy of tyrosine kinase inhibitors via enhanced drug metabolism (van Erp et al. 2011).

As previously mentioned, there is evidence that monotherapy with tyrosine kinase inhibitors causes compensatory hyperactivation of other signaling pathways, explaining the lack of efficacy in many patients (Stommel et al. 2007). In two ACC cell lines, Lin et al. (2012) confirmed the activation of multiple tyrosine kinases under treatment with sunitinib, with ERK as the most activated tyrosine kinase. In line with this finding, the authors found an additive antiproliferative effect when sunitinib was given in combination with the ERK inhibitor PD98059.

**WNT signaling pathway** Activation of the Wnt/β-catenin pathway plays an important role in sporadic adrenocortical tumorigenesis (see the ‘Molecular markers’ section). The most widely investigated Wnt inhibitor is CWP232291, which is currently in a Phase I trial for refractory acute myeloid leukemia (AML) (NCT01398462). CWP232291 can promote β-catenin degradation. The first results of effectiveness of targeting the Wnt signaling pathway in ACC comes from in vitro inhibition of ACC cell proliferation by the small-molecule inhibitor PKF115-S84 (Doghman & Lalli 2012). IGF2 stimulates H295 cell proliferation by increasing the average size of colonies (A) as well as the surviving fraction (B). Both these effects are efficiently antagonized by the coadministration of sirolimus. Data are expressed as percentage of control and represent the mean ± S.D. Control is set as 100%. The right panel (C) shows a representative photograph of the wells containing treated and untreated cells as used to perform colony-forming experiments. ***P < 0.001 vs control. Reproduced, with permission, from De Martino MC, van Koetswold PM, Feelders RA, Sprij-Mooij D, Waaijers M, Lamberts SWJ, de Herder WW, Colao A, Pivonello R & Hofland LJ (2012) The role of mTOR inhibitors in the inhibition of growth and cortisol secretion in human adrenocortical carcinoma cells. Endocrine-Related Cancer 19:351–364.

**Figure 4** Effects of 3-week treatment with IGF2 (10⁻⁸ M) and/or sirolimus (5 × 10⁻⁹ M) on colony formation and growth of the human ACC cell line H295. Left panel: IGF2 stimulates H295 cell proliferation by increasing the average size of colonies (A) as well as the surviving fraction (B). Both these effects are efficiently antagonized by the coadministration of sirolimus. Data are expressed as percentage of control and represent the mean ± S.D. Control is set as 100%. The right panel (C) shows a representative photograph of the
Other tyrosine kinase inhibitors  Novel treatment options are primarily based on inhibition of protein kinases involved in signal transduction, not only in the IGF and VEGF pathway. Interest in targeting the EGFR in ACC comes from the fact that not EGFR itself, but the transforming growth factor α (TGFα), is expressed at high levels in ACC (Sasano et al. 1994). TGFα can bind the EGFR family. To assess the efficacy of targeting this pathway in ACC, to date two clinical studies have been performed (Table 6; Samnotra et al. 2007, Quinkler et al. 2008). Both did not show significant response (Samnotra et al. 2007, Quinkler et al. 2008). During use of imatinib, a platelet-derived growth factor receptor (PDGFR) inhibitor, progressive disease occurred in 4/4 patients with ACC (Table 6; Gross et al. 2006).

Metomidate  As already mentioned in the section ‘Diagnosis of ACCs’, [123I]IMTO has a very high uptake in some adrenocortical lesions (Hahner et al. 2008). The rationale of [131I]IMTO therapy is that patients with high uptake of [123I]IMTO in their tumor lesion are suitable for treatment, given the sensitivity of the adrenal to radio-nuclide therapy and the specific uptake of [123I]IMTO in the tumor (Hahner et al. 2012). Eleven patients receiving up to 20GBq [131I]IMTO were recently evaluated, of which six patients reached stable disease or even partial response for several months (Hahner et al. 2012).

Chemotherapeutics  Research focuses on the investigation of novel chemotherapeutics in preclinical models of ACC. Gemcitabine in vitro demonstrated to be an active cytotoxic agent in ACC cells. Interestingly, efficacy in combination with mitotane was dependent on mitotane sensitivity of the ACC cell line (Germano et al. 2014). In addition, the RRM1 gene appears to play a role in sensitivity to gemcitabine, independent of mitotane (Germano et al. 2014).

MDR/P-Glycoprotein  Expression of the multidrug resistance gene 1 (MDR1), which encodes the P-glycoprotein (P-gp), is found in normal adrenals and ACCs (Flynn et al. 1992). The significant chemoresistant character of ACCs has been associated with the presence of P-gp, which actively pumps cytotoxic agents out of the cell (Flynn et al. 1992). Mitotane is in vitro, already at very low concentrations, known to interfere with the MDR1 gene, leading to reversion of chemoresistance (Bates et al. 1991, Gagliano et al. 2014). However, the lack of efficacy of the combination of mitotane with different cytotoxic drugs indicates that resistance to...
chemotherapy in ACC is mediated by other mechanisms as well. Further studies have to investigate the efficacy of MDR-1 inhibitors in ACC.

Other potential therapeutic targets and compounds  Expression of the steroidogenic factor-1 (SF-1) has already been proposed as a diagnostic tool (see the ‘Differential gene expression’ section). After experiments in transgenic mice, Doghman and colleagues assessed the effect of SF-1 inverse agonists on the SF-1 expressing cell line H295R and the SF-1 negative cell line SW13 (Doghman et al. 2009). Dependent on the class of inhibitors, alkoxyphenol or isoquinolinone, inhibitory effects were seen in both SF-1 positive and negative cells or only in SF-1 positive H295R cells respectively. These results depict the potential therapeutic possibilities of SF-1 targeting drugs (Doghman et al. 2009).

Van Koetsveld et al. (2013) demonstrated the inhibitory effect of interferon-β in vitro on ACC cell lines and primary cultures of human ACC. Interestingly, the sensitivity of ACC cells for mitotane increased if INF-β was administered concomitantly (van Koetsveld et al. 2013).

Three other compounds investigated in preclinical ACC models are thiazolidinediones (TZDs), heat shock protein 90 (HSP90) inhibitors, and decitabine, a DNA methyltransferase inhibitor (Betz et al. 2005, Suh et al. 2010, Cerquetti et al. 2011, Huang et al. 2014). All showed inhibition of ACC cell proliferation and other anti-cancer effects. Recovery of two genes (NDUF8 and PRDX5) at 11q13, which are known to be silenced in ACC, was given as a possible mechanism of efficacy of decitabine by (Suh et al. 2010).

Jain et al. (2013) investigated the potential of targeting topoisomerase alpha 2 (TOP2A), a gene consistently over-expressed in ACC. By silencing TOP2A in ACC cell lines, it was shown that TOP2A is involved in cellular invasion. Jain et al. (2013) confirmed overexpression of TOP2A in ACC and showed efficacy of several TOP2A inhibitors on proliferation and tumor spheroid size in vitro, with aclarubicin as most promising compound. Aclarubicin is already approved as a second-line therapy for acute myelocytic leukemia.

Based on the finding of overexpression of the interleukin-13 receptor alpha2 (IL13Ra2) in ACCs compared to ACAs and normal adrenals (Jain et al. 2012), a phase I study was recently conducted with systemic interleukin-13-Pseudomonas exotoxin in patients with metastatic ACC (Liu-Chittenden et al. 2015). Overall, 1/5 patients reached stable disease for 5.5 months before disease progression.

Prognostic and predictive markers The clinical presentation of patients with ACC as well as the biological behavior of ACCs can be very heterogeneous. Research focuses on the identification of subpopulations of patients in which certain therapies can be effective and increase survival rates. There is an urgent need for markers to improve outcome stratification in patients with ACC. In addition, identifying patients who will respond to treatment will prevent overtreatment, unnecessary adverse effects, and will save costs. To date, several potential factors have been identified for these two purposes.

Transcriptome studies have not only focused on discriminating adrenal adenomas from carcinomas, but also on understanding the pathophysiology and finding prognostic markers for patients with ACC. Two subgroups have been reported based on transcriptome characteristics: cluster C1A and cluster C1B, the latter one with a remarkable better 5-years survival rate (20 vs 91%) (de Reynies et al. 2009, Giordano et al. 2009, Laurell et al. 2009, Assie et al. 2010). The clusters included different genes, where for example genes associated with cell cycle predominated in the poor outcome group. Giordano et al. (2009) demonstrated that the poor-outcome group contained mainly tumors with a high histologic grade. Ragazzon et al. (2010) showed that all TP53 and CTNNB1 mutations, the genes with most frequent somatic genetic alteration in ACCs, were exclusively observed in the poor-outcome (C1A) ACC group. The poor prognosis group was further divided into three subgroups, with inactivated p53 (C1A-p53), activated β-catenin (C1A-β-catenin) and one with a still unidentified molecular alteration (C1A-x) (Ragazzon et al. 2010). Validation of these microarray-based prognostic factors is required. Assie et al. (2014) recently reported, in a study integrating different genomic approaches, that also DNA methylation and microRNAs were different in the C1A and C1B group. A higher number of mutations was also correlated with a worse 5-year survival rate, higher WS and higher ENSAT stage. Correlation of TOP2A, Ki67, EXH2 and cyclin B1 staining with overall survival was validated by Ip et al. (2015) whereas BARD1 was a newly identified prognostic factor in this study.

Barreau et al. (2013) made the first correlation between DNA methylation levels and patient outcome in ACC. Unsupervised clustering of DNA methylation profiles identified two groups of carcinomas, one with a higher methylation compared to ACAs, which was termed the CpG island methylation phenotype (CIMP) group. CIMP had already been reported in other types of cancer, like
colorectal cancer (Toyoda et al. 1999). The CIMP group was further divided into two subgroups, with different levels of methylation (CIMP-high and CIMP-low; Barreau et al. 2013), which was confirmed by Assie et al. (2014). Hypermethylation was associated with a poor survival. Interestingly, the two subgroups of ACC with poor prognosis presenting with a molecular signature (C1A-p53 and C1A-x), showed a CIMP. In contrast, in the third poor prognosis subgroup (C1A-β-catenin) and the good-prognosis C1B group, a non-CIMP pattern was observed (Barreau et al. 2013). This finding suggests that different mechanisms are responsible for the differential transcriptome classification. The fact that not all poor prognosis groups show a CIMP could potentially mean that the prognostic value of methylation patterns is less effective compared to gene expression.

Other factors which are reported to associate with poor prognosis in patients with ACC include overexpression of the pituitary tumor transforming gene 1 (PTTG) (Demeure et al. 2013), low expression of the transforming growth factor β signaling mediator SMAD and diminished expression of GATA-6 (Parviainen et al. 2013), and cyclin E overproduction (Tissier et al. 2004).

Two reports with a total of 274 patients suggested that patients with cortisol secreting ACCs showed decreased overall survival (Berruti et al. 2005, Abiven et al. 2006). In the study of Abiven et al. (2006), disease-free survival in patients with cortisol-secreting tumors did increase after treatment with mitotane postsurgery, whereas this was not the case in the whole population. The same tendency was reported by Bertherat et al. (2007). Berrutti et al. found in a total of 524 patients a correlation between cortisol excess and recurrence-free survival and overall survival, independent of mitotane use (Berrutti et al. 2014).

Recently, several studies have identified potential factors associated with response to mitotane, such as CYP2W1 (Ronchi et al. 2014b). Patients with tumors that had CYP2W1 immunoreactivity showed, when adjusted for ENSAT stage, a longer overall survival and time to progression when treated with mitotane monotherapy. This difference was not present in patients who only underwent follow-up (Ronchi et al. 2014b). Ribonucleotide reductase large subunit 1 (RRM1) gene expression was associated with a shorter disease-free survival and overall survival (Volante et al. 2012). Thereby, patients with low RRM1 expression who received adjuvant mitotane had a significantly longer disease-free survival compared to patients who only received follow-up, whereas this was not the case in patients with high RRM1 expression. As a possible mechanism, Germano et al. (2015) showed that the RRM1 gene interferes with mitotane metabolism in ACC cells. Ronchi et al. (2009) investigated protein expression of excision repair cross complementing group 1 (ERCC1) as a predictor for response to platinum-based chemotherapy in patients with ACC. High ERCC1 expression was correlated with a worse overall survival in patients treated with platinum-based chemotherapy.

Conclusion

There have been advances in diagnosis and treatment of ACC over the past years. The efforts mentioned in this review all aim to improve management of ACC and ACC patient care. Nevertheless, ACC remains a disease with a poor prognosis. Larger molecular studies have greatly expanded our knowledge in the field of pathogenesis, (epi)genetic, chromosomal, transcriptome, and molecular aberrations in adrenocortical cancer. These studies have found different molecular phenotypes for benign and malignant adrenocortical tumors. Also, new imaging techniques, specific immunohistochemical markers (e.g. Ki-67 and reticulin staining), and the measurement of urine metabolomics, have been proposed as new diagnostic tools for ACC. Further research is necessary to validate these findings.

From the molecular studies, we can conclude that ACC does not harbor one ‘driver’, but ACCs are heterogeneous cancers with many different abnormalities compared to ACAs. Studies focusing on prognostic markers now mainly identify large subgroups of patients with different survival rates. These studies, aiming to find prognostic or diagnostic markers, necessitate further validation of the most promising abnormalities in order to be able to extrapolate such large data to the individual patient.

Despite the fact that some in vitro and preclinical data of novel agents are promising, efficacy of targeted therapies in clinical practice have mainly been disappointing. An important consideration is that ACC pathogenesis is considered to be a multi-molecular event and often results in aggressive cancer, making monotherapy unlikely to be effective. As another consequence of the heterogeneity, most of the therapies are only efficient in a subgroup of patients with ACC. Research should focus on identifying patients with response to therapy by performing individualized tumor analysis. The fact that the first large international and multicenter collaborative studies have been conducted recently gives hope for the future as it comes to the recruitment of ACC patients for new clinical trials. These clinical trials may investigate efficacy of new agents or already known compounds for the
treatment of ACC. When designing clinical trials in the future, it is crucial to account for many well-established combinations of therapies, including effects of drugs on cellular processes, pharmacokinetics and dynamics, as well as side effects and interactions between compounds.

Declaration of interest
RA Feelders, LJ Hofland, and WW de Herder have received research grants from Ipsen and Novartis.

Funding
This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

Author contribution statement
All authors significantly contributed to the content of this review.

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Received in final form 12 October 2015
Accepted 15 October 2015
Made available online as an Accepted Preprint 16 October 2015