RESEARCH

The IGF2 methylation score for adrenocortical cancer: an ENSAT validation study

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Abstract

Adrenocortical carcinoma (ACC) is diagnosed using the histopathological Weiss score (WS), but remains clinically elusive unless it has metastasized or grows locally invasive. Previously, we proposed the objective IGF2 methylation score as diagnostic tool for ACC. This multicenter European cohort study validates these findings. Patient and tumor characteristics were obtained from adrenocortical tumor patients. DNA was isolated from frozen specimens, where after DMR2, CTCF3, and H19 were pyrosequenced. The predictive value of the methylation score for malignancy, defined by the WS or metastasis development, was assessed using receiver operating characteristic curves and logistic and Cox regression analyses. Seventy-six ACC patients and 118 patients with adrenocortical adenomas were included from seven centers. The methylation score and tumor size were independently associated with the pathological ACC diagnosis (OR 3.756 95% CI 2.224–6.343; OR 1.467 95% CI 1.202–1.792, respectively; Hosmer–Lemeshow test $P = 0.903$), with an area under the curve (AUC) of 0.957 (95% CI 0.930–0.984). The methylation score alone resulted in an AUC of 0.910 (95% CI 0.866–0.952). Cox regression analysis revealed that the methylation score, WS and tumor size predicted development of metastases in univariate analysis. In multivariate analysis, only the WS predicted development of metastasis (OR 1.682 95% CI 1.285–2.202; $P < 0.001$). In conclusion, we validated the high diagnostic accuracy of the IGF2 methylation score for diagnosing ACC in a multicenter European cohort study. Considering the known limitations of the WS, the objective IGF2 methylation score could potentially provide extra guidance on decisions on postoperative strategies in adrenocortical tumor patients.

Key Words

- adrenocortical carcinoma
- biomarker
- DNA methylation
- *IGF2* methylation score

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Introduction

Adrenal tumors occur at high frequencies in the general population and are often detected incidentally. Autopsy studies show a prevalence of 1.0–8.7% (Kloos *et al.* 1995, Grumbach *et al.* 2003). Radiological studies report a frequency of clinically unapparent adrenal masses of less than 1% for patients under 30 years of age, a percentage which increases up to 10% in those 70 years of age or older (Barzon *et al.* 2003, Bovio *et al.* 2006, Fassnacht *et al.* 2016). Several CT characteristics, like a large diameter (>6 cm), lack of a well-defined margin, and increased heterogeneity, can point towards a malignant adrenal mass, but these collective findings will not always indicate a clear differential diagnosis (Nieman 2010). Only in rare cases, the adrenal tumor has malignant potential. Adrenocortical carcinoma (ACC) is a highly malignant tumor with 5-year-survival ranging from 16 to 38% (Kebebew *et al.* 2006, Fassnacht *et al.* 2013). The Weiss score (WS), consisting of nine histopathological criteria, is the most frequently used scoring system to differentiate between benign and malignant adrenocortical tumors (Weiss *et al.* 1989, Aubert *et al.* 2002) and is also recommended in the European clinical guidelines on ACC (Fassnacht *et al.* 2018). A tumor is classified as ACC at the presence of three or more Weiss criteria. The WS can be ambiguous when a score of 2 or 3 is obtained, as metastasized cases have been reported with a WS as low as 2 (Pohlink *et al.* 2004, Lau & Weiss 2009, Tissier 2010, de Krijger & Papathomas 2012). In addition, the WS has been challenged due to interobserver variability and subjectivity and may be difficult to apply in specific circumstances, even for experienced pathologists (Papotti *et al.* 2011, Tissier *et al.* 2012). Consequences of malignant disease are significant, since prognosis is poor and adjuvant mitotane treatment is recommended in ACC patients after curative resection, particularly in the case of patients with tumors harbouring high recurrence risk (Allolio & Fassnacht 2006, Fassnacht *et al.* 2018). Research is focusing on bias-free molecular markers to identify adrenocortical tumors with malignant potential. Since the diagnosis of malignancy is clinically elusive in non-metastasizing adrenocortical tumors, this can be challenging.

Recently, we showed that methylation patterns of IGF2 regulatory regions discriminate ACC from adrenocortical adenoma (ACA) with a sensitivity of 94% and a specificity of 96% (Creemers *et al.* 2016*b*). This IGF2 methylation score is based on the most frequent molecular alteration in ACC, that is, increased IGF2 expression (Erickson *et al.* 2001, Giordano *et al.* 2003, de Fraipont *et al.* 2005, Almeida *et al.* 2008, Wang *et al.* 2014). The *IGF2* gene is an imprinted gene whose expression largely varies within ACC (Schmitt *et al.* 2006, Wang *et al.* 2014). The proposed methylation score consists of the mean standard deviation score of three different IGF2 regulatory regions compared to methylation in normal adrenals (Creemers *et al.* 2016*b*). The original study, however, only included two limited cohorts with a total of 33 ACCs and 27 ACAs. The major objective of the present study is to validate the diagnostic role of the IGF2 methylation score in a multicenter cohort study via the European Network for the Study of Adrenal Tumors (ENS@T, www.ensat.org). Second aim is to correlate the IGF2 methylation score with follow-up clinical characteristics in patients with adrenocortical tumors.

Methods

Patients and data collection

Patients with ACC or ACA from whom DNA from a snap-frozen specimen from the primary adrenocortical tumor was available were included. Inclusion of both ACC and ACA was mandatory for each individual center, and cases that were included in our previous study investigating the IGF2 methylation score were not included in this study (Creemers *et al.* 2016*b*). Data collected included: age at diagnosis, sex, initial tumor size, steroid secretion pattern, the WS with individual parameters, ENSAT tumor stage, follow-up duration and clinical status at the end of the follow-up period. According to availability at the participating centers, frozen specimens or 200 ng DNA isolated from frozen specimens were collected at Erasmus University Medical Center (EMC). Ten patients had to be excluded because of insufficient DNA yield. Diagnosis was based on the WS determined by the local pathologists, with a threshold of malignancy of ≥3 criteria present in the tumor. Two paediatric patients were excluded because of uncertain ACC diagnosis based on the WS. Of these two patients, no follow-up data were available. This study, that uses residual material, was approved by the Medical Ethics Committee of the Erasmus Medical Center and furthermore inclusion of patients was approved by the local ethics committees. Approval for use of tissues for research purposes was obtained at the coordinating center.

DNA isolation and pyrosequencing

Processing of adrenocortical tumors and DNA isolation, when necessary, was performed as previously described

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using the Wizard® Genomic DNA Purification Kit (Promega), according to manufacturer's protocol (Creemers *et al.* 2016*b*). Bisulfite conversion, PCR reactions, and pyrosequencing were also performed as previously described (Creemers *et al.* 2016*b*). Briefly, after binding of the PCR product to streptavidin-coated Sepharose beads (GE Healthcare), the template was washed, made single-stranded and neutralized. Pyrosequencing assays of previously reported CpGs involved in the expression of IGF2 (DMR2, CTCF3 and the H19 promoter) were designed using Pyromark Assay Design. Pyrosequencing was performed using the PyroGold SQA reagent kit (Qiagen) according to manufacturer's protocol and analyses were performed on the Pyromark Q24 system. DNA quality and quantity was assessed using the NanoDrop 2000c (ThermoFisher). PCR and corresponding sequencing primers are listed in Table 1.

Statistical analysis

Statistical analysis was performed using SPSS24 and Graphpad Prism 6.0. The methylation percentages in the three regions were transformed into a mean standard deviation score (SDS) compared to methylation in normal adrenals, as previously described (Creemers *et al.* 2016*b*). Correlation between parameters was assessed using the Spearman's correlation coefficient. To assess significant differences in methylation between ACC and ACA, the non-parametric Mann–Whitney *U*-test was used.

Logistic regression analysis was used to assess the predictive value of the IGF2 methylation score for the pathological diagnosis of ACC, adjusted for tumor size. The Hosmer–Lemeshow test was used to evaluate the goodness of fit of the model. To determine a clinically relevant cutoff value for the methylation score and to assess the discrimination of the fitted logistic regression model, Receiver Operating Characteristic (ROC) curves were constructed, followed by calculation of the area under the curve (AUC). Hazard ratios (HR) for development of metastases during follow-up were estimated using Cox proportional hazards regression models. Time to metastasis was defined as the time from pathological diagnosis until the time metastasis occurred. The proportional hazards assumption was assessed with interaction of variables with time. Kaplan–Meier curves were constructed and compared using the Logrank test. In an attempt to resemble the clinical situation in which the methylation score could be valuable, patients with an already proven ACC at diagnosis, that is, with metastasized disease (ENSAT stage IV), were excluded from these analyses. For regression analyses, independent variables with a P less than 0.1 in univariate analyses were intended to be included in multivariate analysis. Data are presented as mean ± S.E.M., unless specified otherwise. A two-sided value of $P < 0.05$ was considered statistically significant.

Results

Study population

In total, 76 patients with ACC and 118 ACA patients were included from seven clinical specialist referral centers participating in ENS@T (Netherlands 3, Italy 2, Germany 1, Spain 1; Table 2). From four centers, DNA isolated from snap-frozen specimens was collected, whereas from the remaining three centers frozen specimens were shipped to the coordinating center. The location at which the DNA isolation procedure was performed did not influence the results and the predictive value of the methylation score. Clinical and tumor characteristics of the patients included in this study are listed in Table 2.

The median tumor size was 10 cm for ACC and 3.4 cm for ACA. The proportion of functional tumors (all hormones) was similar between ACC and ACA, whereas there was a clear difference between frequency of androgen and precursor secreting tumors, whose proportions were higher in ACC (both $P < 0.0001$ vs ACA). The proportion of mineralocorticoid overproduction was lower in ACC compared to ACA ($P < 0.0001$ vs ACA).

Of the tumors indicative of ACC on the basis of the WS, and with an available ENSAT stage ($n = 66$), 26% had metastasized disease at diagnosis (ENSAT stage IV) and 45% of tumors with follow-up data available and no metastases at diagnosis were clinically proven to be malignant by development of metastasis during follow-up. The patients with histological suspected ACC who did not metastasize at diagnosis or during follow-up had a median follow-up of 41.5 months (IQR 21.5–72.3).

Predictive value of the IGF2 methylation score for the pathological diagnosis of ACC

For all three regions, a different methylation pattern was observed for ACC compared to ACA (Fig. 1A, B and C). The IGF2 methylation score was significantly higher in ACC compared to ACA (Fig. 1D; $P < 0.0001$). Within ACC, no correlation was found between the methylation score and the WS ($\rho = 0.017$, $P = 0.897$). For analysis of the

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Table 1 PCR and sequencing primers that were used in the present study.

Region	Accession no.	Nucleotide position	PCR primers (5′–3′)	Product length	Sequencing primers (5′–3′)	CpGs
DMR2	AC130303	155440–155238	*Forw*:5′-AGTGGGAAAGGGGTTTAG-3′ *Rev*:5′-[Btn]ATACTATTTCCCCAACTATAACCTAACCCT-3′	127	5′-GAAAGGGGTTTAGGAT-3′	2–4
CTCF3	AF125183	5591–5812	*Forw*:5′-GGTATTTGGTTTGGGTGATT-3′ *Rev*:5′-[Btn]TTCCCCCTTCTATCTCACCAC-3′	160	5′-GGTTGTGATGTGTGAG-3′	5–7
H19	AF125183	9811–10000	*Forw*:5′-GAGGGGAGATAGTGGTTTG-3′ *Rev*:5′-[Btn]ACCCCCCCAAAACCCACCT-3′	190	5′-ATGGGGTAATGTTTAGTT-3′	1–3

Primers were designed using Pyromark Assay Design (Qiagen).
[Btn], biotynilated; DMR, differentially methylated region; Forw, forward; Rev, reverse.

diagnostic accuracy of the IGF2 methylation score for the pathological diagnosis of ACC and for the prediction of metastases development, confirmed ACC with metastases at diagnosis were initially excluded. The IGF2 methylation score and the tumor size appeared to be independently associated with the pathological diagnosis of ACC, with an OR of 3.756 (95% CI 2.224–6.343; $P < 0.001$) and 1.467 (95% CI 1.202–1.792; $P < 0.001$), respectively (Table 3; Hosmer–Lemeshow test, $P = 0.943$).

The methylation score alone predicted the diagnosis on the basis of the WS (59 ACC, 118 ACA) with an AUC of 0.910 (Fig. 2A; 95% CI 0.867–0.953). When applying a cutoff value of 2.13 for the IGF2 methylation score, a sensitivity of 86% and a specificity of 84% was obtained for the pathological diagnosis of ACC. In case ENSAT stage III ACC were also excluded, an AUC of 0.898 (95% CI 0.846–0.949) was obtained for discriminating ACC from ACA. ROC curve of the fitted logistic regression model including the IGF2 methylation score and tumor size resulted in an AUC of 0.957 (Fig. 2C; 95% CI 0.930–0.984).

Towards clinically useful cutoff values

To provide further insights into the discriminative performance of this quantitative test, sensitivity and specificity for different cutoff values are presented in Fig. 2B. In this graph, we also demonstrate a zone that could be interpreted as a grey area, of which the implementation assures high diagnostic accuracy when the IGF2 methylation score is above or below this zone (Fig. 2B, striped area; score 1.28–3.15). Below the grey zone (<1.28), the negative predictive value is 97%, whereas a methylation score above the grey zone (>3.15) results in a positive predictive value of 87%. Tumors with a methylation score between 1.28 and 3.15 should then be classified as inconclusive. Overall, in our series, 75 of the 118 ACA (64%) could be diagnosed as ACA with a sensitivity of 97% and thus had an IGF2 methylation score below 1.28. On the other hand, 33 of the 59 ACC (56%) could be diagnosed as ACC with high diagnostic accuracy. Sixty-two tumors (35%) had a methylation score in the grey zone and were therefore classified as inconclusive diagnosis on the basis of the IGF2 methylation score. Of these cases, 61% were classified as ACA based on the WS (median WS 0, IQR 0–0), whereas 39% had a WS of 3 or more (median 6, IQR 3–8). Of the patients with clinically proven ACC as indicated by metastatic disease either at diagnosis or during follow-up, 21 (58%) of the 36 were diagnosed as ACC according to the IGF2 methylation score, 2 as ACA, and 13 had an IGF2 methylation score in the grey zone.

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Table 2 Clinical and tumor characteristics of patients included in the present study.

	All tumors, $n = 194$	ACC, $n = 76$	ACA, $n = 118$
Age at diagnosis, mean (years, range)	53 yrs (16–83) years	54 (23–83) years	53 (16–79) years
Sex (male, %)	77/194 (40%)	33/76 (43%)	44/118 (37%)
Tumor size (cm)			
Range	0.5–30 cm	2.1–30 cm	0.5–22 cm
Mean	6.8 cm	11.6 cm	3.8 cm
Median	5.0 cm	10.0 cm	3.4 cm
Steroid secretion			
Androgens	24/176 (14%)	21/69 (30%)	3/107 (3%)
Glucocorticoids	88/180 (49%)	40/71 (56%)	48/109 (44%)
Mineralocorticoids	32/180 (18%)	3/71 (4%)	29/109 (27%)
Precursors	13/166 (8%)	13/66 (20%)	0/98 (0%)
Estradiol	1/159 (1%)	1/61 (2%)	0/98 (0%)
Non-secreting	47/175 (27%)	19/71 (27%)	28/104 (27%)
Weiss score			
Range	0–9	3–9	0–2
Mean	2.5	5.8	0.31
Median	1	6	0
ENSAT			
I	-	8/66 (12%)	-
II	-	25/66 (38%)	-
III	-	16/66 (24%)	-
IV	-	17/66 (26%)	-
Metastasis during follow-up (*n*)	17/111 (15%)	17/38 (45%)	0/73 (0%)
Follow-up months, median (IQR)	M1: 13 (4–24) M0: 27 (16–53)	M1: 13 (4–24) M0: 41.5 (21.5–72.3)	M1: - M0: 23 (14.5–45.5)

For the data on follow-up, only patients with available follow-up data were included (ACA $n = 73$, ACC $n = 38$), and for the data concerning occurrence of metastases during follow-up, ENSAT tumor stage IV patients were excluded ($n = 17$).
ENSAT, European Network for the Study of Adrenal Tumors; M0, no metastases during follow-up; M1, metastases at diagnosis or during follow-up.

When focusing on ACC with a WS of 3 in our series ($n = 12$), five appeared to have a methylation score above the grey zone and would therefore be classified as ACC according to the IGF2 methylation score. The other seven ACC with a WS of 3 would be classified as inconclusive diagnosis based on the methylation score. The median follow-up of patients with a tumor harbouring a WS of 3 was 37 months (IQR 21–49), with one patient who developed metastasis after 21 months (IGF2 methylation score 1.66; grey zone). Of the six ACA with a WS of 2, four received a concluding diagnosis of an ACA on the basis of the methylations score, with a total median follow-up of 5 months (IQR 0–22). The two other ACA cases had an IGF2 methylation score in the grey zone.

The predictive value of the IGF2 methylation score for malignancy as defined by metastatic ACC

As secondary outcome, we aimed to assess the predictive value of the IGF2 methylation score and other variables for predicting metastases. When tumors were divided into two groups based on the methylation score, a higher IGF2 methylation score was associated with the development of metastases (Fig. 2D, $P = 0.005$). In univariate Cox regression analysis, not only the IGF2 methylation score but also the WS and tumor size were predictive for development of metastases (Total $n = 118$; 16 cases, 112 censored; Table 4). In multivariate analysis, however, only the WS was independently associated with metastatic disease (Table 4; HR 1.682, 95% CI 1.285–2.202, $P < 0.001$). The same finding was obtained when only ACC (total $n = 53$; 16 cases, 37 censored) were included for both analyses: only the WS was independently associated with development of metastases (OR 1.443, 95% CI 1.050–1.984; $P = 0.024$).

Discussion

In this study, we externally validated the predictive value of methylation of IGF2 regulatory regions for the diagnosis of malignancy of adrenocortical tumors in a multicenter European cohort study and confirmed that the IGF2 methylation score can serve as an objective diagnostic tool with a high sensitivity to detect adrenocortical malignancy.

Currently, the histopathological Weiss score is the most important diagnostic tool to establish adrenal malignancy. The WS harbours multiple challenges (Weiss *et al.* 1989, Aubert *et al.* 2002), as its diagnostic applicability is low

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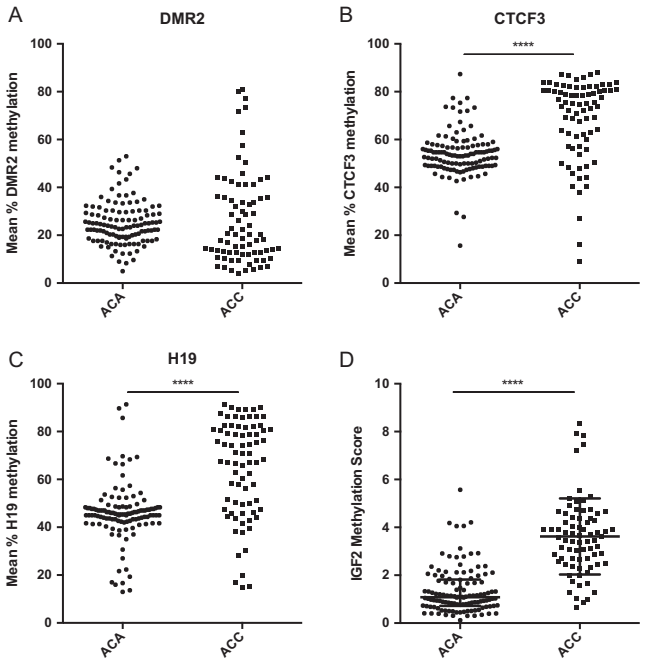

Figure 1
Mean methylation percentages in the three IGF2 regulatory regions DMR2 (A), CTCF3 (B), and the H19-promoter (C), and the IGF2 methylation score (D) for adrenocortical adenomas (ACA, $n = 118$) and carcinomas (ACC, $n = 76$). Every dot represents a patient. Lines represent medians with inter quartile range. DMR, differentially methylated region. ****$P < 0.0001$.

among non-expert pathologists and a group of borderline cases with a WS of 2 or 3 exist with an uncertain outcome (Papotti *et al.* 2011). Inter-observer agreement rates in previous studies are heterogeneous. In a study by Aubert and colleagues, a high inter-observer agreement was found for the total WS ($r = 0.94$) (Aubert *et al.* 2002). In another study using a virtual microscopy reading, a kappa statistic of 0.70 was obtained for the diagnosis of ACC in 50 adrenocortical tumors scored by 12 pathologists (Tissier *et al.* 2012). The inter-observer reproducibility increased after a coaching meeting to a kappa statistic of 0.75 (Tissier *et al.* 2012). It has thereby been shown in the German ACC registry that in 13% ($n = 21/161$) of cases a diagnosis of ACC had to be revised by a reference pathologist, also containing misdiagnosis of metastases from extra-adrenal cancers and pheochromocytoma (Johanssen *et al.* 2010). In addition, after histopathological review of a large Italian series it was demonstrated that the diagnosis was changed from ACC to ACA or *vice versa* upon review in 3% ($n = 9/200$) of the adrenocortical tumors (Duregon *et al.* 2015). Other disagreements were present in an additional 17 cases in this study, concerning, in particular, the discrimination between ACC and pheochromocytoma or metastases (Duregon *et al.* 2015). Taking this into account, considering the retrospective design of the present study, this might have led to changes during follow-up in the current study as well. Studying new diagnostic tests is associated with important concerns and limitations, since diagnosis of adrenal malignancy is only definite in case of locoregional invasive tumor growth or metastatic disease and thus may require long-term follow-up. Consequently, the IGF2 methylation score is of particular interest in adrenocortical tumors with inconclusive diagnoses, that is, ENSAT stage I and II. The importance of studying accurate diagnostic tools for adrenal malignancy lies especially in the early decision on postoperative therapeutic strategies, that is, adjuvant treatment with mitotane, and prognosis stratification.

In recent decades, research has focused on epigenetic changes in ACC (Fonseca *et al.* 2012, Barreau *et al.* 2013). Previously, these genome-wide approach studies were primarily used to identify subgroups of patients with ACC (Rechache *et al.* 2012, Creemers *et al.* 2016*a*), whereas the present study demonstrates a clinically useful cutoff value. Interest in the *IGF2* gene originates from the association of ACC with the Beckwith–Wiedemann syndrome (Wiedemann 1983), and for over 20 years, IGF2 overexpression is the most frequently detected molecular alteration in ACC. IGF2 has also been shown to be an important factor for tumor growth in the majority of ACC cases (Guillaud-Bataille *et al.* 2014). The IGF2 methylation score could be regarded as a measure

Table 3 Predictive value of the IGF2 methylation score and tumor size for the diagnosis of adrenocortical tumors on the basis of the Weiss score.

	Univariate analysis		Multivariate analysis	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
IGF2 methylation score	4.954 (3.130–7.840)	**< 0.001**	3.756 (2.224–6.343)	**< 0.001**
Tumor size	1.733 (1.447–2.076)	**< 0.001**	1.467 (1.202–1.792)	**< 0.001**

The Weiss score as determined by the local pathologist was used, resulting in 57 ACC and 115 ACA. Patients with proven ACC at diagnosis, that is, metastatic disease, were excluded from analyses ($n = 17$). For this analysis, one outlier was excluded (ACA of 22 cm), but exclusion did not influence significance. Hosmer–Lemeshow test, $P = 0.903$. Bold indicates statistical significance.
OR, odds ratio.

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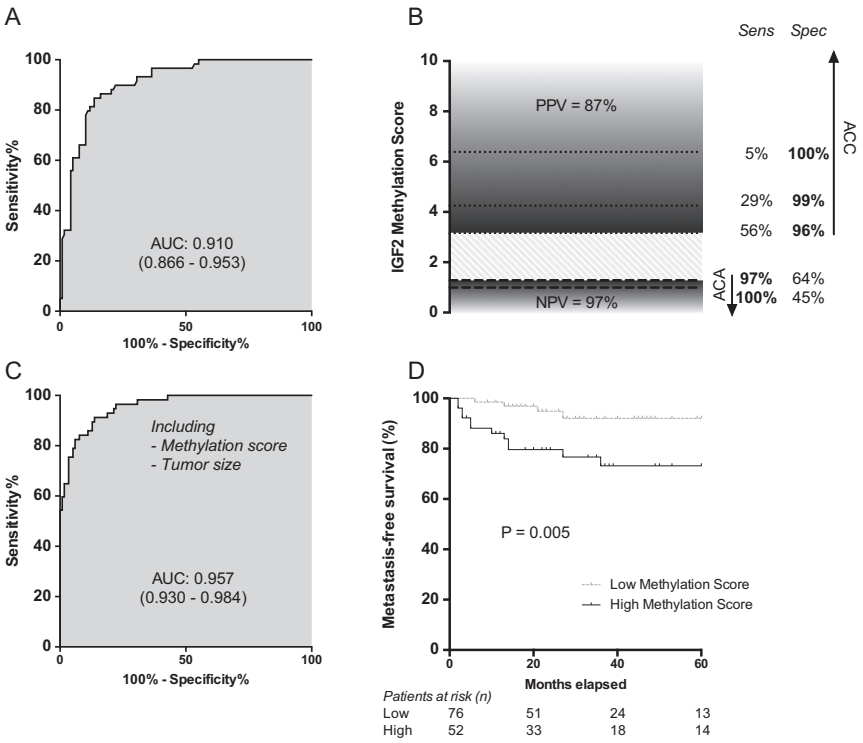

Figure 2
Discriminative value of the IGF2 methylation score for discrimination between adrenocortical adenoma (ACA, $n = 118$) and adrenocortical carcinoma (ACC, $n = 59$). ENSAT tumor stage IV patients were excluded from analyses ($n = 17$). (A) ROC curve of the IGF2 methylation score for prediction of the pathological diagnosis of ACC. (B) Sensitivity and specificity for specific cutoff values of the IGF2 methylation score for the pathological diagnosis of ACC. The striped area represents a grey zone of the methylation score with less diagnostic accuracy. PPV and NPV for the cutoff value below (1.28) or above (3.18) the grey zone. (C) ROC curve of the logistic regression model including the methylation score and tumor size for predicting the pathological diagnosis of ACC. (D) Kaplan–Meier curve for two groups based on the IGF2 methylation score for development of metastases. The two groups were divided based on an IGF2 methylation score of 2.45, which was based on the best discriminative value for the development of metastases calculated using ROC analysis. AUC, area under the curve; NPV; negative predictive value; PPV, positive predictive value; ROC, receiver operating characteristic; Sens, sensitivity; Spec, specificity.

of instability or dysregulation of this system, explaining involvement in ACC. The IGF2 regulatory regions used in the previous study were identified on the basis of known associations with IGF2 expression or malignancy of adrenocortical tumors (Creemers *et al.* 2016*b*). We have now externally validated the IGF2 methylation score in a multicenter European study. Together with the application of the WS as determined by the participating centers, this largely increases the generalizability of our findings. The performance of the IGF2 methylation score is high with a sensitivity and specificity of 86% and 84%, respectively, which is slightly less accurate compared to the previous study (Creemers *et al.* 2016*b*). When we apply the threshold of 2.442 as determined in our first study, we found a sensitivity of 80% and a specificity of 90% in this fully independent set of tumors (Creemers *et al.* 2016*b*). The most important advantage of the IGF2 methylation score as proposed in our study is that it is an easily applicable non-expensive objective measurement, which is not biased by inter-observer variability. Most quantitative diagnostic tests do not perfectly discriminate between groups of patients, often resulting in a significant overlap between distributions of test results for patients with and without a particular disease (Coste & Pouchot 2003). This also applies to the WS, where a score of 2 or 3 can be considered a grey zone (Pohlink *et al.* 2004*a*, Lau & Weiss 2009, Tissier 2010, de Krijger & Papathomas 2012). Although the diagnostic accuracy of the IGF2 methylation score is already high when applying one single cutoff value, we believe that the methylation score is especially useful when the value is below or above the grey zone as presented in this study

Table 4 Cox regression model for the development of metastases during follow-up.

	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
IGF2 methylation score	1.380 (1.070–1.780)	**0.013**	0.861 (0.571–1.298)	0.476
Weiss score	1.702 (1.308–2.216)	**< 0.001**	1.682 (1.285–2.202)	**< 0.001**
Tumor size (cm)	1.110 (1.049–1.174)	**< 0.001**	1.022 (0.940–1.111)	0.613
Patient age (years)	1.034 (0.996–1.074)	0.081	1.035 (0.988–1.083)	0.147

Patients with ENSAT tumor stage IV disease at diagnosis were excluded ($n = 17$). Patients for whom follow-up time was available were included in this analysis. In multivariate analysis, 16 patients developed metastases during follow-up, whereas 102 patients were censored. Bold indicates statistical significance.
HR, hazard ratio.

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(65% of cases in this study), eventually assuring a higher diagnostic accuracy. This indicates, however, that the performance of the methylation score is lower in 35% of the cases with a value in the grey zone, which is a limitation of the clinical applicability.

In this study, we show that also in part of the cases with a WS of 3, which in clinical practice is interpreted as a less solid diagnosis of malignancy compared to a higher WS, a high IGF2 methylation score could potentially help to opt for toxic mitotane treatment. As demonstrated in this study, the diagnostic accuracy of the IGF2 methylation score improves when it is combined with tumor size. Further research could focus on the combination of the IGF2 methylation score with imaging characteristics, other clinical data or image analyses from histopathology, like the Ki67 index, in order to determine the optimal combination. These studies should also aim to further elucidate the diagnostic accuracy of the IGF2 methylation score in the clinically most relevant group of adrenocortical tumors with a WS in the grey zone (WS of 2 or 3).

We have to acknowledge that this test is and will be applied to preselected adrenocortical tumors, with a relative high pre-test probability of malignancy. Adrenocortical tumors are surgically removed in case malignancy is suspected based on imaging characteristics or because of hormonal activity (Creemers *et al.* 2015, 2016*a*). In this respect, assessment of the urinary steroid metabolomic profile seems a promising new tool in the decision-making on surgery in patients with adrenal masses (Arlt *et al.* 2011). To improve practicality and increase availability of samples, further research could focus on the possibility of these analyses in DNA isolated from formalin-fixed paraffin embedded tissues. Previous research has already shown that pyrosequencing of DNA isolated from FFPE tissues and snap-frozen specimens provides highly comparable results (Bock *et al.* 2016).

Besides the retrospective design, a limitation of this study is that we did not have access to executed pre-operative diagnostic tests, like various imaging techniques important for the decision on adrenalectomy. Another consideration is that patients with adrenal tumors classified as adenomas have shorter follow-up time compared to ACC patients, which makes it possible that development of metastases is underestimated in this group of patients. Development of metastases after years of follow-up have been previously reported in patients with a resected adrenocortical tumor originally classified as benign (Pohlink *et al.* 2004, Tan *et al.* 2005). In our study, the occurrence of metastases in the total group of patients probably represent underestimations, considering the median follow-up time of 27.5 months. Thereby, regarding our secondary aim, that is, the prediction of metastases occurring during follow-up, we acknowledge that the number of cases is very limited and the analyses should therefore be interpreted with caution. In univariate analyses, the IGF2 methylation score, the WS, and the tumor size were associated with the development of metastases. A limitation of this study is the lack of availability of the Ki67 index, which is to date the most important prognostic factor within ACC (Beuschlein *et al.* 2015). In our study, the WS was the only independent predictive factor for metastases, although this might be affected by the limited statistical power due to a small sample size. Prospective studies are needed to further validate the diagnostic value of the IGF2 methylation score and evaluate the potential role in prediction of metastases.

In conclusion, we externally validated the high diagnostic accuracy of the previously proposed IGF2 methylation score for confirming the pathological diagnosis of ACC in a multicenter European cohort study. Considering the known limitations in clinical applicability of the WS, the objective IGF2 methylation score could provide extra guidance to multidisciplinary teams on decisions regarding postoperative strategies in patients with adrenal masses.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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