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Transcriptome sequencing reveals two subtypes of cortisol-secreting adrenocortical tumours in dogs and identifies CYP26B1 as a potential new therapeutic target

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Abstract

Cushing's syndrome (CS) is a serious endocrine disorder that is relatively common in dogs, but rare in humans. In ~15%–20% of cases, CS is caused by a cortisol-secreting adrenocortical tumour (csACT). To identify differentially expressed genes that can improve prognostic predictions after surgery and represent novel treatment targets, we performed RNA sequencing on csACTs ($n = 48$) and normal adrenal cortices (NACs; $n = 10$) of dogs. A gene was declared differentially expressed when the adjusted p-value was <.05 and the $\log_2$ fold change was >2 or < −2. Between NACs and csACTs, 98 genes were differentially expressed. Based on the principal component analysis (PCA) the csACTs were separated in two groups, of which Group 1 had significantly better survival after adrenalectomy ($p = .002$) than Group 2. Between csACT Group G1 and Group 2, 77 genes were differentially expressed. One of these, cytochrome P450 26B1 (*CYP26B1*), was significantly associated with survival in both our canine csACTs and in a publicly available data set of 33 human cortisol-secreting adrenocortical carcinomas. In the validation cohort, *CYP26B1* was also expressed significantly higher ($p = .012$) in canine csACTs compared with NACs. In future studies it would be interesting to determine whether CYP26B1 inhibitors could inhibit csACT growth in both dogs and humans.

KEYWORDS
adrenocortical carcinoma, canine diseases, Cushing's syndrome, RNA-seq

1 | INTRODUCTION

Hypercortisolism, also known as Cushing's syndrome (CS), is a serious endocrine disorder that results in significant morbidity and mortality if left untreated.[1] In dogs, CS is a relatively common disorder with a

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prevalence of 1 per 400.[2] In humans, CS is a rare disorder with a prevalence of 39–79 per million.[3] In both humans and dogs, ~15%–20% of CS cases are caused by a cortisol-secreting adrenocortical tumour (csACT).[4,5] If metastases are not detected, the treatment of choice for a csACT is surgical removal (adrenalectomy).[6]

A csACT can be classified as benign (adrenocortical adenoma; ACA) or malignant (adrenocortical carcinoma; ACC).[7] Although the histopathological classification of ACA or ACC has been well-described in humans,[7–9] this is less the case in dogs. For both humans and dogs, the histopathological parameters that are commonly used to make this classification are known to have high interobserver variability.[8,10,11] Moreover, using this traditional histopathological classification of ACA or ACC, several studies observed no significant differences in survival times of dogs after adrenalectomy.[12,13] We therefore recently developed a new histopathological scoring system, the Utrecht score, to predict the prognosis of dogs with a csACT after adrenalectomy.[14] The Utrecht score is based on histopathological parameters, including the Ki67 proliferation index, that had low intra- and interobserver variability and were associated with survival times after adrenalectomy,[14] and is comparable to the Helsinki score that is used in human ACTs.[8] In addition to histopathology, gene expression profiling has been shown to be related to patient outcome in many solid tumour types,[15] including ACTs in humans,[16] and could therefore further improve prognostic predictions for dogs with csACTs.

In addition to improving prognostic predictions, gene expression profiling could also provide more insight into potential therapeutic targets. We previously identified three genes of which the expression was significantly associated with survival after adrenalectomy in dogs with csACTs: Steroidogenic factor-1, topoisomerase II alpha and pituitary tumour-transforming gene-1.[17] However, in that study, we used a candidate gene approach with only 14 genes. We hypothesized that many more genes are associated with canine csACTs and survival after adrenalectomy. These genes or their products could represent potential novel therapeutic targets in dogs. Because dogs with spontaneously occurring csACTs represent a potential animal model for humans, the identified genes would also be interesting to study in human csACTs. In this study, we performed RNA sequencing (RNA-seq) on 48 csACTs and 10 normal adrenal cortices (NACs) of dogs.

2 | METHODS

2.1 | Patients

For the discovery cohort (RNA-seq), 49 canine csACTs were collected between 2002 and 2017. For the validation cohort (RT-qPCR), 25 canine csACTs were collected between 2017 and 2020. Permission to use the csACT tissues for research purposes was obtained from all dog owners. The suspicion of CS was based on clinical signs and routine laboratory findings that were consistent with hypercortisolism. The presence of non-suppressible hypercortisolism was detected with the low-dose dexamethasone suppression test, or with urinary corticoid: creatinine ratios (UCCRs) in combination with a high-dose dexamethasone suppression test.[5] The presence of an adrenal mass was visualized using abdominal ultrasonography, computed tomography, or both. All dogs underwent adrenalectomy, after which the tissues were formalin-fixed and paraffin-embedded for histopathology, and either snap-frozen or first stored in RNAlater™ Stabilization Solution (Invitrogen™, ThermoFisher Scientific). The tissues were stored at −70°C until RNA isolation. Histopathology confirmed the adrenocortical origin of the adrenal masses in all cases. The Utrecht score was assessed as previously described (Ki67 proliferation index +4 if ≥33% of cells have clear/vacuolated cytoplasm +3 if necrosis is present).[14] Of the included csACT samples, 36 (of which one was excluded from further analyses) were also included in our previously published studies on prognostic markers in canine csACTs.[14,17] Adrenal cortices of healthy dogs were used as reference material, these dogs were euthanized for reasons unrelated to this study, approved by the Ethical Committee of Utrecht University conform Dutch legislation. All methods were performed in accordance with the relevant guidelines and regulations.

2.2 | Extraction, library preparation and RNA sequencing

RNA was isolated from the csACTs or from the adrenal cortex of normal adrenal glands using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA concentrations were measured with the Qubit™ RNA high sensitivity Assay Kit (ThermoFisher Scientific). The quality of RNA samples was assessed using the 2100 Bioanalyzer Instrument (Agilent Technologies) using RNA Nano Chips (Agilent Technologies).

CEL-Seq2[18] was used to generate sequencing libraries. The methodology captures 3′-end of polyadenylated RNA species and includes unique molecular identifiers (UMIs), which allow direct counting of unique RNA molecules in each sample; 20 ng of total RNA was precipitated using isopropanol and washed with 75% ethanol. After removing ethanol and air-drying the pellet, primer mix containing 5 ng primer per reaction was added, initiating primer annealing at 65°C for 5 min. Subsequent RT reaction was performed; first strand reaction for 1 h at 42°C, heat-inactivated for 10 min at 70°C, second strand reaction for 2 h at 16°C, and then put on ice until proceeding to sample pooling. The primer used for this initial reverse-transcription (RT) reaction was designed as follows: an anchored polyT, a unique 6 bp barcode, a unique molecular identifier (UMI) of 6 bp, the 5′ Illumina adapter and a T7 promoter. Each sample now contained its own unique barcode due to the primer used in the RNA amplification, making it possible to pool together up to 10 cDNA samples per pool. The cDNA was cleaned using AMPure XP beads (Beckman Coulter), washed with 80% ethanol, and resuspended in water before proceeding to the in vitro transcription (IVT) reaction (AM1334; ThermoFisher) incubated at 37°C for 13 h. Next, primers were removed by treating with Exo-SAP (Affymetrix, Thermo-Fisher) and amplified RNA (aRNA) was fragmented and then cleaned with RNAClean XP (Beckman-Coulter), washed with 70% ethanol, air-dried, and

resuspended in water. After removing the beads using a magnetic stand, RNA yield and quality in the suspension were checked by Bioanalyzer (Agilent). The cDNA library construction was then initiated by performing an RT reaction using SuperScript II reverse transcriptase (Invitrogen/Thermo-Fisher) according to the manufacturer's protocol, adding randomhexRT primer as a random primer. Next, PCR amplification was done with Phusion High-Fidelity PCR Master Mix with HF buffer (NEB) and a unique indexed RNA PCR primer (Illumina) per reaction, for a total of 11–15 cycles, depending on aRNA concentration, with 30 s elongation time. PCR products were cleaned twice with AMPure XP beads (Beckman Coulter). Library cDNA yield and quality were checked by Qubit fluorometric quantification (ThermoFisher) and Bioanalyzer (Agilent), respectively.

2.3 | Sequencing read mapping and quality filtering

Libraries were sequenced on the Illumina Nextseq500 platform; a high output paired-end run of 2 × 75 bp was performed (Utrecht Sequencing Facility). The reads were demultiplexed and aligned to canine cDNA reference build CanFam3.1 using the BWA (0.7.13) by calling 'bwa aln' with settings -B 6 -q 0 -n 0.00 -k 2 -l 200 -t 6 for R1 and -B 0 -q 0 -n 0.04 -k 2 -l 200 -t 6 for R2, 'bwa sampe' with settings -n 100 -N 100. Multiple reads mapping to the same gene with the same unique molecular identifier (UMI, 6 bp long) were counted as a single read. The raw read counts were corrected for UMI sampling (corrected_count = −4096*(ln(1- (raw_count/4096)))).

2.4 | RNA-seq data analysis

The raw data files are available in the Gene Expression Omnibus (GEO) repository, accession number GSE196108. TMM normalized counts and fold changes were calculated in edgeR. P-values were calculated with the Mann–Whitney *U* test, which were corrected for multiple comparisons using the FDR Benjamini-Hochberg method.

ToppFun was used for functional enrichment analysis based on functional annotations and protein interactions networks,[19] including genes with a $\log_2$ fold change of >1.5 or < −1.5 and FDR-corrected *p*-values of <.05. Vulcano-plots were generated using EnhancedVulcano (R package version 1.12.0).[20]

2.5 | RT-qPCR validation cohort

After isolation of RNA, cDNA was synthesized with the iScript cDNA Synthesis Kit (Bio-Rad, Veenendaal, The Netherlands) according to the manufacturer's instructions, and subsequently diluted to 1 ng/μL. RT-qPCR reactions were performed using SYBR-green Supermix (Bio-Rad) in a CFX384 Touch Real-Time PCR Detection System (Bio-Rad). Primers for CYP26B1 (forward: TCA CCT CTT CGA AGT CTA CC; reverse: AGT AGT CCT TGC CCT GG) were designed with PerlPrimer software,[21] checked for secondary structures with the Mfold web server,[22] and ordered from Eurogentec (Maastricht, The Netherlands). The mRNA expression levels of four reference genes were analysed for data normalization: signal recognition particle receptor, succinate dehydrogenase complex subunit A, ribosomal protein S19, and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta.[23,24] All reactions were performed in duplicate. The geNorm[25] method was used to analyse the pairwise variance and stability of reference gene expression, which justified their use. The relative expression of *CYP26B1* was calculated using the $2^{-\Delta\Delta CT}$ method.[26]

2.6 | Statistical analyses

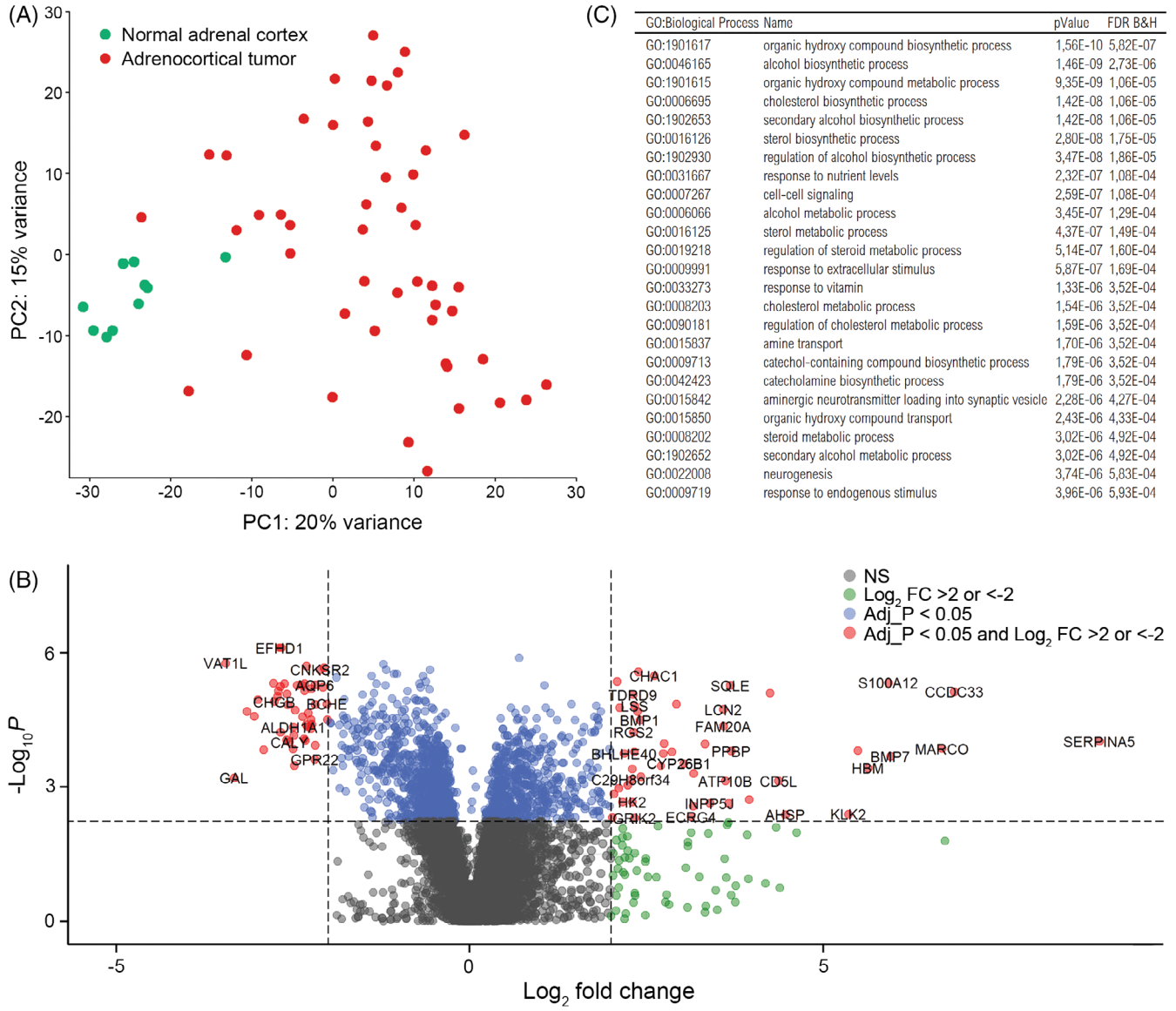
In all dogs, assessment of UCCRs was routinely performed 2 months after surgery to assess remission. In the follow-up, endocrine testing (either UCCRs or low-dose dexamethasone suppression test) was performed when hypercortisolism-related clinical signs were present. Recurrence was confirmed when endocrine testing confirmed the clinical suspicion. In some dogs with recurrence, metastases or regrowth of the csACT were confirmed with diagnostic imaging and/or fine needle aspiration biopsy. The owners of all dogs that were still alive at the end of the study were asked to submit a urine sample of their dog for UCCR assessment. For the survival analyses, dogs were considered to have had an event when they were euthanized because of recurrence of hypercortisolism, resulting from either metastases or regrowth of the csACT. Dogs that died from an unrelated cause, were still alive at the end of the study, or were lost to follow-up, were censored at the timepoint they were last known to be alive. Survival times were recorded as the time between adrenalectomy and censoring or death due to recurrence. Continuous variables were analysed using the Cox proportional hazards model, while bivariate variables were analysed using the Kaplan–Meier product-limit method with the log-rank test. To determine optimal cut-off values for group classification, receiver operating characteristic curves were used to find the value with highest Youden's index (sensitivity + specificity −1).

For comparisons between groups, the Mann–Whitney *U* test was used. Survival analyses and Mann–Whitney *U* tests were performed using IBM SPSS Statistics (Version 25, IBM Corp.). *p*-values of <.05 were considered significant.

3 | RESULTS

3.1 | ACTs versus NACs

For the initial analyses, we compared gene expression profiles between NACs ($n = 10$) and csACTs ($n = 49$). The clinical data of all included dogs can be found in Data S1. The RNA isolated from all samples was analysed with a Bioanalyzer, which gave a mean RNA integrity number (RIN) value of 8.7 (SD±0.8). The number of



GO:Biological Process	Name	pValue	FDR B&H
GO:1901617	organic hydroxy compound biosynthetic process	1,56E-10	5,82E-07
GO:0046165	alcohol biosynthetic process	1,46E-09	2,73E-06
GO:1901615	organic hydroxy compound metabolic process	9,35E-09	1,06E-05
GO:0006695	cholesterol biosynthetic process	1,42E-08	1,06E-05
GO:1902653	secondary alcohol biosynthetic process	1,42E-08	1,06E-05
GO:0016126	sterol biosynthetic process	2,80E-08	1,75E-05
GO:1902930	regulation of alcohol biosynthetic process	3,47E-08	1,86E-05
GO:0031667	response to nutrient levels	2,32E-07	1,08E-04
GO:0007267	cell-cell signaling	2,59E-07	1,08E-04
GO:0006066	alcohol metabolic process	3,45E-07	1,29E-04
GO:0016125	sterol metabolic process	4,37E-07	1,49E-04
GO:0019218	regulation of steroid metabolic process	5,14E-07	1,60E-04
GO:0009991	response to extracellular stimulus	5,87E-07	1,69E-04
GO:0033273	response to vitamin	1,33E-06	3,52E-04
GO:0008203	cholesterol metabolic process	1,54E-06	3,52E-04
GO:0090181	regulation of cholesterol metabolic process	1,59E-06	3,52E-04
GO:0015837	amine transport	1,70E-06	3,52E-04
GO:0009713	catechol-containing compound biosynthetic process	1,79E-06	3,52E-04
GO:0042423	catecholamine biosynthetic process	1,79E-06	3,52E-04
GO:0015842	aminergic neurotransmitter loading into synaptic vesicle	2,28E-06	4,27E-04
GO:0015850	organic hydroxy compound transport	2,43E-06	4,33E-04
GO:0008202	steroid metabolic process	3,02E-06	4,92E-04
GO:1902652	secondary alcohol metabolic process	3,02E-06	4,92E-04
GO:0022008	neurogenesis	3,74E-06	5,83E-04
GO:0009719	response to endogenous stimulus	3,96E-06	5,93E-04

FIGURE 1 Differences in transcriptomic profiles of canine cortisol-secreting adrenocortical tumours (csACTs; $n = 48$) compared with normal adrenal cortices (NACs; $n = 10$). Principal component analysis (A) shows that the majority of csACTs cluster apart from the NACs. The volcano plot (B) shows the differentially expressed genes with FDR-corrected p-values <.05 and $\log_2$ fold changes of >2 ($n = 50$ genes) or < −2 ($n = 48$ genes) in red. The enrichment analysis (C) based on Gene Ontology: Biological Processes shows the top 25 of most affected pathways

reads per sample after quality control ranged from 993 628 to 5 385 270 (mean = 2 078 649), on which a correction for library size was performed. The Trimmed Mean of M-values (TMM) normalized counts can be found in Data S2. One csACT was determined an outlier based on the principal component analysis (PCA) plot and was therefore excluded from further analyses, leaving a total of 48 csACTs. In the PCA plot, the csACT cohort largely clustered apart from the NAC cohort (Figure 1A). After false discovery rate (FDR) correction of the obtained P-values, 1452 genes were differentially expressed between csACTs and NACs (Data S3). Considering not only p-values but also (stringent) effect size measures, 98 of these differentially expressed genes had a $\log_2$ fold change of >2 or < −2, of which 50 were upregulated in ACTs compared with NACs, and 48 were downregulated (Figure 1B; Data S4). The top 25 pathways that were affected in csACTs compared with NACs are shown in Figure 1C.

3.2 | csACT classification

Of the 48 included csACTs, follow-up information was available for 35 dogs (Data S1), which were also included in our previous studies.[14,17] We divided these 35 csACTs in two groups: those with a histopathological Utrecht score of <6 ($n = 13$; low risk of recurrence tumours) and those with a score of ≥6 ($n = 22$; moderate-to-high risk of recurrence tumours).[17] As shown in the PCA plot in Figure 2A, the samples with Utrecht scores <6 showed a potential tendency to form a different cluster from those with scores of ≥6, but the groups

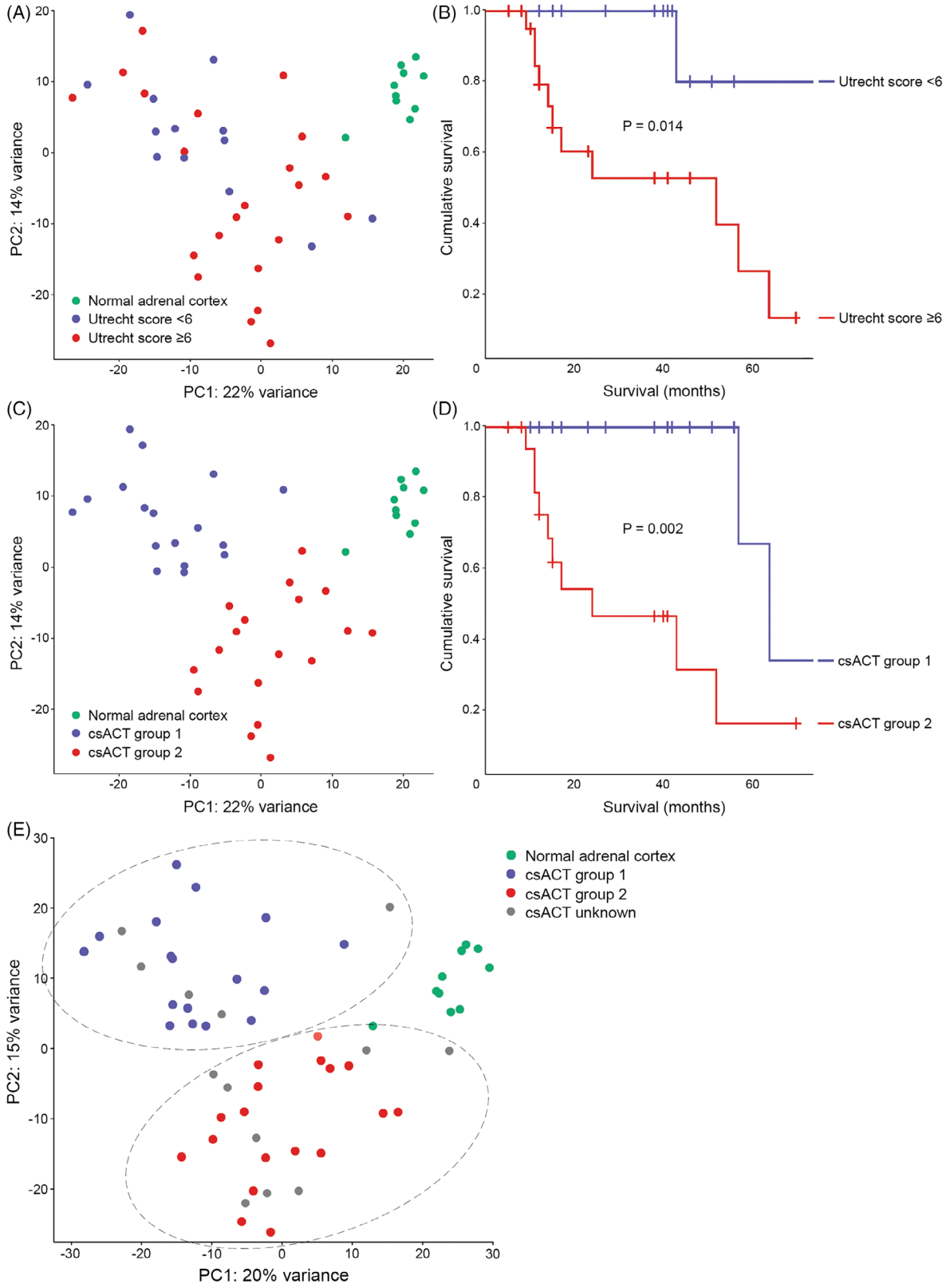
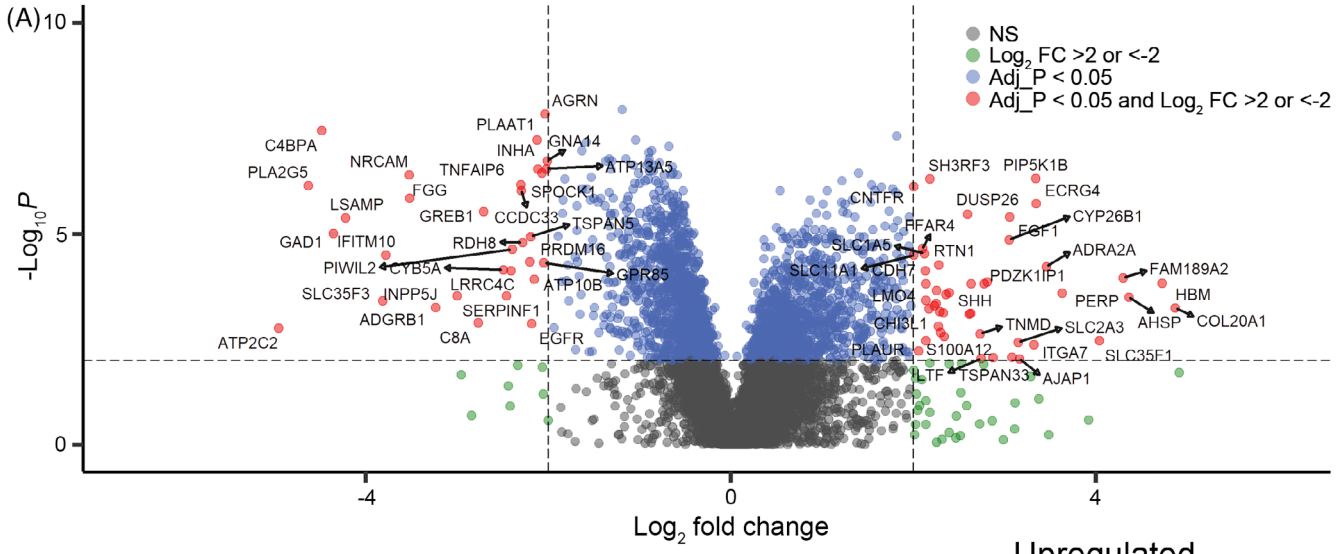


FIGURE 2 Classification of canine cortisol-secreting adrenocortical tumours. The samples with follow-up data ($n = 35$) were initially classified as having histopathological Utrecht scores of <6 ($n = 13$) or ≥6 ($n = 22$), which did not completely separate these groups in principal component analysis (A) and had significantly different survival times after adrenalectomy (B). The samples were subsequently classified based on their principal component analysis profile (C) as csACT Group 1 ($n = 17$) and csACT Group 2 ($n = 18$), which had significantly different survival times after adrenalectomy (D). The samples without follow-up data ($n = 13$) were subsequently added to either csACT Group 1 ($n = 5$, group total $n = 22$) or csACT Group 2 ($n = 8$, group total $n = 26$) based on their transcriptome profile in the principal component analysis (E). csACT, cortisol-secreting adrenocortical tumour



(B)

GO:Biological Process	Name	pValue	FDR B&H
GO:0007267	cell-cell signaling	3.57E-12	1.41E-08
GO:0046903	secretion	5.12E-10	1.01E-06
GO:0140352	export from cell	3.75E-09	4.95E-06
GO:0032940	secretion by cell	5.06E-09	5.01E-06
GO:0050804	modulation of chemical synaptic transmission	3.77E-08	2.56E-05
GO:0099177	regulation of trans-synaptic signaling	3.88E-08	2.56E-05
GO:0098916	anterograde trans-synaptic signaling	5.17E-08	2.56E-05
GO:0007268	chemical synaptic transmission	5.17E-08	2.56E-05
GO:0099537	trans-synaptic signaling	6.29E-08	2.77E-05
GO:0099536	synaptic signaling	9.67E-08	3.83E-05
GO:0022610	biological adhesion	1.50E-07	5.42E-05
GO:0007155	cell adhesion	4.16E-07	1.37E-04
GO:0010038	response to metal ion	4.91E-07	1.50E-04
GO:0045055	regulated exocytosis	9.44E-07	2.67E-04
GO:0023061	signal release	2.01E-06	5.30E-04
GO:0000902	cell morphogenesis	3.65E-06	9.03E-04
GO:0016050	vesicle organization	4.40E-06	1.02E-03
GO:0051240	positive regulation of multicellular organismal process	6.14E-06	1.35E-03
GO:0006906	vesicle fusion	7.25E-06	1.51E-03
GO:0090174	organelle membrane fusion	8.00E-06	1.54E-03
GO:0006869	lipid transport	8.85E-06	1.54E-03
GO:0006887	exocytosis	9.36E-06	1.54E-03
GO:0140029	exocytic process	9.36E-06	1.54E-03
GO:0099500	vesicle fusion to plasma membrane	9.36E-06	1.54E-03
GO:0022600	digestive system process	9.69E-06	1.54E-03

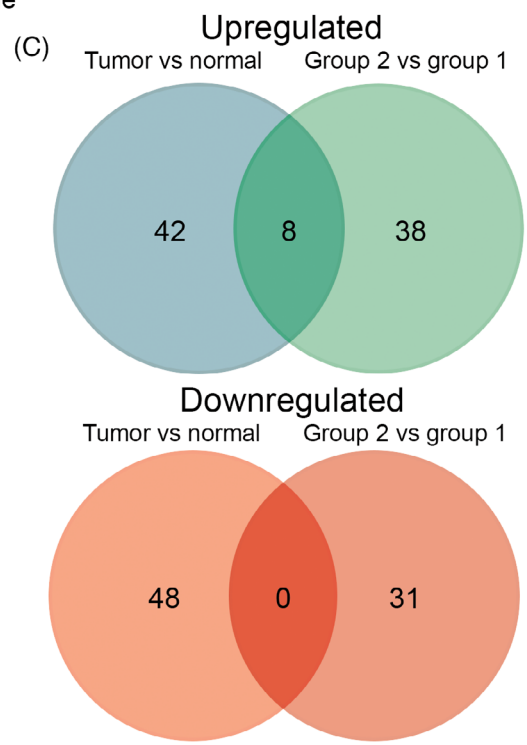


FIGURE 3 Differences in transcriptomic profile between csACT Group 1 ($n = 22$) and csACT Group 2 ($n = 26$). The volcano plot (A) shows the differentially expressed genes with FDR-corrected p-values <.05 and $\log_2$ fold changes of >2 ($n = 46$ genes) or < −2 ($n = 31$ genes) in red. The enrichment analysis (B) based on Gene Ontology: Biological Processes shows the top 25 of most affected pathways in csACT Group 1 compared with csACT Group 2. The pie-charts in (C) show the overlap in genes that were either up- or downregulated both in csACTs compared with NACs (left) as well as in csACT Group 2 compared with csACT Group 1 (right). csACT: cortisol-secreting adrenocortical tumour.

overlapped to a large extent. As expected, based on their inclusion in our previous study concerning the Utrecht score, these samples had significantly different ($p = .014$) survival times after adrenalectomy when classified as Utrecht score of <6 or ≥6 (Figure 2B).

Because the classification based on Utrecht scores <6 or ≥6 did not result in clearly separated clusters, we divided these 35 samples into two groups based on their PCA profile: csACT group 1 ($n = 17$) and Group 2 ($n = 18$; Figure 2C). In survival analysis, these groups had significantly different ($p = .002$) survival times (Figure 2D), with a p-value that was lower than when separating the samples based on having an Utrecht score of <6 or ≥6.

We subsequently added the 13 additional samples of which we had little to no follow-up information on survival in the PCA. Based on their positions in the PCA plot, we allocated these samples to either csACT group 1 ($n = 5$, group total $n = 22$) or csACT Group 2 ($n = 8$, group total $n = 26$; Figure 2E).

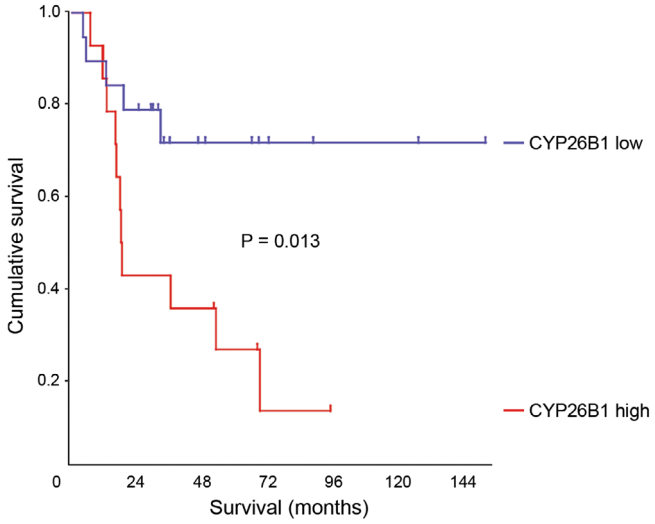


FIGURE 4 Kaplan–Meier curve of human patients with adrenocortical carcinomas with known excessive cortisol secretion (either alone or in combination with other hormones, $n = 33$) classified as having either high ($n = 14$) or low ($n = 19$) *CYP26B1* mRNA expression. Data set of The Cancer Genome Atlas as described by Zheng et al. (2016)[27] and uploaded in the web-based genomic analysis and visualization platform R2 (http://r2platform.com).[28] Figure acquired from the R2 platform and slightly adjusted for consistent visualization

3.3 | csACT Group 2 versus Group 1

When comparing csACT Group 2 with csACT Group 1, a total of 2277 genes were differentially expressed after FDR correction (Data S3). Of these, 77 had a $\log_2$ fold change of >2 ($n = 46$) or < −2 ($n = 31$) in csACT Group 2 compared with csACT Group 1 (Figure 3A; Data S4). The top 25 pathways that were affected in csACT Group 2 compared with csACT Group 1 are shown in Figure 3B.

As a therapeutic target, a gene would be most interesting when it is up- or downregulated in csACTs compared with NACs, as well as in csACT Group 2 (poor prognosis) compared with csACT Group 1 (favourable prognosis). We found eight genes that were upregulated with $\log_2$ fold change >2 in csACTs compared with NACs, as well as in csACT Group 2 compared with csACT Group 1, but no genes that were downregulated with $\log_2$ fold change <−2 in both comparisons (Figure 3C). Of these eight upregulated genes, three were significantly associated with survival after surgery in our cohort of 35 patients with follow-up data: CD5 antigen-like (*CD5L*; $p = .026$, hazard ratio [HR] = 1.030), Cytochrome P450 26B1 (*CYP26B1*; $p = .031$, HR = 1.010), and Oesophageal Cancer-Related Gene 4 (*ECRG4*; $p = .013$, HR = 1.046).

3.4 | Comparison with human ACCs

To determine whether *CD5L*, *CYP26B1* and *ECRG4* could also be relevant in human ACTs, we consulted a RNA-seq data set of The Cancer Genome Atlas (TCGA) including 79 human ACCs, as described by Zheng et al. (2016)[27] and uploaded in the web-based genomic analysis and visualization platform R2 (http://r2platform.com).[28] To make the human data set more comparable to our canine data set, we selected only those ACCs with known excessive cortisol secretion (either alone or in combination with other hormones, $n = 33$). When dividing these patients in R2 based on high or low expression of the respective genes in the ACCs, calculated using their optimal cut-off values, there were no significant differences in overall survival for *CD5L* ($p = .174$) or *ECRG4* ($p = .091$; Figure S1), but there was a significant difference ($p = .013$) in survival of patients with high ($n = 14$) or low ($n = 19$) *CYP26B1* expression (Figure 4).

Because of its potential as a therapeutic target based on its function[29] and expression profile in both human and canine csACTs, and the availability of CYP26B1 inhibitors,[30] we decided to more closely study *CYP26B1* expression.

3.5 | Canine csACT validation cohort *CYP26B1*

When comparing samples with high *CYP26B1* expression ($n = 13$) to those with low *CYP26B1* expression ($n = 23$) in our cohort of 35 dogs with follow-up data, we found that those with high *CYP26B1* expression had a significantly worse prognosis than those with low expression ($p = .004$; Figure 5A). To validate our RNA-seq findings in the discovery cohort (10 NACs and 48 csACTs), we performed RT-qPCR for *CYP26B1* mRNA expression on an independent patient cohort (8 NACs and 25 csACTs). As in the discovery cohort, *CYP26B1* mRNA was expressed significantly higher in csACTs than in NACs in the validation cohort (Figure 5B).

4 | DISCUSSION

This is the first study in which RNA-seq was performed on canine csACTs to identify new prognostic markers and potential therapeutic targets for adrenal-dependent CS. We have found many genes and biological processes that were dysregulated in csACTs compared with NACs. Additionally, we identified two transcriptionally distinct groups of csACTs that had significantly different survival times after adrenalectomy and identified CYP26B1 as a potential new therapeutic target for both dogs and humans with csACTs.

Based on our previously developed histopathological Utrecht score,[14] we initially classified the tumours based on having an Utrecht score of <6 or ≥6. Although this classification showed a potential tendency for separation of samples in the PCA plot, there was substantial overlap between the groups. We therefore classified the samples based on their position in the PCA plot as either csACT Group 1 or csACT Group 2 and found that this classification improved the significance of the survival analysis. This suggests that there is still room for improvement in the histopathological analysis of csACTs. In future studies it would be interesting to determine whether assessment of additional markers that are upregulated in csACT Group 2 compared with csACT Group 1, such as CYP26B1, CD5L and ECRG4, could

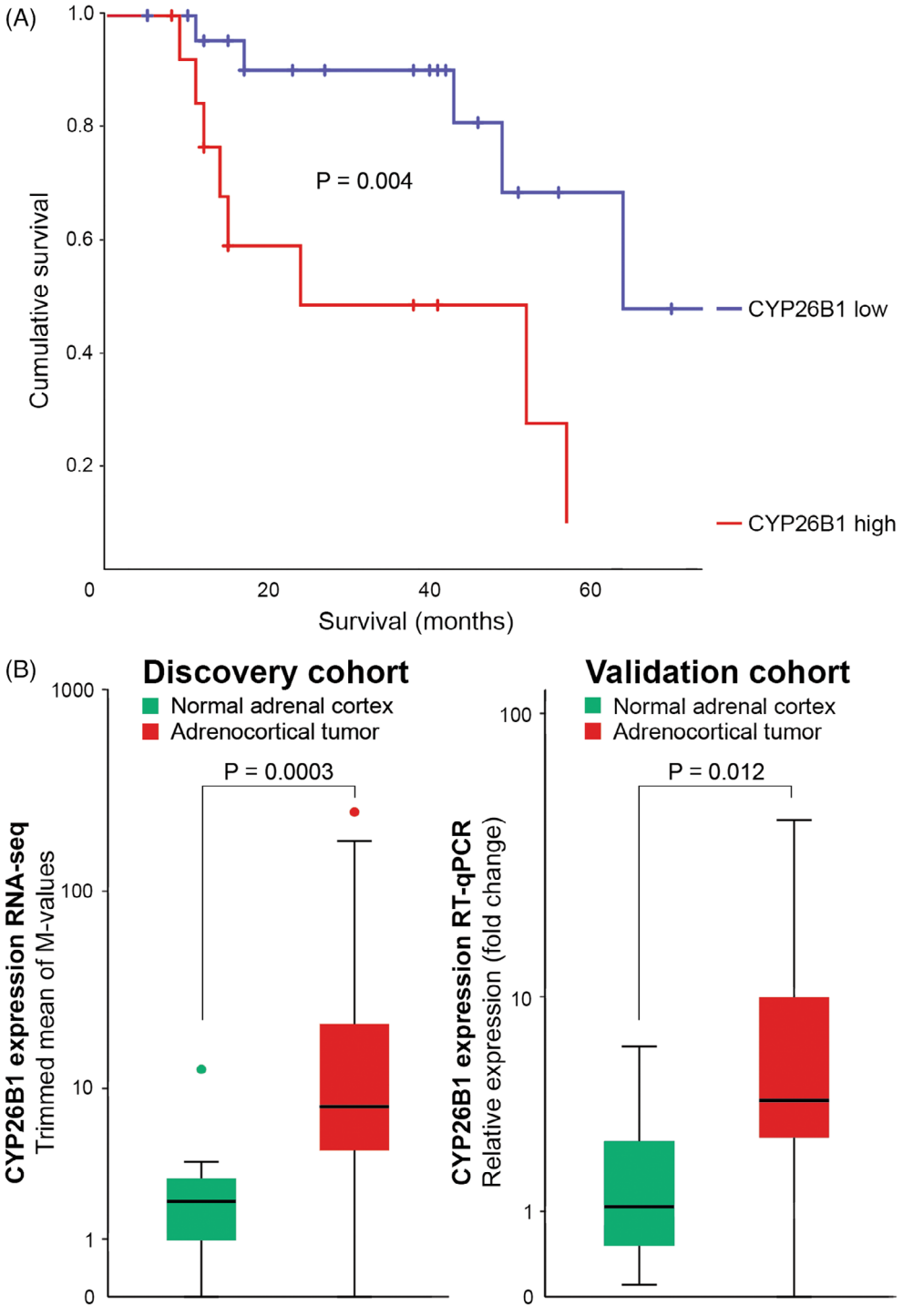


FIGURE 5 *CYP26B1* mRNA expression. (A) When the csACTs with follow-up data ($n = 35$) were classified as having either high (Trimmed mean of M-values [TMM] ≥12.2; $n = 13$) or low (TMM <12.2; $n = 22$) CYP26B1 expression, these groups had significantly different survival times after surgery. (B) To validate the significant differences in CYP26B1 mRNA expression between cortisol-secreting adrenocortical tumours ($n = 48$) and normal adrenal cortices (NACs) in the discovery cohort (RNA-seq), we performed RT-qPCR on CYP26B1 in an independent validation cohort with cortisol-secreting adrenocortical tumours ($n = 25$) and normal adrenal cortices ($n = 8$), which also showed significant differences. ***$p < .001$; *$p < .05$

improve the prognostic prediction of dogs with csACTs after adrenalectomy. The survival analyses, however, do have some limitations. The follow-up monitoring after surgery was not standardized for all dogs, because some were monitored in our University clinic, while some were monitored in external specialist clinics. Although assessment of UCCRs was routinely performed 2 months after surgery for all dogs, the follow-up period after this was not standardized. Therefore, it is possible that cases with recurrence but with limited clinical signs of hypercortisolism could have been missed. In addition, for the dogs with recurrence, it was not always determined whether this was caused by metastases or by regrowth of the tumour.

While csACT Group 1 has a more favourable prognosis compared with csACT Group 2, it does not cluster more closely with the NACs in the PCA plots. In human ACTs, transcriptomic analyses showed that ACAs cluster closely together with NACs, while ACCs cluster apart from the NAC/ACA groups.[16] CsACT Group 1 and csACT Group 2 might therefore not represent the classical ACA versus ACC classification. The underlying cause for the different gene expression

patterns in these groups is currently still unknown. It would be interesting to perform, for example, whole genome sequencing on these samples to determine whether the different gene expression patterns and survival times could be explained by different tumour-driving mutations.

In the normal adrenal cortex, progenitor cells are located in the subcapsular region within the zona glomerulosa.[31] After differentiating into functional hormone-producing zona glomerulosa cells, these cells migrate inward over time, transdifferentiate into cells of the zona fasciculata and subsequently of the zona reticularis, and will eventually undergo apoptosis at the cortical-medullary boundary.[31] Of note is that several zona glomerulosa markers, such as *SHH*[31] and *DAB*,[32] are expressed significantly higher in csACT Group 2 than in csACT Group 1, while the opposite is true for several zona reticularis markers, such as *CYB5A*[33] and *FGG*[34] (Figure 2, Data S3 and S4). These different expression profiles could suggest that the group classification is related to the location in the adrenal cortex from which the csACTs arise. For example, csACT Group 2 tumours could arise from progenitor cells located in the zona glomerulosa, while csACT Group 1 tumours could arise from further differentiated cells near or in the zona reticularis. Alternatively, the csACTs could either de-differentiate towards a more progenitor-like phenotype (csACT Group 2) or further differentiate towards their end-station (csACT Group 1). In both hypotheses, the progenitor-like phenotype of csACT Group 2 compared with the further differentiated phenotype of csACT Group 1 could explain the observed differences in survival times after adrenalectomy.

As a therapeutic target, a gene would be most interesting when it is expressed higher (or lower) in csACTs compared with NACs, as well as in csACTs with poor prognosis compared with those with favourable prognosis. Theoretically, pharmaceutically targeting a product of such a gene could then work in (almost) all csACTs, but even better in those with poor prognosis (which is the most important group in terms of requiring additional therapeutics). We therefore looked at which genes were differentially expressed in both comparisons. We found eight genes that fulfilled this criterium, of which three were significantly associated with survival times after surgery. One of these genes, *CYP26B1*, was not only prognostically relevant in our data set of canine csACTs, but also in a publicly available data set of 33 human cortisol-secreting ACCs. Additionally, *CYP26B1* expression was also increased in an independent validation cohort of 25 csACTs compared with 8 NACs of dogs, as assessed with RT-qPCR.

In addition to its prognostic value, the function of the CYP26B1 protein also makes it an interesting treatment target. CYP26B1 is an enzyme that metabolizes all-trans-retinoic acid (ATRA), the most biologically active metabolite of vitamin A (retinol).[29] ATRA is an important regulator of cell differentiation, proliferation and apoptosis[35] and has been reported to inhibit cancer development by inducing differentiation and/or apoptosis.[36] CYP26B1 can metabolize ATRA into less biologically active intermediates,[29] and therefore reduces ATRA-induced differentiation or apoptosis.[35] Previous studies have shown that increased expression of CYP26A1, an enzyme that is closely related to and has similar functions as CYP26B1, increases the resistance of cells to apoptogenic factors.[35] Increasing CYP26 expression could therefore be a protection mechanism of tumour cells against the influence of ATRA. Indeed, studies in other tumour types such as neuroblastoma have shown that in response to ATRA, cells can increase their CYP26A1 expression.[37] Subsequently, inhibition of CYP26B1 activity could be a valuable treatment strategy. CYP26B1 has also been identified as a prognostic marker in other cancer types, such as colorectal cancer.[29]

In human ACTs, a previous meta-analysis study identified disturbed retinoic acid (RA) signalling as a major pathogenetic pathway.[38] To our knowledge, this has not previously been linked to CYP26B1. In a subsequent study, these researchers tried to exploit this finding by treating xenograft mice inoculated with an ACC cell line with 9-cis retinoic acid (9-cisRA), an isomer of ATRA. They found that 9-cisRA significantly reduced the tumours' Ki67 proliferation index.[39] Although this was promising, the usefulness of ATRA therapy is limited because resistance can rapidly emerge, partly due to accelerated RA metabolism.[40] A strategy to overcome this is to inhibit the enzymes that metabolize RA, also referred to as retinoic acid metabolism blocking agents (RAMBAs).[40] Especially in tumour types where CYP26B1 seems to be involved in tumorigenesis, such as in canine and human csACTs, using RAMBAs specifically aimed at CYP26B1 seem particularly useful to inhibit tumour growth. CYP26B1 inhibitors are available and are under investigation for several diseases.[30,41,42]

5 | CONCLUSION

With this study, we have gained important insights into the molecular pathways that underly tumorigenesis in canine csACTs. We have identified two transcriptionally distinct groups of csACTs. These groups do not seem to represent the classical ACA versus ACC classification, but rather a more progenitor-like phenotype versus a further differentiated phenotype. It would be interesting to determine whether the classification of these groups is related to their mutational background. We have identified *CYP26B1* as a novel prognostic marker and as a promising potential therapeutic target in both canine and human csACTs. This represents an important proof-of-concept of research in spontaneously-occurring ACTs of dogs that could lead to novel discoveries in ACTs of humans, highlighting the importance of this comparative model. In future in vitro and in vivo studies, it would be interesting to determine whether CYP26B1 inhibitors can inhibit tumour growth in both dogs and humans with ACTs.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

The raw data files are available in the Gene Expression Omnibus (GEO) repository, accession number GSE196108. The analysed data can be found in the Data S1.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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