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A proteome-based classification of paediatric adrenocortical tumours links functional tumour states to clinical outcome and therapeutic vulnerabilities



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Summary

Background Paediatric adrenocortical tumours (pACT) are rare endocrine neoplasms characterised by marked biological heterogeneity and variable clinical behaviour. Current risk stratification relies largely on histopathology and clinical staging, which incompletely capture underlying tumour biology. We aimed to define proteome-based subtypes of pACT and evaluate their biological, clinical, and therapeutic relevance.

Methods In this retrospective cohort study, we performed mass spectrometry-based proteomic profiling of 83 pACT and adjacent non-tumour adrenal tissues. Differential abundance analysis, pathway enrichment, consensus clustering, survival analyses, and clinicopathological correlations were performed. A minimal protein classifier was derived using forward feature selection and leave-one-out cross-validation. Cluster-specific therapeutic vulnerabilities were assessed using a curated druggability framework.

Findings Tumours displayed profound proteomic differences compared with adjacent non-tumour adrenal cortex, characterised by activation of cholesterol biosynthesis, steroidogenic, MYC, mTORC1, and cell-cycle programs and suppression of extracellular matrix, adhesion, and immune-related pathways. Unsupervised clustering identified four proteome-defined tumour states that only partially overlapped with histopathological diagnosis. These subtypes differed significantly in endocrine phenotype, vascular invasion, Ki67 index, necrosis, and overall survival (log-rank $p = 0.00036$). Distinct biological programs included stromal-immune-enriched, mitochondrial steroidogenic, IGF/mTOR-driven anabolic, and highly proliferative chromatin-remodelled states. A five-protein classifier (DAAM2, CIP2A, TSC2, PALS2, and P3H1) reproduced cluster assignments with 92.8% leave-one-out

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cross-validation accuracy. Cluster-specific analyses identified distinct therapeutic target landscapes, including IGF-axis and epigenetic vulnerabilities.

Interpretation Proteomic profiling defines biologically and clinically distinct pACT subtypes that provide prognostic information beyond histopathology. This framework enables proteome-based stratification, identifies candidate therapeutic vulnerabilities, and can be approximated by a five-protein classifier.

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Keywords: Paediatric adrenocortical tumour; pACT; Proteomics; Classification; Subtypes

Research in context

Evidence before this study

We reviewed the literature before undertaking this study by searching PubMed from 2020 to 2025. Search terms included "paediatric adrenocortical tumour", "childhood adrenocortical carcinoma", "adrenocortical tumour proteomics", "adrenocortical carcinoma proteome", "IGF2", "TP53", "methylation", "transcriptomics", and "molecular classification". We also screened reference lists of relevant reviews and original research articles. We considered studies in paediatric and adult adrenocortical tumours that reported genomic, transcriptomic, methylation, proteomic, clinical, or prognostic data. We did not restrict by language. Previous studies have established that paediatric adrenocortical tumours (pACT) are biologically heterogeneous and frequently associated with *TP53* alterations, *IGF2* overexpression, and changes in DNA methylation. Transcriptomic studies have suggested prognostic gene expression patterns, and adult proteomic studies have described altered metabolism, steroid hormone pathways, and mitochondrial programs in adrenocortical carcinoma. However, most proteomic studies were exploratory, focused mainly on tumour-vs.-control comparisons, and did not define clinically annotated tumour subtypes. Overall, the available evidence suggested that current histopathological classification does not fully capture the biological diversity of pACT.

Added value of this study

This study applies deep proteomic profiling to clinically annotated pACT and matched adrenal tissue. We show that

tumour proteomes differ strongly from adjacent non-tumour adrenal cortex and are characterised by activation of lipid, steroid, growth, and cell-cycle programs together with suppression of extracellular matrix and immune-related pathways.

Using unsupervised analysis, we identify four proteome-defined tumour states that only partially overlap with standard histopathological categories. These states differ in endocrine activity, proliferation, vascular invasion, necrosis, and survival. We further show that the proteome-based classification can be approximated by a compact five-protein classifier and that each tumour state is associated with distinct, potentially druggable pathways.

Implications of all the available evidence

Together with previous genomic, transcriptomic, and methylation studies, our findings suggest that pACT should be understood as a biologically diverse group of diseases rather than as a single entity defined only by histology. Proteomic profiling adds functional information about the proteins and pathways active in each tumour state, which may improve biological interpretation and future risk stratification.

The proposed protein classifier and therapeutic target landscape are not yet ready for routine clinical use and require independent validation. However, they provide a framework for future studies aiming to develop practical diagnostic tools, refine prognostic assessment, and design biology-informed treatment strategies for children and adolescents with pACT.

Introduction

Adrenocortical tumours (ACT) in children and adolescents are exceedingly rare, yet represent one of the most challenging paediatric endocrine malignancies. Their incidence is estimated at ~0.2–0.3 cases per million children annually worldwide. There is a striking

geographic clustering in southern Brazil due to a founder *TP53* variant (p.R337H).^{1,2} Paediatric ACT (pACT) encompass a clinical spectrum ranging from benign adenomas (ACA) to aggressive adrenocortical carcinomas (ACC). More than 80% are hormonally active, typically presenting with virilisation, Cushing

syndrome, or combined androgen–cortisol excess, which not only leads to significant morbidity but can also accelerate tumour detection.^{1,3–5}

Despite decades of research, accurate diagnosis and risk stratification remain major hurdles in pACT management. Histopathological classification, traditionally adapted from adult scoring systems such as Weiss and Van Slooten, has limited reliability in children and adolescents. The paediatric-specific Wieneke criteria improved discrimination between ACA and ACC, but prognostic accuracy remains suboptimal.^{6–8} Clinical factors such as age, stage, and extent of resection strongly influence outcome, yet patients with similar clinicopathological profiles may follow divergent clinical courses.^{9,10} Current systemic therapies for advanced disease rely on mitotane and cisplatin-based regimens largely extrapolated from adult ACC protocols, with dismal long-term survival in metastatic disease (5-year survival <20%).^{1,11} There is an urgent need for molecularly informed diagnostic and prognostic biomarkers, as well as novel therapeutic targets.¹²

Genomic and transcriptomic studies have substantially advanced our understanding of pACT biology.^{3,13–16} Comprehensive sequencing revealed recurrent alterations in *TP53*, *ATRX*, and *CTNNB1*, frequent loss of heterozygosity at 11p, and nearly universal IGF2 overexpression.³ These discoveries underscore the developmental and genetic context of pACT, including links to hereditary cancer syndromes and imprinting disorders. More recently, transcriptomic profiling has identified prognostic gene expression signatures and pathway dependencies, including a 15-gene hub signature associated with 5-year event-free survival.¹⁴ In adult ACC, integrated multi-omic characterisation has defined molecular subtypes with distinct outcomes.^{17,18} Nevertheless, transcriptional profiles do not fully capture the regulatory complexity that dictates tumour phenotype. Post-transcriptional modifications, protein abundance, and signalling pathway activation remain incompletely resolved at the RNA level.^{19,20}

Proteomic approaches provide complementary insight, capturing the functional layer of molecular biology most proximal to cellular phenotype.^{21,22} In adult ACT, mass spectrometry-based studies have revealed proteomic alterations, including upregulation of metabolic enzymes, cytoskeletal proteins, and mitochondrial pathways, as well as candidate prognostic markers.^{23–26} Recent studies identified the IGF2 protein (juxtanuclear Golgi pattern) in 80% of carcinomas vs. 0% of adenomas, establishing it as the most discriminating diagnostic biomarker at the protein level.²⁶ Additionally, a mass spectrometry study identified 64 upregulated and 48 downregulated proteins distinguishing ACC from adenoma, with evidence of Warburg effect metabolic reprogramming.²⁵ Jang et al.²⁷ further demonstrated that proteomic profiles predict survival in ACC, highlighting their potential for clinical translation. In

contrast, pACT have not yet been studied at the proteomic level.

Together, these gaps motivated a systematic proteomic analysis of pACT. We prespecified the objectives of this retrospective cohort study as follows: first, to define protein abundance differences between pACT and adjacent non-tumour adrenal cortex; second, to determine whether tumour-only proteomic profiles identify proteome-based subtypes beyond histopathological classification; third, to evaluate associations between proteome-defined subtypes and clinicopathological features, including outcome; and fourth, to explore whether subtype-specific protein programs nominate diagnostic markers and therapeutic vulnerabilities.

Here, we report a comprehensive proteomic analysis of pACT integrating quantitative protein abundance data with clinicopathological annotation to address these objectives.

Methods

This proteomics study of pACT included patient samples based on availability, without selection for sex. Both male and female patients were represented in the cohort; however, the study was not specifically powered or designed to assess sex-based differences. Given the rarity of pACT, inclusion was driven by sample accessibility to maximise cohort size. While sex-specific biological differences cannot be excluded, the molecular mechanisms investigated are expected to be broadly relevant across sexes, as they reflect core tumour biology rather than sex-specific processes. Data on race, ethnicity, and disability were not collected and did not inform patient selection.

Study design and ethical approval

We obtained the proteomic profiling cohort of pACT specimens through the German Malignant Endocrine Tumour (MET)-Registry (n = 83 samples from n = 72 patients) including matched non-tumour adrenal controls (n = 43 specimens). Tumour specimens included ACT subtypes annotated as ACC, ACA, and adrenocortical neoplasms of uncertain malignant potential (ACX). Clinicopathological variables included sex, age at diagnosis, functional activity/endocrine phenotype, Ki67 index, Children's Oncology Group (COG) stage, pathological tumour (pT) stage, and follow-up parameters including death status and overall survival time ([Supplementary Table S1](#)). All samples and associated clinical data were pseudonymised.

Patient material, clinical data, and written informed consent for data and biospecimen use in scientific projects were obtained from all included patients and/or legal guardians at the time of registry enrolment, in accordance with national regulations. The MET studies were conducted in accordance with the Declaration of

Helsinki and were approved by the ethics committees of the University of Lübeck (IRB 97125) and Otto-von-Guericke University Magdeburg (IRBs 174/12 and 52/22), Germany. The “Molecular basis of Paediatric Adrenocortical Tumours” (MoPACT) project (IRB 23-0351) was approved by the Ethics Committee of the Ludwig-Maximilians-Universität (LMU) Munich, Germany.

Processing of tissue samples

Tissue samples were fixed in buffered 10% formalin after surgical resection and processed into formalin-fixed, paraffin-embedded (FFPE) tissue blocks after extended fixation (typically 12–15 days). FFPE tissue was sectioned at 10 µm and mounted on adhesive glass slides (TOMO IHC Adhesive Glass Slide, Matsunami) or membrane slides (1.0 PEN, Zeiss). Representative tissue sections were deparaffinised and stained with haematoxylin and eosin for histopathological assessment. Tumour regions were inspected and marked by board-certified pathologists, and the annotated tumour regions were subsequently macrodissected (scalpel-based) to enrich for tumour content prior to proteomic analysis; adjacent non-tumour adrenal regions were selected analogously from non-tumour tissue. Macrodissection was performed from a standardised tissue area of 25 mm² per sample.^{28,29}

Proteomics measurement and primary quantification

Macrodissected FFPE tissue sections were collected into a 96-well format (AFA-TUBE TPX Plate, #520291, Covaris), with edge wells left empty to minimise evaporation or temperature gradient effects. Tissue was lysed and proteins extracted from FFPE sections using a TEAB/DDM-based lysis buffer [70 mM triethylammonium bicarbonate (#T7408, Sigma Aldrich) and 0.013% n-Dodecyl β-D-maltoside (#D4641, Sigma Aldrich)] at 90 °C for 1 h, followed by Adaptive Focused Acoustics (AFA) sonication on a Covaris LE220Rsc (Covaris). Acetonitrile (#83640.290, VWR) was added to a final concentration of 12.5%, and samples were incubated at 75 °C for 1 h. Proteins were digested overnight at 37 °C with 1 µg each of trypsin (#T6567, Sigma Aldrich) and LysC (#129-02541, Wako), and digestion was quenched by acidification to 1% TFA (trifluoroacetic acid, #80457, Merck).^{28,29}

LC–MS/MS analysis was performed on a Thermo Orbitrap Astral mass spectrometer (RRID:SCR_026205, ThermoFisher, DIA mode 4th isolation windows with a maximum injection time of 5 ms) coupled to an Evosep One system (60 samples/day, 21-min gradient), equipped with a FAIMS Pro interface (–40 V). Approximately 200 ng of peptides were injected and separated on an IonOpticks Aurora Rapid 8 cm × 150 µm C18 column (#AUR3-80150C18-XT, IonOpticks) at 50 °C. Raw data were analysed using DIA-NN (v1.8.1, RRID:SCR_022865) in library-free mode, with a deep-learning

predicted spectral library from the UniProt human reference proteome, with precursor and protein group FDR controlled at 1%.³⁰

Primary data analysis, quality control and normalisation

Downstream processing was performed in R (v4.5.2). Per-sample completeness was assessed; empty controls were used for quality control only and excluded from analyses. Protein filtering and imputation followed a dual-filter strategy adapted from our previous workflow²⁸; multivariate and visualisation analyses used proteins detected in ≥50% of samples within every group (these methods being sensitive to missingness), whereas differential-abundance testing used a more permissive filter (≥3 valid values in ≥1 group) that preserves group-specific proteins a stricter filter would discard. Intensities were log₂-transformed, and missing values in the liberally filtered matrix were imputed sample-wise from a down-shifted Gaussian distribution (width 0.3 SD, down-shift 1.8 SD) as in that study. Results were robust to these thresholds: sensitivity analyses across strict (40–70% detection per group) and liberal (2–5 valid values) settings gave a stable differentially abundant protein set (Jaccard ≈ 0.89) and a four-cluster assignment that remained highly concordant with the reported clustering (adjusted Rand index 0.79–0.94).³¹ For analyses requiring standardised features, row-wise Z-score normalisation was applied to the strictly filtered log₂ matrix. Missingness was predominantly abundance-dependent (detection rate increased with mean intensity; Spearman rho = 0.75), consistent with a left-censored (MNAR) mechanism; remaining values in the liberally filtered matrix were imputed by a single sample-wise down-shifted Gaussian draw, and the differentially abundant proteins were robust to this choice (94% recovered under an alternative MNAR-aware imputation, QRILC).

Bioinformatics and statistical analysis

Exploratory analyses were performed on the filtered dataset (tumour and control; empty controls excluded). PCA (prcomp) and UMAP (umap R package; default parameters, fixed seed) were computed from row-wise Z-score-normalised protein profiles.³² To rank proteins by their overall tumour enrichment, the row-wise Z-score-normalised abundances (standardised across all samples) were averaged across the tumour samples for each protein, and proteins were ordered by descending mean tumour Z-score. Sample similarity was visualised as hierarchically clustered Euclidean distance heatmaps (Ward’s D2 linkage). For display heatmaps, the 500 most variable proteins [by median absolute deviation (MAD)] were selected. Separation of tumour and control samples in the unsupervised proteome was tested by PERMANOVA (adonis2, vegan; Euclidean distance on Z-scored profiles, 999

permutations), with homogeneity of multivariate dispersion assessed by betadisper.

Differential protein abundance (tumour vs. control) was tested using limma on the imputed, liberally filtered log₂ matrix.³³ A cell-means model ($\sim 0 + \text{Group}$) with a tumour–control contrast was fitted, and moderated t-statistics were obtained via eBayes with robust empirical Bayes moderation. p-values were adjusted by Benjamini–Hochberg (BH) correction; significance thresholds were $\text{FDR} < 0.05$ and $|\log_2 \text{FC}| \geq 1$.

Functional enrichment combined over-representation analysis (ORA) and pre-ranked gene set enrichment analysis (GSEA).³⁴ ORA was run separately on proteins with increased and decreased abundance against GO Biological Process (GO:BP), KEGG, and Reactome databases, with the full set of quantified proteins as background. The GO:BP category–protein network (cnetplot) visualises this over-representation result for the upregulated proteins: term nodes represent enriched GO:BP categories, and protein nodes the contributing members drawn from the significantly increased proteins ($\text{FDR} < 0.05$, $|\log_2 \text{FC}| \geq 1$). For GSEA, a ranked list was generated from moderated t-statistics and tested against MSigDB Hallmark (H), C2 Canonical Pathways (C2:CP), oncogenic signatures (C6), and chemical/genetic perturbation (C2:CGP) collections.³⁵ To identify the most robust biological signals, all significant results (adjusted $p < 0.05$) were integrated across databases using two complementary approaches: (i) a multi-source convergence analysis using Active-Pathways (Brown’s method for combining correlated p-values across sources, FDR-corrected); and (ii) extraction of leading-edge proteins from all GSEA results to identify proteins recurrently driving enrichment across multiple independent gene sets. A composite priority score was computed to rank biological themes by their breadth of cross-database support, effect size, and mechanistic centrality.

Supervised tumour subgroup analyses were performed on samples classified as ACC, ACA, or ACX. An omnibus limma moderated F-test identified discriminating proteins, followed by pairwise contrasts. A limma trend model with ordinal coding (ACA = 1, ACX = 2, and ACC = 3) tested for monotonic abundance changes along the putative malignancy spectrum. Directed GSEA (ACC vs. ACA) was performed against GO:BP and Reactome.

Unsupervised tumour stratification used consensus clustering (ConsensusClusterPlus; Ward’s D2, Euclidean distance, $k = 2\text{--}6$, 1000 iterations, 80% sample resampling, all features) on the full tumour Z-score matrix.³⁶ The number of clusters ($k = 4$) was selected using the consensus cumulative distribution function (CDF) delta-area criterion, corroborated by a non-negative matrix factorisation rank survey (ranks 2–6, 30 runs per rank, Brunet algorithm) and by silhouette analysis across k . Cluster-defining proteins were

identified by limma one-vs.-rest contrasts (BH-adjusted $p < 0.05$, $\log_2 \text{FC} > 0.5$). Cluster associations with clinical variables were tested by Fisher’s exact (categorical) or Kruskal–Wallis (continuous) tests. Composite pathway and immune-signature scores were likewise compared across clusters by Kruskal–Wallis, with Dunn’s post-hoc test (Benjamini–Hochberg) for significant immune signatures. Overall survival was analysed by Kaplan–Meier estimation with log-rank testing.

A minimal immunohistochemistry (IHC) panel was derived by greedy forward selection with leave-one-out cross-validated nearest-centroid classification (panel size 5–20; significance assessed by 1000-permutation test).

Adrenal zonation was inferred from curated marker panels for the zona glomerulosa (ZG), zona fasciculata (ZF), and zona reticularis (ZR) (Supplementary Table S2). Markers were mapped to quantified protein groups (retaining the highest-variance group per gene symbol), and each per-sample zone score was the mean Z-scored abundance of these markers, requiring at least two detected markers per zone. The three zone scores were then converted to compositional proportions ($p_{\text{ZG}} + p_{\text{ZF}} + p_{\text{ZR}} = 1$) by softmax transformation (Supplementary Methods) and visualised as ternary diagrams. Analyses aimed at clinical translation comprised: (i) drug repurposing via CMap-proxy GSEA (C2:CGP perturbation signatures); (ii) druggable proteome annotation against a curated database of ~360 targets across 20 pharmacological families (Supplementary Table S3); (iii) kinase activity inference by single sample (ss)GSEA on KEGG/Reactome signalling pathways; (iv) pathway-specific analyses of IGF2/IGF1R, Wnt/ β -catenin, and TP53 signalling (Supplementary Tables S4–S6); (v) immune microenvironment estimation using curated cell-type markers (Supplementary Table S7); (vi) foetal-vs.-adult adrenal signature scoring (Supplementary Table S8); and (vii) metabolic vulnerability profiling (ssGSEA on KEGG/Hallmark metabolic pathways).

Detailed descriptions of all analyses including the key packages used in R v4.5.2 (R Core Team, 2025) are available in the Supplementary Methods and Supplementary Table S9. All tests were two-sided; BH correction at FDR 5% was applied unless stated otherwise.

Role of the funding source

The funders had no role in the design, data collection, data analysis, interpretation, and reporting of this study.

Results

Study cohort and proteome profiling of paediatric adrenocortical tumours and adjacent non-tumour adrenal tissue

We analysed a clinically annotated cohort of children and adolescents diagnosed between 1999 and 2019,

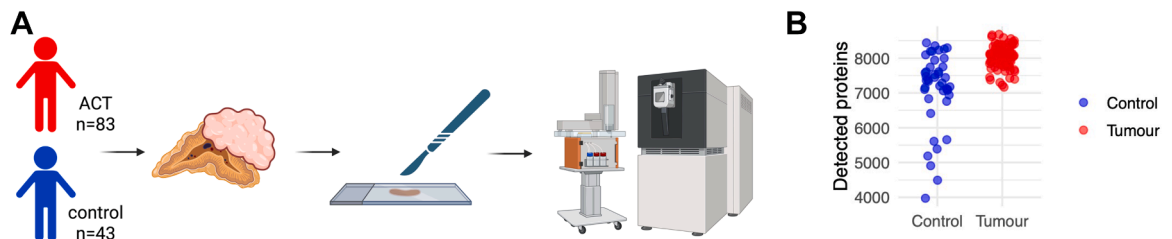


Fig. 1: Cohort overview, sample processing workflow and proteome depth. (A) Schematic overview of cohort composition and proteomics workflow. Formalin-fixed, paraffin-embedded (FFPE) tissue from paediatric adrenocortical tumour specimens (tumour; $n = 83$) and matched non-neoplastic adrenal cortex (control; $n = 43$) were collected at surgery, followed by standardised macrodissection, protein extraction and digestion, and quantitative liquid chromatography - mass spectrometry (LC-MS)-based proteome profiling. Partly created with BioRender. (B) Number of protein groups detected per sample, shown for adjacent non-tumour adrenal cortex (control; blue) and tumour tissue (red). Each dot represents one sample. Protein counts are derived from the unfiltered raw expression matrix prior to any quality or completeness filtering.

comprising 83 pACT samples from 72 patients and 43 adjacent non-tumour adrenal cortex specimens from the same patients (Fig. 1A). Tumours were classified according to the Wieneke criteria by centralised reference pathology review into ACC ($n = 42$), ACA ($n = 25$), and ACX ($n = 16$). Median age at diagnosis was 3.5 years (range 0.3–17.8), with a female-to-male ratio of 2.3:1. The majority of tumours were hormonally functional (77%; 54/70 with available endocrine phenotype data), with virilisation as the predominant phenotype ($n = 34$), followed by combined virilisation and Cushing syndrome ($n = 13$), Cushing syndrome alone ($n = 7$), and non-functional tumours ($n = 16$). Clinical and pathological characteristics are summarised in [Supplementary Table S1](#).

Proteome profiling yielded a mean of 8064 proteins per tumour sample (range 7165–8686) and 7123 per adjacent non-tumour adrenal cortex sample (range 3973–8442). In total, 10,714 non-redundant protein groups were quantified across the cohort. Tumour samples displayed significantly higher proteome depth than adjacent non-tumour adrenal tissue (mean difference: 941 proteins; $p < 0.001$; Fig. 1B, [Supplementary Figure S1A](#)), with greater inter-sample variability in control tissue (SD 1068 vs. 328). The wider range of protein identifications among non-tumour adrenal samples likely reflects their intrinsic zonal heterogeneity together with a variable admixture of lower-cellularity peri-adrenal tissue (e.g. adipose and connective tissue), whereas tumour samples were generally more cellular and compositionally homogeneous. Of 10,714 protein groups, 216 (2.0%) were removed due to insufficient detection across both sample groups. A strict completeness filter ($\geq 50\%$ of samples in each group) retained 7035 proteins for all multivariate and visualisation analyses. A parallel liberal filter retained an additional 3463 group-specific proteins, predominantly tumour-exclusive, yielding 10,498 proteins available for differential abundance analysis ([Supplementary Figure S1B and C](#)). Raw and $\log_2$ -transformed

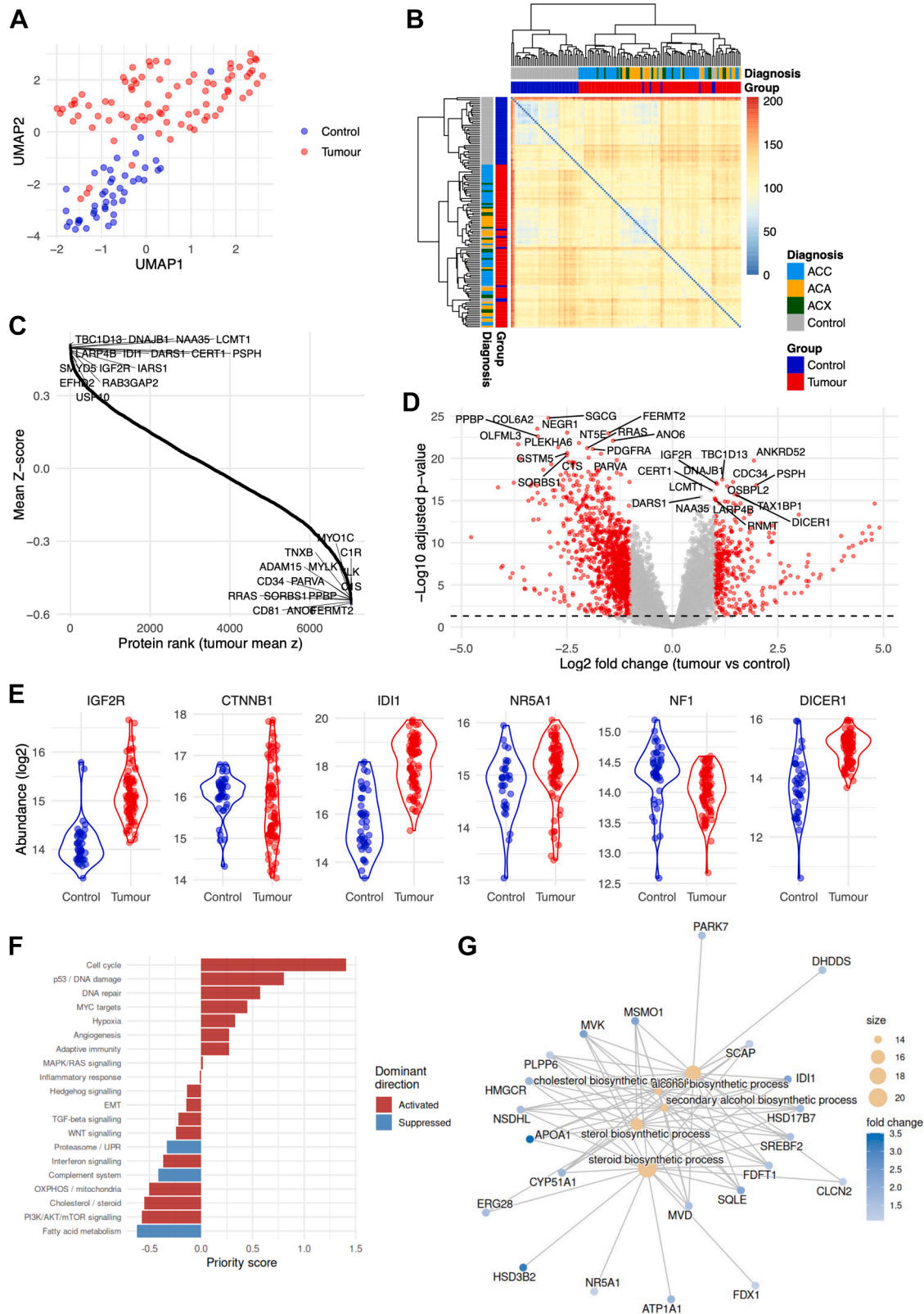
intensity distributions were consistent across the cohort ([Supplementary Figure S1D and E](#)).

Tumour and adjacent non-tumour adrenal tissues show distinct global proteome structure and broad differential protein abundance

We first examined whether global protein abundance profiles distinguish tumours from adjacent non-tumour adrenal cortex. Unsupervised visualisation and clustering of all samples revealed a clear separation of tumour and control tissues. UMAP projection showed that the dominant axes of proteomic variation align with the tumour identity (Fig. 2A), and sample-to-sample distance profiling together with hierarchical clustering identified two major branches corresponding to tumour and matched adrenal control samples (PERMANOVA on proteome distances, $R^2 = 0.10$, $p = 0.001$; comparable multivariate dispersion between groups, $\text{betadisper } p = 0.39$; Fig. 2B). Clustering of the 500 most variable proteins further emphasised coherent expression programs separating tumours from adjacent non-tumour adrenal cortex ([Supplementary Figure S2A](#)).

To identify proteins consistently altered in tumours, we ranked all proteins by their mean tumour-centred Z-score. This analysis highlighted a set of proteins uniformly elevated across tumours, including TBC1D13, DNAJB1, LCMT1, CERT1, IGF2R, and IDI1, and a corresponding group consistently reduced, including FERMT2, SORBS1, RRAS, and TNXB (Fig. 2C), providing reference candidates for subsequent analyses.

Differential abundance analysis using a linear model with empirical Bayes moderation quantified the extent of tumour-control divergence. At $\text{FDR} < 0.05$ and $|\log_2\text{FC}| \geq 1$, 342 proteins were significantly increased and 956 significantly decreased in tumours, yielding 1298 differentially abundant proteins in total. The volcano plot demonstrated widespread dysregulation with strong statistical support (Fig. 2D). The most



significantly increased proteins included ANKRD52, TBC1D13, CDC34, IGF2R, and DNAJB1 (adjusted $p < 10^{-17}$), whereas the most significantly decreased proteins, SGCG, COL6A2, NEGR1, FERMT2, and OLFML3 (adjusted $p < 10^{-22}$), were predominantly linked to extracellular matrix (ECM) organisation and adhesion programs. Representative examples, some linked to pACT pathogenesis, illustrate the magnitude and consistency of protein differences are shown in Fig. 2E.

Pathway-level analyses condensed the protein-level alterations into coherent biological themes, revealing a fundamental metabolic and signal transduction rewiring in pACT. An integrated pathway priority analysis identified p53/DNA damage, DNA repair, PI3K/AKT/mTOR signalling, TGF- β signalling, Wnt signalling, MYC targets, cell cycle, cholesterol/steroid metabolism, and OXPHOS/mitochondria and hypoxia programs as coordinated activated, while proteasomal programs, fatty acid metabolism, and complement system were suppressed (Fig. 2F; [Supplementary Methods](#)).

Central to this reprogramming was a pronounced activation of the mevalonate–cholesterol–steroid biosynthesis axis. GO biological process ORA of proteins increased in tumours revealed over-representation of small molecule and steroid biosynthetic processes, alcohol and sterol metabolic processes, cholesterol metabolic and biosynthetic processes, response to reactive oxygen species, DNA replication initiation, and platelet aggregation ([Supplementary Figure S2B](#)). Reactome ORA further confirmed activation of steroid metabolism, cholesterol biosynthesis, SREBP/SREBF-regulated cholesterol biosynthesis and gene

expression, lanosterol biosynthesis, ATR activation in response to replication stress, ECM reorganisation and integrin cell surface interactions ([Supplementary Figure S2C](#)), and single-sample GSEA of KEGG metabolic pathways showed a concordant increase in metabolic pathway activity in tumours ([Supplementary Figure S2D](#)). Network visualisation demonstrated that these cholesterol- and steroid-related processes were driven by a highly interconnected enzyme core, including enzymes of the mevalonate pathway (HMGCR, MVK, MVD, IDI1, FDFT1, SQLE, and PLPP6), post-squalene cholesterol biosynthesis (CYP51A1, MSMO1, NSDHL, HSD17B7, and ERG28), the cholesterol sensor/transcription factor complex (SREBF2 and SCAP), steroidogenesis (NR5A1, HSD3B2, and FDX1), and cholesterol transport (APOA1), collectively indicating coordinated activation of the mevalonate–cholesterol–steroid axis (Fig. 2G).

This metabolic shift was accompanied by parallel rewiring of proliferative and stress-response signalling. GSEA using MSigDB Hallmark gene sets confirmed activation of MYC targets, mTORC1 signalling, cholesterol homeostasis, unfolded protein response, and E2F targets, alongside suppression of epithelial–mesenchymal transition, apical junction, complement, inflammatory response, and angiogenesis pathways in tumours relative to adjacent non-tumour adrenal tissue ([Supplementary Figure S2E](#)).

Collectively, these analyses define pACT as tumours characterised by a coupled metabolic and signal transduction reprogramming: coordinated activation of the mevalonate–cholesterol–steroid biosynthesis network is coupled with engagement of MYC-, mTORC1-, and cell cycle-driven proliferative programs, while ECM

Fig. 2: Global proteome structure, differential abundance, and functional enrichment in tumours vs. adjacent non-tumour adrenal tissue. Unless otherwise noted, visualisation panels (A–C, E) use the strictly filtered matrix (7035 proteins; $\geq 50\%$ detection in each group); differential abundance and enrichment panels (D, F, and G) use the liberally filtered, imputed matrix (10,498 proteins; ≥ 3 valid values in at least one group). (A) Uniform Manifold Approximation and Projection (UMAP) of the analysis-only dataset (tumour and matched adrenal control samples; empty controls excluded). UMAP was computed on the Z-score-normalised protein abundance matrix. Each point represents one sample; colour denotes group (tumour vs. control). (B) Sample-to-sample Euclidean distance heatmap with hierarchical clustering. Annotation bars indicate group (tumour/control) and diagnosis category (ACC/ACA/ACX/control). (C) Tumour-centred protein ranking plot based on mean tumour Z-scores. For each protein, abundances were Z-score normalised across samples and averaged across tumour samples; proteins were ranked by this mean. Selected proteins are labelled. (D) Volcano plot of differential protein abundance between tumours and controls (limma; liberally filtered, imputed matrix). The x-axis shows $\log_2$ fold change (tumour vs. control) and the y-axis $-\log_{10}$ adjusted p-value (Benjamini–Hochberg); points are coloured by significance ($FDR < 0.05$, $|\log_2 FC| \geq 1$). (E) $\log_2$ -transformed abundance for selected proteins from the tumour–control comparison: IGF2R, CTNNA1, IDI1, NR5A1, NF1, and DICER1. Violin shapes depict the distribution; individual points represent samples. Observed values only (no imputation). (F) Multi-source convergence priority ranking of biological themes in tumour vs. control adrenocortical tissue. Horizontal bar plot of the heuristic composite priority score for each a priori-defined biological theme, ranked in descending order. Bar colour indicates the dominant direction of regulation across significant pathways assigned to that theme (red, Activated; blue, Suppressed; based on mean normalised enrichment score, NES). The priority score is a dimensionless composite Z-score integrating cross-database convergence (number of independent enrichment sources with at least one significant pathway for the theme) and effect magnitude (mean absolute NES across significant GSEA pathways for the theme). Unlike the individual per-database analyses shown separately in [Supplementary Figure S2B–E](#) (GO:BP ORA, S2B; Reactome ORA, S2C; KEGG ssGSEA, S2D; Hallmark GSEA, S2E), this panel integrates enrichment results across databases into a single convergence-based theme prioritisation. (G) GO Biological Process category–protein association network (cnetplot) for proteins with increased abundance in tumours. Term nodes represent enriched GO:BP categories; protein nodes represent contributing members; colour encodes $\log_2$ fold change. The network highlights the interconnected enzymes of the mevalonate–cholesterol–steroid biosynthetic axis.

organisation, stromal adhesion, immune surveillance, and developmental signalling programs are concurrently dismantled.

Proteomic heterogeneity across tumour subgroups and adrenal zonation features

Having established robust global differences between tumours and adjacent non-tumour adrenal cortex, we next examined whether proteomic variation also stratifies histological tumour subgroups. Tumour-only samples were compared across ACC, ACA, and ACX using a linear model with empirical Bayes variance moderation (overall F-test). The top 50 discriminating proteins revealed partially overlapping abundance patterns across ACC, ACA, and ACX, with incomplete separation between diagnostic categories (Fig. 3A). Many top-ranked proteins mapped to proteostasis, mitochondrial, and biosynthesis pathways, suggesting differences in cellular state across diagnostic categories despite substantial inter-group overlap.

A focused pairwise contrast between ACC and ACA delineated a carcinoma-specific proteomic signature (Fig. 3B). ACC tumours exhibited a coordinated increase in proteins involved in oxidative phosphorylation, ATP synthesis, ribosome biogenesis, and RNA processing. GSEA using ranked moderated t-statistics confirmed enrichment of mitochondrial respiration and translational programs in ACC relative to ACA and reinforced activation of biosynthesis and energy-generating pathways (Supplementary Figure S3A and B). These findings indicate that carcinomas are characterised by enhanced metabolic and proliferative capacity compared with adenomas.

We next explored whether protein abundance changes follow a graded trajectory across the histological sequence ACA-ACX-ACC. Ordinal trend modelling identified proteins with monotonic increases or decreases, including ABHD14B, ACSF2, COQ8A, ECHDC2, KCTD21, RANBP9, STAT5B, and SYNE3 (Fig. 3C). To assess whether these trends reflect a global

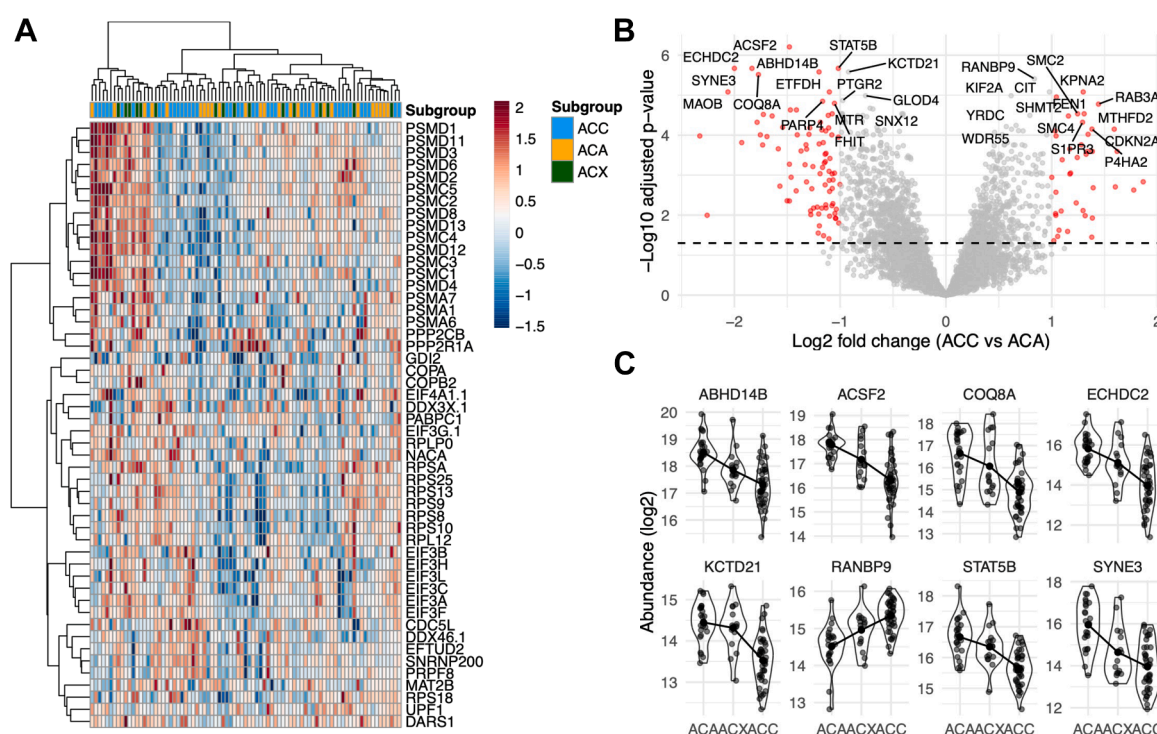


Fig. 3: Proteomic heterogeneity across histopathological subgroups (tumour-only analysis). All panels use the strictly filtered, log2-transformed tumour-only matrix (7035 proteins; ≥50% detection in each group), without imputation. (A) Heatmap of the top 50 proteins discriminating tumour subgroups (F-test across ACC/ACA/ACX, BH-adjusted). Proteins were selected by F-statistic and displayed as Z-score-normalised values; hierarchical clustering of samples and proteins used Ward's method. Annotation bar indicates diagnosis (ACC blue, ACA orange, ACX green). (B) Volcano plot for the pairwise ACC vs. ACA contrast (limma with empirical Bayes moderation; tumour-only matrix). The x-axis shows log2 fold change (ACC vs. ACA) and the y-axis -log10 adjusted p-value (BH). Points are coloured by significance (FDR < 0.05, |log2 FC| ≥ 1); the 15 most significant proteins in each direction are labelled. (C) Violin/jitter plots of the top 8 proteins with monotonic abundance trends across the ordered ACA → ACX → ACC sequence (ordinal limma model, BH-adjusted). Each panel shows log2 abundance per subgroup; the overlaid line connects subgroup means. Proteins shown: ABHD14B, ACSF2, COQ8A, ECHDC2, KCTD21, RANBP9, STAT5B, and SYNE3.

gradient, we tested the correlation between the first ten principal components and ordinal subgroup coding (Spearman, BH-adjusted). PC1 (10.5% variance) showed no significant association ($\rho = 0.19$, adj. $p = 0.29$; [Supplementary Figure S3C and D](#)), confirming that the dominant proteomic variance axis is not aligned with the ACA–ACX–ACC sequence. In contrast, PC2 (9.1% variance; $\rho = 0.64$, adj. $p < 0.0001$) and PC5 (3.9%; $\rho = 0.40$, adj. $p = 0.00086$) captured significant monotonic gradients. These relationships are also directly apparent in two-dimensional sample projections on the PC1/PC2 and PC2/PC5 axes ([Supplementary Figure S3E](#)). Proteins driving the PC2 gradient toward ACC were enriched for DNA replication, repair, and chromatin remodelling (e.g. DNMT1, FEN1, SMC2/4, and MCM2–6), while those decreasing toward ACC were dominated by mitochondrial and organic acid metabolism (e.g. TK2, MCEE, LACTB, and CYP27A1; [Supplementary Figure S3F and G](#)). Thus, ACA-to-ACC progression is associated with a gain of proliferative and genome-maintenance capacity coupled with a loss of differentiated metabolic programs, but this gradient is embedded in secondary variance components rather than dominating the overall proteomic landscape.

Finally, we examined whether inter-tumour heterogeneity relates to adrenal cortical lineage features. Zonation marker scores for zona glomerulosa (ZG), zona fasciculata (ZF), and zona reticularis (ZR) were calculated as the mean Z-score of the best-detected markers per zone ([Supplementary Table S2](#)). Unsupervised clustering of these markers demonstrated clear separation of zonal blocks ([Supplementary Figure S4A](#)), with heterogeneous but structured patterns across tumours. Proportional zonation compositions derived via softmax transformation revealed mixed zone signatures in most tumours ([Supplementary Figure S4B](#)), while ternary plots illustrated subgroup-associated shifts in zonal representation, with ACC tumours exhibiting comparatively constrained patterns ([Supplementary Figure S4C](#)). These observations suggest partial retention and/or reconfiguration of adrenal cortical lineage identity within pACT. This retained cortical identity was carried by a conserved core of adrenocortical steroidogenic and identity proteins—the master steroidogenic transcription factor NR5A1 (SF-1) together with the cholesterol-delivery proteins STAR and SCARB1 and the steroidogenic electron-transfer proteins POR, FDX1, and CYB5A—all of which were more abundant than in non-tumour cortex in both ACC and ACA and did not differ significantly between the two histotypes (BH-adjusted Wilcoxon $p > 0.05$; [Supplementary Figure S4D](#)). This indicates a shared adrenocortical steroidogenic lineage underlying both histotypes, distinct from the zonation composition and proliferative programs that differentiate them.

Collectively, while histological categories capture discrete proteomic differences, the absence of a dominant global gradient across the tumour spectrum suggests additional layers of tumour-intrinsic heterogeneity beyond morphology.

Unsupervised clustering defines four proteome-defined tumour subtypes with distinct functional programs and lineage features

To resolve tumour-intrinsic heterogeneity independent of histopathological diagnosis, we applied consensus clustering to the tumour-only proteome. The consensus cumulative distribution function (CDF) and delta area plots identified a four-cluster solution as the most stable partition ([Supplementary Figure S5A–C](#)), yielding clusters C1–C4 comprising 25, 14, 27, and 17 tumours, respectively, and an independent non-negative matrix factorization (NMF) rank survey supported the same four-cluster solution ([Supplementary Figure S5D](#)). The mean silhouette width of 0.84 indicated robust separation between clusters ([Supplementary Figure S5E and F](#)). In UMAP space, these proteome-defined clusters segregated clearly, while diagnostic labels were distributed non-uniformly across clusters ([Fig. 4A](#)), demonstrating that cluster structure does not recapitulate histopathology.

Cluster composition revealed only partial overlap with histopathological diagnosis. C1 and C2 were enriched for ACA (56% and 57%, respectively), C3 was predominantly ACC (67%), and C4 comprised almost exclusively ACC tumours (88%), with only two ACX cases and no ACA. Structured expression programs underlying these groups were evident in the heatmap of the most variable proteins ([Fig. 4B](#)), with expanded marker panels and enrichment analyses provided in [Supplementary Figure S6](#).

Pathway-level interrogation revealed distinct functional states across clusters. Composite scores of key pACT pathways quantified these distinctions ([Fig. 4C–E](#)). IGF signalling activity differed significantly across clusters (Kruskal–Wallis $p < 0.0001$), peaking in C3 and being lowest in C4. p53 target scores (Kruskal–Wallis $p = 0.0003$) were highest in C1 (and lowest in C3), while Wnt pathway scores (Kruskal–Wallis $p = 0.0004$) were elevated in C1 and lowest in C2. Furthermore, C1 was characterised by enrichment of phagocytosis and actin cytoskeleton remodelling programs alongside relative suppression of sterol biosynthesis and mTORC1 signalling. C2 displayed strong activation of mitochondrial organisation, oxidative phosphorylation, and respiratory chain programs, consistent with enhanced bioenergetic capacity. C3 was distinguished by elevated levels of cholesterol and sterol biosynthesis proteins and mTORC1 activation, consistent with anabolic steroidogenic programs. C4 exhibited the most proliferative phenotype, with enrichment of E2F targets, G2M checkpoint, MYC targets, and chromatin organisation pathways, accompanied by relative

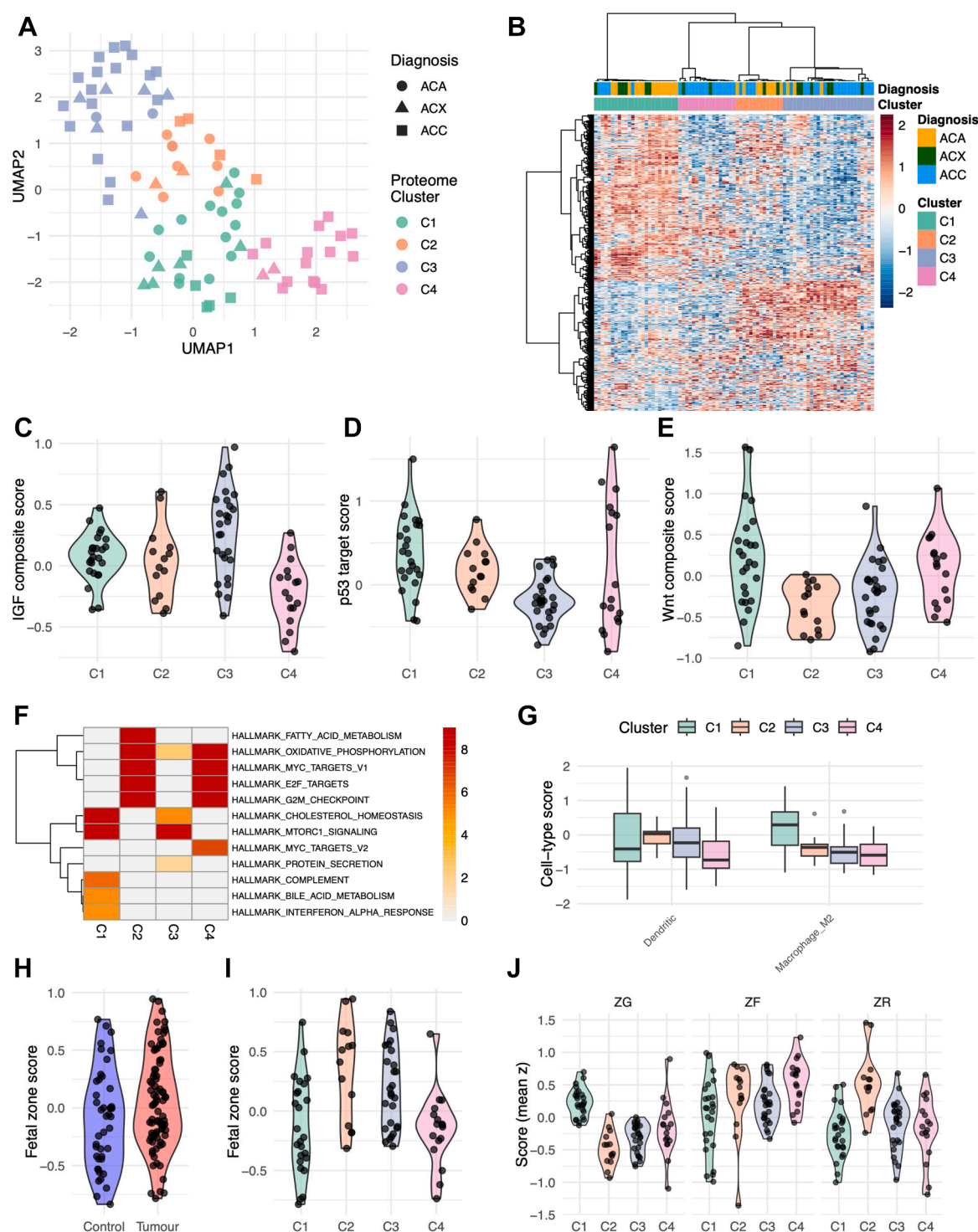


Fig. 4: Proteome cluster-specific functional programs, oncogenic pathway scores, immune microenvironment, and adrenal zonation features. All panels use the strictly filtered, log2-transformed tumour-only matrix (7035 proteins), without imputation. Consensus clustering was performed on all strictly filtered tumour proteins (ConsensusClusterPlus, Ward's D2, Euclidean distance, 1000 resamplings); $k = 4$ was selected based on consensus CDF and delta-area criteria (Supplementary Figure S5). (A) UMAP of the tumour-only proteome coloured by proteome cluster (C1–C4); diagnosis is encoded by point shape (circle = ACA, triangle = ACX, square = ACC). (B) Heatmap of the most variable proteins across all tumour samples, displayed as Z-score-normalised values. Hierarchical clustering of samples used Ward's method.

suppression of TCA cycle activity, consistent with a highly dedifferentiated carcinoma state (Fig. 4F; Supplementary Figure S6A–E). Immune-related features were comparatively limited. Among evaluated immune cell signatures, only M2 macrophage scores varied significantly across clusters (Kruskal–Wallis $p < 0.0001$; post-hoc Dunn’s tests (Benjamini–Hochberg-adjusted) showed M2 scores were significantly higher in C1 than in C2, C3, and C4 ($p = 0.027$, 0.0002 , and 0.0004 , respectively), with no significant differences among C2, C3, and C4 (Fig. 4G). Dendritic cell signatures did not differ significantly (Kruskal–Wallis $p = 0.36$). The enrichment of M2-associated markers in C1 aligns with its phagocytosis and cytoskeletal remodelling profile and likely reflects differences in stromal composition rather than classical tumour-intrinsic immune activation.

Of the 7 foetal adrenal panel proteins, 4 were quantified (IGF2, CYP17A1, CYB5A, and SULT2A1). Foetal adrenal scores showed a trend toward higher values in tumours compared with adjacent non-tumour adrenal tissue (Wilcoxon $p = 0.066$; Fig. 4H), suggesting that this overall difference may be driven by a subset of tumours. Indeed, when stratified by proteome cluster, foetal scores differed significantly (Kruskal–Wallis $p = 0.00055$; Fig. 4I), with clusters C2 and C3 showing the highest values. A mature cortex score could not be computed as most panel members (including CYP11B2, MC2R, and MRAP) fell below the detection limit of the label-free workflow.

Finally, adrenal zonation marker scoring revealed distinct lineage biases (ZG, ZF, and ZR zone scores each differed across clusters; Kruskal–Wallis $p < 0.0001$, $p = 0.005$, $p < 0.0001$, respectively; Fig. 4J; Supplementary Figure S7). C1 exhibited a ZG-like predominance, C2 showed a ZR-like identity (lowest ZG representation), and C3 and C4 were ZF-dominant, with C4 demonstrating the most pronounced fasciculata-like signature. These lineage patterns suggest that proteome-defined tumour subtypes integrate functional metabolic programs with retained or reconfigured adrenal cortical lineage identity.

Collectively, these analyses define four proteome-based tumour subtypes integrating metabolic

specialisation, proliferative capacity, immune context, and adrenal lineage features, extending histopathological classification into a multidimensional molecular framework.

Proteome-based clusters reflect distinct clinical characteristics

Having defined four proteome-based tumour subtypes, we next examined their clinical correlates. Cluster membership was significantly associated with histological diagnosis (Fisher’s exact $p < 0.0001$; Fig. 5A, Supplementary Figure S8A), yet the distribution confirmed only partial concordance between morphology and proteome-defined subtypes. Age at diagnosis differed markedly across clusters (Fisher’s exact $p < 0.0001$; Fig. 5B): C1 and C3 were strongly enriched for young children aged 0–3 years (71% and 58%, respectively), whereas C2 and C4 comprised predominantly older patients (>10 years; 62% and 64%, respectively). In contrast, sex distribution did not differ significantly (Fisher’s exact $p = 0.600$; Fig. 5C), with females representing the majority in all clusters (62–85%).

Tumour stage parameters showed more nuanced patterns. Neither COG stage nor pT stage reached statistical significance across clusters (Fisher’s exact $p = 0.420$ and $p = 0.092$; Fig. 5D and E), although C4 displayed a trend toward higher-stage disease (79% COG stage 3–4; 43% pT4). In contrast, endocrine phenotype differed significantly (Fisher’s exact $p = 0.0005$; Fig. 5F). C2 and C3 were enriched for virilisation (69% and 75% of evaluable cases), whereas C1 had the highest proportion of non-functional tumours (47%), consistent with its relative suppression of steroid biosynthesis programs.

Markers of aggressive tumour behaviour were strongly cluster-associated. Vascular invasion varied significantly across clusters (Fisher’s exact $p = 0.0002$; Fig. 5G), being largely absent in C1 and C2 (75% and 85% without vascular invasion) but frequent in C3 and C4 (54% and >80%). Ki67 proliferation index and necrosis both differed robustly (Kruskal–Wallis $p < 0.0001$ and Fisher’s exact $p < 0.0001$; Fig. 5H, Supplementary Figure S8B). C1 and C2 showed low proliferative

Annotation bars indicate proteome cluster (C1–C4) and diagnosis (ACC/ACA/ACX). (C–E) Composite pathway proteomic scores, shown as violin plots stratified by cluster. IGF signalling (C), p53 target activity (D), and Wnt/ β -catenin signalling (E) were selected a priori as the pathways most recurrently altered in adrenocortical tumours (IGF2 overexpression, TP53 alteration, CTNNB1/Wnt activation). Scores were computed as the mean Z-score of curated pathway member proteins detected in the dataset (Supplementary Tables S4–S6). (F) MSigDB Hallmark gene set enrichment heatmap across clusters. Enrichment was performed per cluster (cluster vs. rest, limma; BH-adjusted). Colour encodes normalised enrichment direction and significance; rows represent Hallmark gene sets, columns represent clusters. (G) Immune cell type marker scores per cluster, shown as boxplots. Only dendritic cell and M2 macrophage signatures were detected at sufficient marker coverage (≥ 2 markers per cell type) for reliable scoring. (H) Foetal adrenal cortex proteomic scores per sample, shown as violin plots stratified by tumour vs. control. Scores were computed as the mean Z-score of detected marker proteins. (I) Foetal adrenal cortex proteomic scores per sample, shown as violin plots stratified by proteomic cluster. Scores were computed as the mean Z-score of detected marker proteins. (J) Individual ZG, ZF, and ZR zone scores per sample shown as violin plots stratified by cluster, illustrating the cluster-specific lineage biases underlying the ternary compositions in Supplementary Figure S7B.

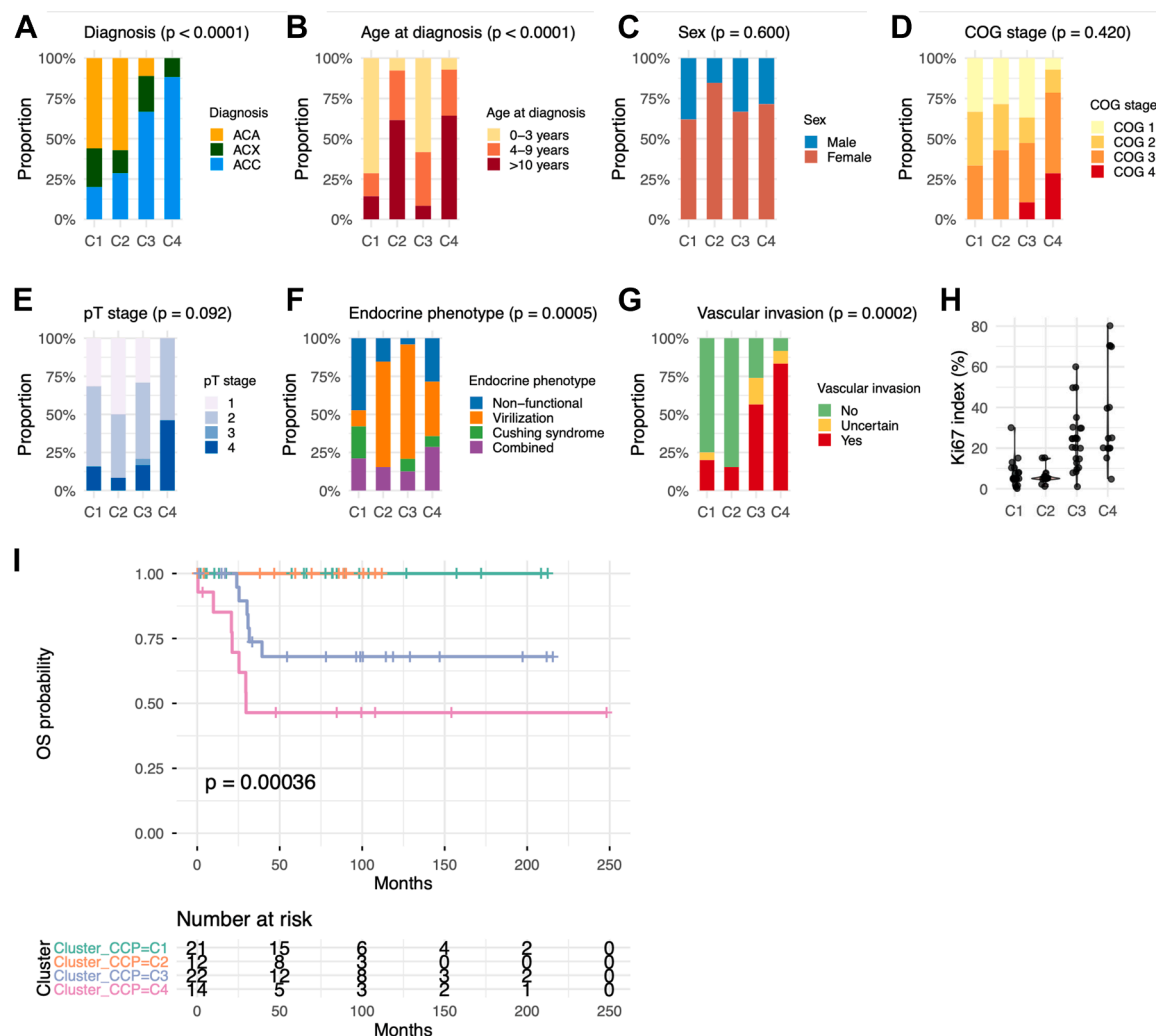


Fig. 5: Clinical and pathological correlates of proteome-based cluster membership. Clinical variables were assessed across the four proteome clusters (C1–C4) using Fisher's exact test for categorical variables and Kruskal–Wallis test for continuous variables. (A) Proportion of histological diagnoses (ACA, ACX, ACC) within each proteome cluster (C1–C4), shown as stacked proportion bars (Fisher's exact $p < 0.0001$). Same format for panels B–G. (B) Age at diagnosis stratified by cluster, categorised as 0–3 years, 4–9 years, and ≥ 10 years (Fisher's exact $p < 0.0001$). (C) Sex distribution across clusters (Fisher's exact $p = 0.605$). (D) COG stage distribution across clusters (Fisher's exact $p = 0.419$). (E) pT stage distribution across clusters (Fisher's exact $p = 0.090$). (F) Endocrine phenotype distribution across clusters, categorised as non-functional, virilisation, Cushing syndrome, and combined (Fisher's exact $p = 0.0004$). (G) Vascular invasion status across clusters (Fisher's exact $p = 0.0002$). (H) Ki67 proliferation index (%) per cluster shown as violin plots with individual data points. Median Ki67: C1 = 5%, C2 = 5%, C3 = 25%, and C4 = 25% (mean C4 = 37%). Kruskal–Wallis $p < 0.0001$. (I) Kaplan–Meier overall survival curves stratified by proteome cluster. The x-axis shows time in months (0–250); the y-axis shows OS probability (log-rank $p = 0.00036$). Numbers at risk are shown below the plot.

activity (median Ki67 5%) and low rates of necrosis (32% and 18%), C3 displayed intermediate-to-high proliferation (median Ki67 25%) with necrosis in 71% of cases, and C4 exhibited the highest proliferative indices (median 25%, mean 37%) with near-universal necrosis (93%, [Supplementary Figure S8B](#)), consistent with its highly proliferative molecular profile.

Importantly, overall survival differed significantly across clusters (log-rank $p = 0.00036$; [Fig. 5I](#)), with C1

and C2 showing the most favourable outcomes and C4 the poorest prognosis. Notably, survival stratification by histological diagnosis alone yielded weaker separation (log-rank $p = 0.0023$; [Supplementary Figure S8C](#)), indicating that proteome-based clustering provides superior prognostic resolution compared with histopathology alone.

Documented germline *TP53* status was available for only 14/83 tumours (7 mutant, 7 wild-type) and was

informative almost exclusively in ACC (13/14). A formal test against proteome cluster was therefore precluded by the few informative cases and their confounding with diagnosis; mutation-positive tumours fell within clusters C3 and C4, which also contained wild-type cases.

Together, these findings demonstrate that proteome-defined tumour subtypes integrate molecular programs with demographic, endocrine, and pathological features, translating into distinct clinical trajectories.

Clinical translatability of proteome-based tumour subtypes: a compact protein classifier and cluster-specific therapeutic vulnerabilities

To evaluate whether proteome-defined tumour subtypes can be approximated by a clinically tractable marker set, we derived a compact classifier using greedy forward feature selection with leave-one-out cross-validation (LOO-CV) and a nearest-centroid model. Candidate features were drawn from the top 50 one-vs.-rest differentially abundant proteins per cluster ($\text{FDR} < 0.05$, $\log_2\text{FC} > 0.5$), yielding up to 200 non-redundant candidates. The optimal panel size was defined as the smallest set achieving $\geq 95\%$ of maximal cross-validated accuracy.

A five-protein panel comprising DAAM2, CIP2A, TSC2, PALS2, and P3H1 achieved a LOO-CV accuracy of 92.8% (77/83 correctly classified tumours, Fig. 6A). Per-cluster sensitivities were 100%, 93%, 85%, and 94%, with specificities of 91%, 100%, 100%, and 99% for C1–C4, respectively. Classification relies on the combinatorial abundance pattern across all five proteins rather than on individual cluster-exclusive markers: DAAM2 is preferentially abundant in C1, CIP2A and TSC2 in C2, PALS2 in C2 and C3, and P3H1 in C3 and C4 (Supplementary Figure S9A–E). Notably, no single protein distinguished C4; instead, C4 tumours were classified by low abundance of DAAM2, CIP2A, and TSC2 together with high P3H1, a pattern consistent with the dedifferentiated molecular profile of this cluster. Permutation testing (1000 permutations; null mean accuracy 25.1%) confirmed that classification performance significantly exceeded chance ($p < 0.001$; Supplementary Figure S9F). Hierarchical clustering and UMAP based solely on the five-protein matrix recapitulated full-proteome cluster structure (Fig. 6B and C), demonstrating that a minimal marker set preserves the major biological stratification.

Beyond classification, we systematically interrogated cluster-specific proteomes for potentially actionable therapeutic targets using a curated druggability framework stratified by target class, localisation, and development stage (Fig. 6D; Supplementary Figure S10). Distinct therapeutic vulnerabilities emerged across clusters.

C1 displayed the broadest actionable spectrum (24 candidates, including 13 Tier 1 targets), encompassing signalling mediators and transcriptional regulators such as MAPK3, AXL, CTNNB1, STAT3, YAP1, and HDAC4. C2 yielded 8 targets including PTPN11, PRKCA/B, DHODH, and IRAK4, consistent with its kinase-dependent metabolic phenotype. In C3, 13 targets were identified, including 6 surface-expressed and 2 antibody-drug conjugate (ADC)-eligible proteins, notably IGF1R, NAMPT, ADAM10, and GPNMB. The enrichment of IGF1R is particularly relevant in the context of established IGF2 overexpression in pACT. C4 exhibited 11 targets with the highest Tier 1 proportion (9/11), dominated by epigenetic regulators (SMARCA4, HDAC4, DNMT3A, and KDM1A) and DNA replication machinery components (TOP2B and RRM1), aligning with its chromatin remodelling and highly proliferative phenotype.

Collectively, these findings demonstrate that proteome-defined tumour subtypes are not only biologically distinct but can be approximated by a minimal diagnostic panel and linked to cluster-specific therapeutic vulnerabilities, providing a conceptual framework for proteome-guided stratification and hypothesis-driven therapeutic exploration in pACT.

Discussion

ACT are among the most biologically heterogeneous endocrine tumours. This is particularly true in paediatric patients where classic histopathological criteria incompletely capture malignant potential.^{1,9,10,12,37} Over the past decade, genomic and transcriptomic studies have substantially advanced our understanding of ACT biology.^{3,13,14} Integrative analyses in adult ACC, most prominently through The Cancer Genome Atlas (TCGA), identified recurrent alterations in *TP53*, *CTNNB1*, *ZNRF3*, *CDKN2A*, and chromatin-remodelling genes, as well as transcriptome-based subgroups associated with proliferation and CpG island methylator phenotypes.^{17,38,39} In pACT, genomic studies have emphasised germline and somatic *TP53* alterations, particularly the Brazilian founder variant, together with *IGF2* overexpression and methylation-based risk stratification.^{3,13,15,40} More recently, RNA sequencing studies have proposed prognostic gene expression signatures and co-expression networks linked to steroidogenesis and cell cycle activity.¹⁴

Despite these advances, molecular stratification of ACT relies largely on upstream regulatory levels, DNA mutations, methylation, and RNA abundance, while the proteome, which is closest to the disease phenotype and represents the integrated functional output of these processes, has been comparatively understudied.^{19,20} Prior proteomic investigations in adult ACC have predominantly focused on tumour-vs.-control comparisons, reporting dysregulation of steroid biosynthesis,

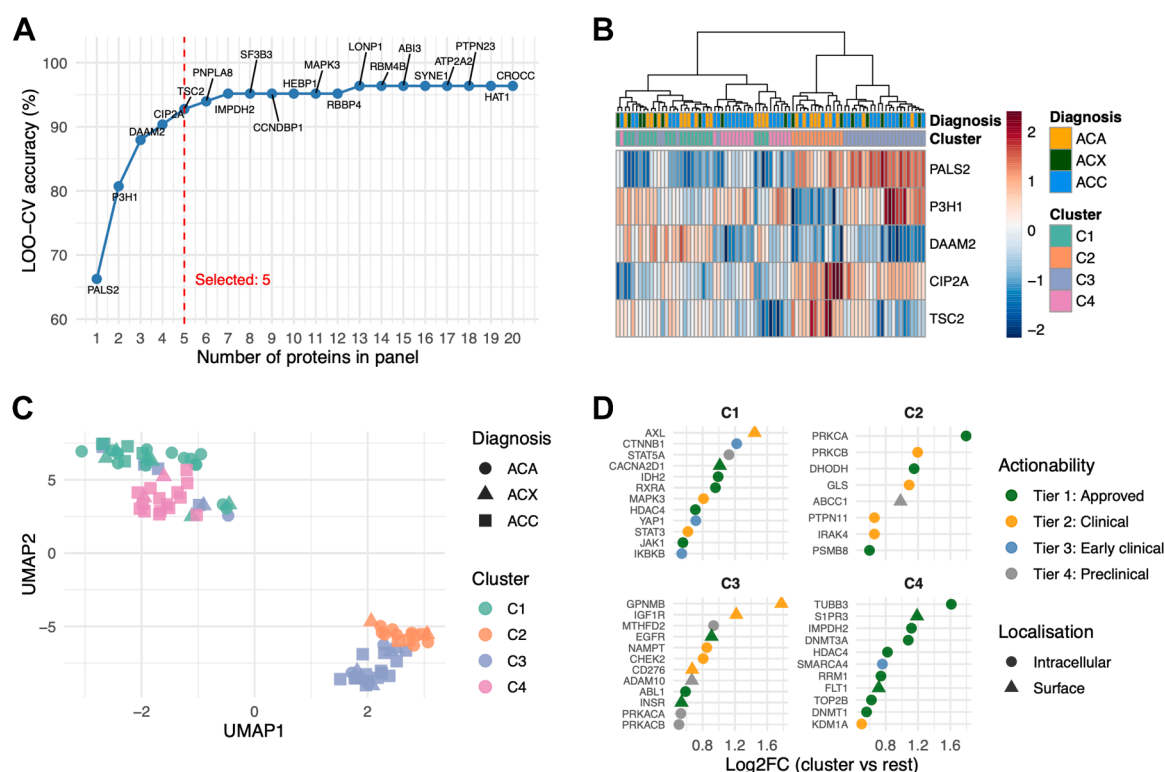


Fig. 6: Compact protein classifier and cluster-specific therapeutic target landscape. (A) LOO-CV classification accuracy (%) as a function of panel size during greedy forward feature selection with a nearest-centroid classifier. Candidates comprised the top 50 differentially abundant proteins per cluster (one-vs.-rest; FDR < 0.05, log₂ FC > 0.5), de-duplicated across clusters. Each point is labelled with the added protein; the red dashed line marks the selected five-protein panel (LOO-CV accuracy = 92.8%). (B) Heatmap of Z-score-normalised abundance of the five panel proteins (DAAM2, CIP2A, TSC2, PALS2, and P3H1) across all tumour samples, hierarchically clustered (Ward's method) with cluster and diagnosis annotation bars. (C) UMAP of tumour samples computed exclusively from the five-protein panel. Colours indicate proteome cluster (C1–C4); shape indicates diagnosis (circle = ACA, triangle = ACX, square = ACC). (D) Cluster-specific therapeutic target landscape: dot plot of log₂ fold change (cluster vs. rest) for actionable proteins in each cluster. Dot colour indicates actionability tier (Tier 1: approved; Tier 2: clinical; Tier 3: early clinical; Tier 4: preclinical); dot shape indicates localisation (surface vs. intracellular).

lipid metabolism, and mitochondrial pathways.^{23–27} However, these studies were limited by smaller cohort sizes and did not define reproducible tumour subtypes with integrated clinical and prognostic annotation. In pACT, no proteomic characterisation has been reported to date.

Here, we demonstrate that whole-proteome profiling resolves four robust tumour subtypes that go beyond histopathological classification and integrate metabolic specialisation, proliferative signalling, immune context, and adrenal lineage identity. Importantly, these proteome-defined clusters were only partially concordant with morphological categories and provided more detailed prognostic stratification than histology alone. This observation suggests that functional molecular states captured at the protein level reflect clinically meaningful biology not fully resolved by current diagnostic frameworks.^{19–21}

Rather than a simple linear malignancy continuum across ACA–ACX–ACC, our analyses revealed

qualitatively distinct tumour subtypes. C1 was enriched for actin remodelling, phagocytosis, and M2 macrophage-associated signatures, suggesting a tumour subtype characterised by enhanced stromal interaction or altered microenvironment composition.⁴¹ The enrichment of immune and cytoskeletal programs in this cluster underscores that proteomic profiling captures not only tumour cell-intrinsic programs but also tumour–microenvironment interplay, an aspect incompletely resolved by transcriptomics alone.⁴² C2 and C3 were characterised by metabolically active steroidogenic programs, enriched for oxidative phosphorylation, mitochondrial organisation, and cholesterol biosynthesis.^{43,44} Although oncocyte adrenal cortical neoplasms may show mitochondrial enrichment,⁴⁵ no C2 tumour had documented oncocyte morphology, supporting interpretation of this signature as a steroidogenic and bioenergetic state. The anabolic and IGF2-enriched phenotype of C3 aligns with established IGF2 overexpression in pACT and may reflect sustained

mTORC1-driven biosynthetic activity.^{3,46,47} In contrast, C4 exhibited a highly proliferative, chromatin-remodelled state marked by E2F and MYC activation and the poorest survival outcomes. This profile complements prior genomic observations implicating chromatin regulators and cell cycle dysregulation in aggressive ACC.^{16,17,48}

These findings suggest that pACT biology is organised around discrete functional states rather than a uniform progression model. Proteomics thus provides a systems-level perspective that integrates metabolic, proliferative, and lineage programs into coherent tumour phenotypes.

Our proteome-defined clusters do not simply recapitulate known genomic or methylation subgroups but instead functionally contextualise them. For example, the IGF1R enrichment observed in C3 corresponds with established *IGF2* overexpression, yet proteomics further reveals downstream anabolic cholesterol biosynthesis and mTORC1 activation as coordinated outputs. Similarly, the chromatin regulator enrichment in C4 complements genomic reports of alterations in epigenetic modifiers, suggesting that proteomic stratification captures the functional consequences of these alterations.

Importantly, while transcriptomic studies frequently stratify ACT by proliferation signatures, our data indicate that proliferative state represents only one dimension of tumour heterogeneity. Metabolic specialisation, steroidogenic capacity, and lineage bias constitute additional independent organising dimensions that are more directly observable at the protein level.

Proteome-defined clusters were strongly associated with clinical parameters, including vascular invasion, necrosis, proliferative index, endocrine phenotype, and overall survival. Survival stratification by cluster exceeded that achieved by histological diagnosis alone, highlighting the potential of proteome-based classification to refine risk assessment.

The derivation of a five-protein classifier achieving 92.8% cross-validated accuracy demonstrates that proteome-defined subtypes can be approximated by a compact candidate marker panel. Because feature selection and classifier derivation were performed within the full tumour cohort, this accuracy should be interpreted as an internally cross-validated performance estimate rather than validation in an independent test set. Public antibody resources indicate that antibody-based detection of the five classifier proteins is technically plausible. However, translation into an immunohistochemical assay will require antibody optimisation, standardised scoring, inter-observer reproducibility assessment, and validation in independent pACT cohorts. Thus, the classifier should be viewed as a translational bridge from discovery proteomics toward routine pathology, rather than as a clinically validated diagnostic assay.

Equally important is the identification of cluster-specific therapeutic vulnerabilities. The enrichment of IGF1R in C3 reinforces the rationale for IGF-axis targeting, whereas the concentration of epigenetic regulators and replication machinery components in C4 suggests potential sensitivity to chromatin-modifying agents or replication stress-targeting strategies.^{9,47,49} Such subtype-specific vulnerabilities support hypothesis-driven precision therapy development in a disease where systemic treatment options remain limited.

This study represents a comprehensive proteomic characterisation of pACT and one of the largest proteomic analyses of ACT to date, with a mean of over 8000 proteins quantified per tumour sample and more than 10,700 protein groups across the cohort. With 83 tumour samples from 72 patients, the cohort is substantial for such a rare paediatric disease. The cohort encompasses the full histological spectrum (ACA, ACX, and ACC) within a richly annotated clinical framework, including endocrine phenotype, Ki67 proliferation index, vascular invasion, tumour staging, and long-term survival follow-up, with adjacent non-tumour adrenal cortex specimens, enabling robust tumour-vs.-control comparisons while controlling for patient-level variation. Whole-proteome profiling enabled simultaneous interrogation of metabolic, proliferative, immune, and lineage programs, providing multidimensional biological resolution. The use of FFPE tissue, the standard archival material in clinical pathology, further supports the translational applicability of this approach.

Nevertheless, several limitations warrant consideration. The retrospective design introduces potential selection bias. Bulk proteomics averages signals across tumour and stromal compartments, limiting resolution of microenvironmental contributions.^{25,42} Furthermore, mass spectrometry preferentially detects abundant proteins, potentially underrepresenting low-abundance transcription factors and signalling intermediates.^{23–25} Missing values were handled by single down-shifted imputation, which suits left-censored missingness but does not propagate imputation uncertainty; although the differential-abundance results were robust to the imputation model and to the filtering thresholds, fully probabilistic dropout models represent a more rigorous alternative. Additionally, protein abundance does not directly measure activation state.²⁵ Phosphoproteomic profiling would be required for precise pathway activity assessment.⁵⁰ Finally, while the five-protein classifier showed strong internal performance, it has not yet been validated in an independent cohort, which is essential before clinical implementation.

A further limitation is the use of adjacent non-tumour adrenal cortex as matched control tissue. These samples may be affected by tumour proximity, altered tissue architecture, or systemic endocrine effects and therefore should not be considered equivalent to healthy age-matched adrenal tissue.

A systematic assessment of *TP53* status across proteome clusters was not possible. Documented germline data were available for only a subset of tumours (14/83), reflecting heterogeneous diagnostic practice and consent for sharing of genetic data across the retrospective study period, and were informative almost exclusively in ACC; *TP53* status was therefore confounded with diagnosis and could not be evaluated independently of the ACC-vs.-other axis that structures the clusters. Somatic *TP53* testing was likewise not routinely performed or systematically captured. Within these constraints we found no evidence that the clusters simply recapitulate *TP53* status, as mutation-positive tumours were distributed across rather than confined to individual clusters; whether proteome-defined clusters are associated with germline or somatic *TP53* alterations therefore remains an important question for future studies integrating comprehensive genetic profiling.

Future integration of proteomic data with genomic, transcriptomic, and epigenomic profiles will be important to clarify how the proteome-defined tumour states identified here relate to established molecular classifications and underlying genetic mechanisms of pACT. Spatial proteomics and phosphoproteomics may further refine this framework by resolving microenvironmental contributions and pathway activation states. Importantly, prospective validation in independent cohorts and, ultimately, clinical trials incorporating proteome-based stratification will be required to determine whether proteome-defined subtypes can guide therapeutic decision-making and improve outcomes in pACT.

In conclusion, unsupervised proteomic profiling of 83 pACT defines four biologically and clinically distinct tumour subtypes integrating metabolic specialisation, proliferative signalling, immune context, and adrenal lineage features. These proteome-defined subtypes provide superior prognostic resolution compared with histopathology alone and can be approximated by a five-protein immunohistochemistry-compatible classifier. Systematic interrogation of cluster-specific proteomes revealed distinct therapeutic vulnerabilities, including IGF-axis activation in steroidogenic subtypes and epigenetic and replication targets in high-risk tumours. This framework establishes a proteomic taxonomy of pACT and a foundation for proteome-informed risk assessment and precision therapy. Prospective validation of both the classifier and the subtype-specific therapeutic vulnerabilities will be critical next steps toward clinical implementation.

Contributors

R. Claus: designed and performed the bioinformatics/computational analyses, Conceptualisation, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Resources, Software, Supervision, Visualisation, and Writing—Original Draft Preparation; A. Metousis: performed the proteomics

analysis, Data Curation, Formal Analysis, Investigation, Methodology, Validation, and Writing—Review & Editing; V.E. Fincke: Validation, and Writing—Review & Editing; M. Kunstreich: Data Curation, Investigation, Resources, and Writing—Review & Editing; S.A. Wudy: Supervision, Validation, and Writing—Review & Editing; E. Jüttner: Investigation, Resources, and Writing—Review & Editing; J. Pons-Kühnemann: Formal Analysis, and Writing—Review & Editing; C. Vokuhl: Investigation, Resources, and Writing—Review & Editing; M.C. Frühwald: Writing—Review & Editing; C. Röcken: Investigation, Resources, and Writing—Review & Editing; L. Schweizer: Investigation, Supervision, and Writing—Review & Editing; P.D. Johann: Supervision, Validation, and Writing—Review & Editing; A. Redlich: Data Curation, Funding Acquisition, Investigation, Project Administration, Resources, and Writing—Review & Editing; M. Mann: provided the proteomics facility, expertise, and financial support, Investigation, Methodology, Resources, and Writing—Review & Editing; M. Kühlen: conceived and designed the study, provided the cohort and material, co-analysed the data, and wrote the manuscript, Conceptualisation, Data Curation, Funding Acquisition, Investigation, Methodology, Project Administration, Resources, Supervision, Validation, and Writing—Original Draft Preparation.

All authors read and approved the final version of the manuscript.

Data sharing statement

Proteomics data are available from PRIDE (<http://www.ebi.ac.uk/pride>; Project accession: PXD080313).

Access to individual-level proteomics in combination with associated clinical data is restricted due to research use agreements. Researchers may contact the corresponding authors (R.C. and M.K.) to request access, subject to institutional and ethical approvals.

Code sharing

The source code, including analyses added during peer review, is available upon request from the first author (R.C.; raimer.claus@uk-augsburg.de).

Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

During the preparation of this work, the authors used OpenEvidence, Nature Research Assistant, ChatGPT, Claude AI and perplexity.ai in order to brainstorm and enhance readability of sections of the text. After using these tools, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

Declaration of interests

Matthias Mann is an indirect investor in Evosep Biosystems.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.jebiom.2026.106504>.

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