

Supplemental material

Delineating pediatric adrenocortical tumors by GC-MS urinary steroid metabolome analysis: observations from the MET study

Stefan A. Wudy¹), Joern Pons-Kühnemann²), Marina Kunstreich^{3),4}), Antje Redlich⁴), Michaela F. Hartmann¹), Michaela Kuhlen^{3),5})

1) Steroid Research & Mass Spectrometry Unit, Laboratory for Translational Hormonal Analytics, Pediatric Endocrinology & Diabetology, Center of Child and Adolescent Medicine, Justus Liebig University Giessen, Feulgenstr. 12, Giessen D-35392, Germany

2) Medical Statistics, Institute of Medical Informatics, Justus Liebig University Giessen, Rudolf-Buchheim-Str. 6, Giessen D-35392, Germany

3) Pediatrics and Adolescent Medicine, Faculty of Medicine, University of Augsburg, Stenglinstr. 2, 86156 Augsburg, Germany

4) Department of Pediatrics, Pediatric Hematology/Oncology, Otto-von-Guericke-University, Leipziger Str. 44, 39120 Magdeburg, Germany

5) Bavarian Cancer Research Center (BZKF), Augsburg, Germany

Table 1: Steroid hormones with abbreviations, systematic names and trivial names

abbreviation	Urinary steroid metabolites
An	5 α -Androstane-3 α -ol-17-one (androsterone)
Et	5 β -Androstane-3 α -ol-17-one (etiocholanolone)
A5-3 β ,17 α	5-Androstene-3 β ,17 α -diol
DHEA	5-Androstene-3 β -ol-17-one (dehydroepiandrosterone)
A5-3 β ,17 β	5-Androstene-3 β ,17 β -diol (androstenediol-17 β)
11-O-An	5 α -androstane-3 α -ol-11,17-dione (11-oxo-androsterone)
E1	estrone
E2	estradiol
T	testosterone
Po-5 β ,3 α	5 β -Pregnane-3 α ,17 α -diol-20-one (17 α -OH-pregnanolone)
11-OH-An	5 α -Androstane-3 α ,11 β -diol-17-one (11-hydroxy-androsterone)
11-OH-Et	5 β -androstane-3 α ,11 β -diol-17-one (11-hydroxy-etiocholanolone)
Po-5 α ,3 α	5 α -Pregnane-3 α ,17 α -diol-20-one
16 α -OH-DHEA	5-androstene-3 β ,16 α -diol-17-one
PD	5 β -Pregnane-3 α ,20 α -diol (pregnanediol)
PT	5 β -Pregnane-3 α ,17 α ,20 α -triol (pregnanetriol)
P5D	5-Pregnene-3 β ,20 α -diol (pregnenediol)
A5T-16 α	5-androstene-3 β ,16 α ,17 β -triol (androstetriol-16 α)
THS	5 β -Pregnane-3 α ,17 α ,21-triol-20-one (tetrahydro-11-deoxycortisol)
TH-DOC	5 β -Pregnan-3 α ,21-diol-20-one (tetrahydro-11-deoxycorticosterone)
E3	estriol
11-O-PT	5 β -Pregnane-3 α ,17 α ,20 α -triol-11-one (11-oxo-pregnanetriol)
P5T-17 α	5-Pregnene-3 β ,17 α ,20 α -triol (pregnenetriol-17 α)
THE	5 β -Pregnane-3 α ,17 α ,21-triol-11,20-dione
THA	5 β -Pregnane-3 α ,21-diol-11,20-dione (tetrahydro-11-dehydro-corticosterone)
THB	5 β -Pregnane-3 α ,11 β ,21-triol-20-one (tetrahydro-corticosterone)
a-THB	5 α -Pregnane-3 α ,11 β ,21-triol-20-one (allo-tetrahydro-corticosterone)
THF	5 β -Pregnane-3 α ,11 β ,17 α ,21-tetrol-20-one (tetrahydro-cortisol)
a-THF	5 α -Pregnane-3 α ,11 β ,17 α ,21-tetrol-20-one (allo-tetrahydro-cortisol)
α -Cl	5 β -Pregnane-3 α ,17 α ,20 α ,21-tetrol-11-one (α -cortolone)
β -C	5 β -Pregnane-3 α ,11 β ,17 α ,20 β ,21-pentol (β -Cortol)
β -Cl	5 β -Pregnane-3 α ,17 α ,20 β ,21-tetrol-11-one (β -cortolone)
α -C	5 β -Pregnane-3 α ,11 β ,17 α ,20 α ,21-pentol (α -cortol)
F	4-Pregnene-11 β ,17 α ,21-triol-3,20-dione (cortisol)
6 β -OH-F	4-Pregnene-6 β ,11 β ,17 α ,21-tetrol-3,20-dione (6 β -hydroxycortisol)
20 α -DHF	4-Pregnene-11 β ,17 α ,20 α ,21-tetrol-3-one (20 α -dihydrocortisol)

Table 2: Absolute concentrations of urinary steroid metabolites (µg/l) in patients with pACAs, pACCs and controls.

	pACA N=21	pACC N=25	Control N=145	P-value
E1	0 0 0	0 0 19	0 0 0	0.200
E2	0.0 0.0 0.0	0.0 0.0 3.2	0.0 0.0 0.0	0.240
E3	0.0 0.0 25.6	0.0 0.0 18.0	0.0 0.0 8.3	0.007
T	0.0 0.0 17.5	0.0 0.0 29.5	0.0 7.8 17.1	0.660
An*	4.3 17.5 62.8	5.4 21.1 35.7	1.4 3.6 6.9	<0.001
Et*	2.6 13.2 28.1	9.9 26.8 44.1	1.1 2.6 5.8	<0.001
DHEA*	7.2e-01 3.0e+02 7.6e+02	9.2e+00 5.0e+02 1.1e+03	7.9e-02 2.3e-01 8.3e-01	<0.001
16α-OHDHEA*	7.1 54.5 383.5	64.5 224.9 476.4	0.2 2.0 3.1	<0.001
A53β17α	17.3 225.1 404.2	40.4 102.0 225.6	3.9 11.6 21.7	<0.001
A53β17β	35 941 2352	234 2132 3585	0 19 53	<0.001
A5T16α*	1.98 12.59 63.20	25.85 79.97 117.23	0.28 0.63 1.63	<0.001
11OHA _n *	9.3 26.0 52.2	10.5 22.6 103.0	2.0 2.8 4.5	<0.001
11OAn	65 150 399	52 132 910	19 28 44	<0.001
11OHEt	99 188 376	73 356 722	22 83 181	<0.001
PD	92 183 328	162 354 673	24 51 110	<0.001
PT	382 835 1539	307 700 1036	155 231 367	<0.001
P5D	238 415 844	300 638 1358	0 15 56	<0.001
P5T17α*	5.65 24.49 32.54	12.22 46.25 75.75	0.12 0.33 0.90	<0.001
Po5β3α	62 174 343	142 313 623	21 37 61	<0.001
Po5α3α	9.2 20.6 51.1	0.0 11.3 19.0	7.2 9.6 13.1	0.014
11OPt	5.7 20.7 32.1	3.6 9.0 19.9	4.5 7.2 11.0	0.044
THS	144 337 734	369 876 4392	39 59 111	<0.001
F	66 142 349	142 428 826	46 63 88	<0.001
THE*	20 32 60	11 31 61	14 22 34	0.022
THF*	8.4 12.6 18.2	5.9 12.9 28.3	4.5 7.1 10.5	<0.001
a-THF	539 770 2404	400 619 966	570 826 1332	0.045
α-Cl	597 996 1872	447 990 1633	494 732 1145	0.059
β-Cl	486 842 1309	332 712 1795	337 497 751	0.003
α-C	112 231 362	140 284 497	72 127 198	<0.001
β-C	267 432 723	176 342 804	191 267 433	0.008
6β-OHF	205 365 729	233 1279 2350	73 119 200	<0.001
20α-DHF	35 77 109	55 101 345	22 27 39	<0.001
THA	0 109 202	0 0 62	59 82 149	<0.001
THB	0 177 767	0 0 52	43 64 119	<0.001
a-THB	110 233 909	0 62 136	130 222 352	<0.001
THDOC	11 33 140	0 83 180	13 15 17	0.003

Abbreviation of steroids see Table 1, supplemental material; pACA pediatric adrenocortical adenoma, pACC pediatric adrenocortical carcinoma; a b c represent the lower quartile a, the median b, and the upper quartile c for continuous variables. Test used: Kruskal-Wallis test. * value times 100. Analyses were performed using R Statistical Software (v4.4.2; R Core Team 2024).

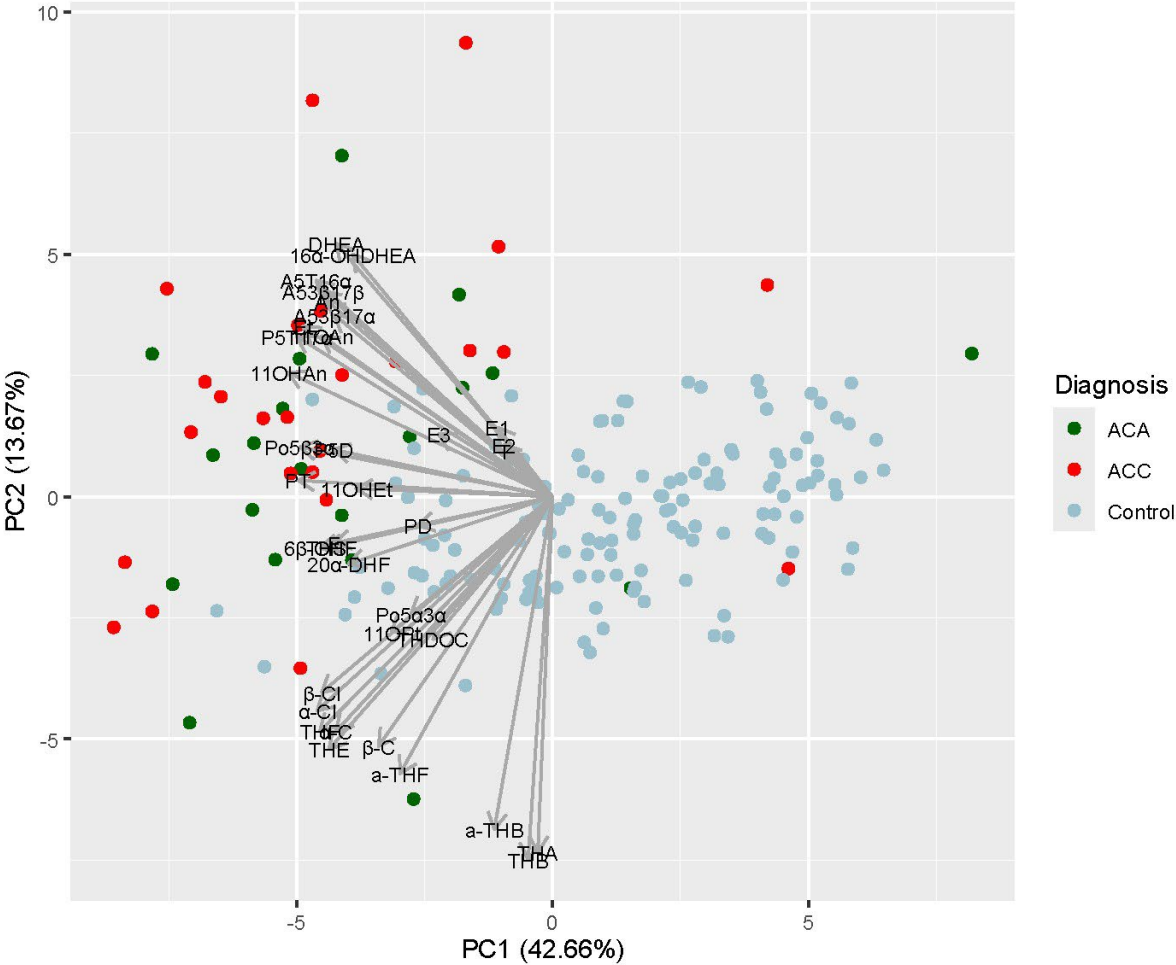
Table 3: Precursor/product ratios of relative enzyme activities of steroid metabolizing enzymes

Labels	precursor/product ratio
r_andro/Fs_1	$(an + et) / (a_thf + thf + the)$
r_andro/Fs_2	$(an + et + a5_3b_17a + a5_3b_17b + dhea + x16a_oh_dhea + a5t_16a) / (a_thf + thf + the)$
r_DHEA/Fs_1	$dhea / (a_thf + thf + the)$
r_DHEA/Fs_2	$(dhea + x16a_oh_dhea + a5t_16a) / (a_thf + thf + the)$
r_DHEA/Fs_3	$(dhea + x16a_oh_dhea + a5t_16a) / x11_oh_et$
r_andro 11-oxy/deoxy_1	$(x11_oh_an + x11_o_an + x11_oh_et) / (an + et + a5_3b_17a + a5_3b_17b + dhea + x16a_oh_dhea + a5t_16a)$
r_andro 11-oxy/deoxy_2	$x11_oh_et / (dhea + x16a_oh_dhea + a5t_16a)$
r_5red_1	an / et
r_5red_2	$x11_oh_an / x11_oh_et$
r_5red_3	a_thf / thf
r_5red_4	a_thb / thb
r_5red_5	$(an + x11_oh_an + a_thf + a_thb + po_5a_3a) / (et + x11_oh_et + thf + thb + po_5b_3a)$
r_5red_6	po_5a_3a / po_5b_3a
r_11HSD_1	$(a_thf + thf) / the$
r_11HSD_2	thf / the
r_11HSD_3	$(a_c + b_c) / (a_cl + b_cl)$ Cortols / cortolones
r_3HSD_1	$dhea / (an + et)$
r_3HSD_2	$(dhea + x16a_oh_dhea + a5t_16a) / (a_thf + thf + the)$
r_3HSD_3	$(dhea + x16a_oh_dhea + a5t_16a) / (an + et)$
r_3HSD_4	$p5t_17a / (a_thf + thf + the)$
r_3HSD_5	$p5t_17a / pt$
r_21hdrox_1	$(pt + po_5b_3a + po_5a_3a) / (a_thf + thf + the)$
r_21hdrox_2	$(x11_o_pt + pt + po_5b_3a + po_5a_3a) / (a_thf + thf + the)$
r_21hdrox_3	$x11_o_pt / (a_thf + thf + the)$
r_21hdrox_4	$x11_o_pt / the$
r_21hdrox_5	po_5b_3a / the

r_21hdrox_6	x11_o_pt/a_cl
r_17HSD_1	a5_3b_17b/dhea
r_17HSD_2	t/dhea
r_17HSD_3	t/x11_oh_an
r_17HSD_4	a5_3b_17b/x11_oh_an
r_11hdrox_1	ths/(a_thf+thf+the)
r_11hdrox_2	(an+et)/(x11_oh_an+x11_oh_et)
r_17hdrox_1	(tha+thb+a_thb)/(an+et)
r_17hdrox_2	pd/(an+et)
r_17hdrox_3	p5d/p5t_17a
r_17hdrox_4	pd/pt
r_17hdrox_5	(tha+thb+a_thb)/(a_thf+thf+the)
r_17hdrox_6	p5t_17a/a5_3b_17b
r_17hdrox_7	pt/(an+et)
r_17hdrox_8	(pt+po_5b_3a+po_5a_3a)/(an+et);
r_17hdrox_9	(a_thf+thf+the)/(an+et)
r_POR_1	pd/the
r_POR_2	pd/(a_thf+thf+the)
r_arom_1	(e1+e2+e3)/t
r_arom_2	(e1+e2+e3)/a5_3b_17b
r_CYP3A4_1	x6b_oh_f/f
r_20HSD_1	x20a_dhf/f
r_20HSD_2	a_cl/b_cl

Abbreviation of steroids see Table 1, supplemental material

Figure 1: Biplot - explorative data analysis using principal component analysis.

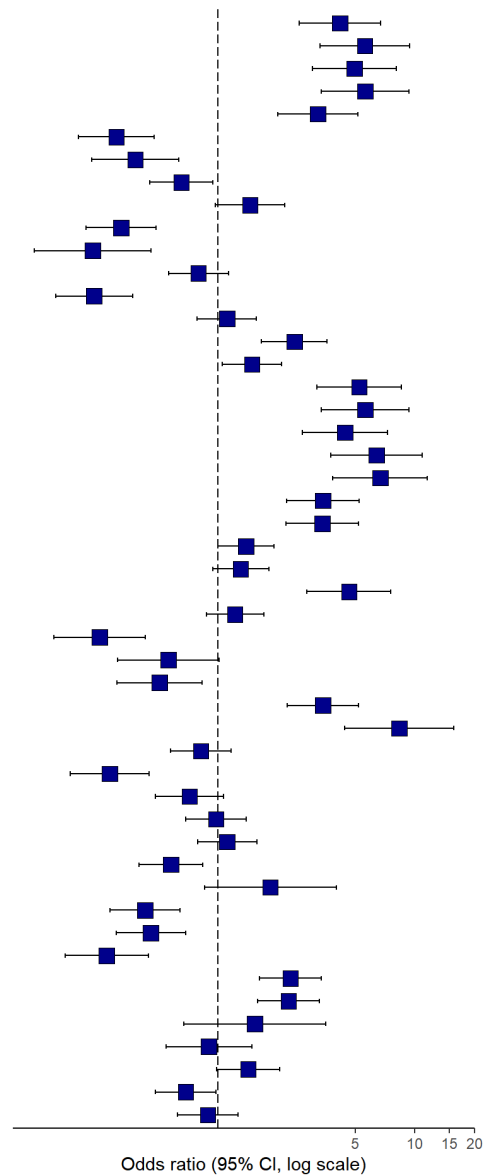


The colored dots resemble the patients according to their diagnoses. The arrows represent the individual metabolites. The longer and the more parallel to principal component 1 (PC1) the arrow of an individual metabolite is, the better it discriminates between ACTs (left) and controls (right). The first and second principal components explain together 55,38% (PC1: 42.66%; PC2: 13.76%) of the overall variability. ACA pediatric adrenocortical adenoma; ACC pediatric adrenocortical carcinoma. Analyses were performed using R Statistical Software (v4.4.2; R Core Team 2024).

Figure 2: Precursor/product ratios and risk of adrenocortical tumor (odds ratios and 95% confidence intervals).

ACT vs. Control: OR (95% CI, p-value)

r_andro/Fs_1	-	4.17 (2.59-6.70, p<0.001)
r_andro/Fs_2	-	5.56 (3.29-9.39, p<0.001)
r_DHEA/Fs_1	-	4.93 (3.03-8.03, p<0.001)
r_DHEA/Fs_2	-	5.59 (3.36-9.31, p<0.001)
r_DHEA/Fs_3	-	3.21 (2.02-5.13, p<0.001)
r_andro 11-oxy/deoxy_1	-	0.31 (0.20-0.48, p<0.001)
r_andro 11-oxy/deoxy_2	-	0.38 (0.23-0.64, p<0.001)
r_5red_1	-	0.65 (0.45-0.94, p=0.023)
r_5red_2	-	1.46 (0.97-2.19, p=0.068)
r_5red_3	-	0.32 (0.21-0.49, p<0.001)
r_5red_4	-	0.23 (0.12-0.46, p<0.001)
r_5red_5	-	0.80 (0.56-1.13, p=0.203)
r_5red_6	-	0.24 (0.15-0.37, p<0.001)
r_11HSD_1	-	1.11 (0.79-1.57, p=0.551)
r_11HSD_2	-	2.44 (1.67-3.58, p<0.001)
r_11HSD_3	-	1.49 (1.05-2.11, p=0.025)
r_3HSD_1	-	5.22 (3.19-8.53, p<0.001)
r_3HSD_2	-	5.59 (3.36-9.31, p<0.001)
r_3HSD_3	-	4.42 (2.69-7.28, p<0.001)
r_3HSD_4	-	6.37 (3.73-10.87, p<0.001)
r_3HSD_5	-	6.66 (3.83-11.58, p<0.001)
r_21hdrox_1	-	3.41 (2.23-5.21, p<0.001)
r_21hdrox_2	-	3.39 (2.22-5.16, p<0.001)
r_21hdrox_3	-	1.39 (1.00-1.93, p=0.047)
r_21hdrox_4	-	1.31 (0.94-1.81, p=0.111)
r_21hdrox_5	-	4.63 (2.84-7.55, p<0.001)
r_21hdrox_6	-	1.22 (0.87-1.71, p=0.240)
r_17HSD_1	-	0.25 (0.15-0.43, p<0.001)
r_17HSD_2	-	0.56 (0.31-1.02, p=0.056)
r_17HSD_3	-	0.51 (0.31-0.83, p=0.007)
r_17HSD_4	-	3.41 (2.25-5.17, p<0.001)
r_11hdrox_1	-	8.31 (4.40-15.70, p<0.001)
r_11hdrox_2	-	0.82 (0.58-1.17, p=0.272)
r_17hdrox_1	-	0.28 (0.18-0.45, p<0.001)
r_17hdrox_2	-	0.72 (0.48-1.07, p=0.102)
r_17hdrox_3	-	0.98 (0.69-1.39, p=0.911)
r_17hdrox_4	-	1.12 (0.79-1.58, p=0.537)
r_17hdrox_5	-	0.58 (0.40-0.84, p=0.004)
r_17hdrox_6	-	1.85 (0.85-3.99, p=0.118)
r_17hdrox_7	-	0.43 (0.28-0.64, p<0.001)
r_17hdrox_8	-	0.46 (0.31-0.69, p<0.001)
r_17hdrox_9	-	0.27 (0.17-0.44, p<0.001)
r_POR_1	-	2.33 (1.62-3.35, p<0.001)
r_POR_2	-	2.28 (1.59-3.27, p<0.001)
r_arom_1	-	1.54 (0.67-3.53, p=0.307)
r_arom_2	-	0.90 (0.55-1.49, p=0.685)
r_CYP3A4_1	-	1.42 (0.99-2.06, p=0.059)
r_20HSD_1	-	0.69 (0.48-0.98, p=0.038)
r_20HSD_2	-	0.89 (0.63-1.27, p=0.519)

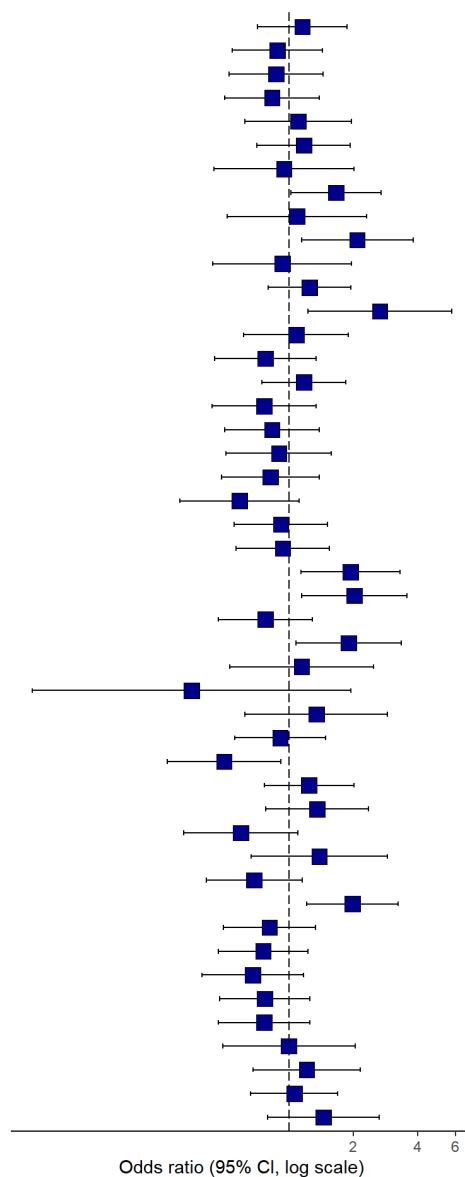


ACT pediatric adrenocortical carcinoma; abbreviation of precursor/product ratios see Table 3, supplemental material. Analyses were performed using R Statistical Software (v4.4.2; R Core Team 2024).

Figure 3: Precursor/product ratios and risk of adrenocortical carcinoma (odds ratios and 95% confidence intervals).

ACA vs. ACC: OR (95% CI, p-value)

r_andro/Fs_1	-	1.15 (0.71-1.87, p=0.560)
r_andro/Fs_2	-	0.88 (0.54-1.43, p=0.610)
r_DHEA/Fs_1	-	0.87 (0.53-1.44, p=0.589)
r_DHEA/Fs_2	-	0.83 (0.50-1.39, p=0.481)
r_DHEA/Fs_3	-	1.10 (0.62-1.96, p=0.735)
r_andro 11-oxy/deoxy_1	-	1.17 (0.71-1.93, p=0.539)
r_andro 11-oxy/deoxy_2	-	0.95 (0.45-2.02, p=0.888)
r_5red_1	-	1.66 (1.02-2.70, p=0.042)
r_5red_2	-	1.09 (0.51-2.31, p=0.824)
r_5red_3	-	2.08 (1.14-3.80, p=0.017)
r_5red_4	-	0.93 (0.44-1.96, p=0.849)
r_5red_5	-	1.25 (0.80-1.95, p=0.333)
r_5red_6	-	2.66 (1.23-5.75, p=0.013)
r_11HSD_1	-	1.08 (0.61-1.90, p=0.792)
r_11HSD_2	-	0.78 (0.45-1.34, p=0.364)
r_11HSD_3	-	1.17 (0.75-1.84, p=0.487)
r_3HSD_1	-	0.76 (0.44-1.34, p=0.346)
r_3HSD_2	-	0.83 (0.50-1.39, p=0.481)
r_3HSD_3	-	0.90 (0.51-1.58, p=0.704)
r_3HSD_4	-	0.82 (0.48-1.38, p=0.451)
r_3HSD_5	-	0.59 (0.31-1.11, p=0.103)
r_21hdrox_1	-	0.91 (0.55-1.51, p=0.728)
r_21hdrox_2	-	0.93 (0.56-1.55, p=0.789)
r_21hdrox_3	-	1.94 (1.14-3.31, p=0.015)
r_21hdrox_4	-	2.02 (1.14-3.55, p=0.015)
r_21hdrox_5	-	0.77 (0.47-1.29, p=0.324)
r_21hdrox_6	-	1.90 (1.08-3.36, p=0.027)
r_17HSD_1	-	1.14 (0.53-2.49, p=0.733)
r_17HSD_2	-	0.35 (0.06-1.95, p=0.231)
r_17HSD_3	-	1.34 (0.62-2.89, p=0.453)
r_17HSD_4	-	0.91 (0.56-1.49, p=0.704)
r_11hdrox_1	-	0.50 (0.27-0.91, p=0.025)
r_11hdrox_2	-	1.24 (0.76-2.01, p=0.384)
r_17hdrox_1	-	1.35 (0.78-2.35, p=0.282)
r_17hdrox_2	-	0.59 (0.32-1.10, p=0.099)
r_17hdrox_3	-	1.39 (0.67-2.88, p=0.381)
r_17hdrox_4	-	0.69 (0.41-1.15, p=0.153)
r_17hdrox_5	-	1.98 (1.21-3.25, p=0.007)
r_17hdrox_7	-	0.81 (0.49-1.33, p=0.406)
r_17hdrox_8	-	0.76 (0.47-1.22, p=0.255)
r_17hdrox_9	-	0.68 (0.39-1.17, p=0.159)
r_POR_1	-	0.77 (0.47-1.25, p=0.288)
r_POR_2	-	0.76 (0.47-1.25, p=0.281)
r_arom_2	-	1.00 (0.49-2.04, p=0.995)
r_CYP3A4_1	-	1.21 (0.68-2.15, p=0.521)
r_20HSD_1	-	1.06 (0.66-1.69, p=0.816)
r_20HSD_2	-	1.45 (0.79-2.64, p=0.229)



ACA pediatric adrenocortical adenoma, ACC pediatric adrenocortical carcinoma; abbreviation of precursor/product ratios see Table 3, supplemental material. Analyses were performed using R Statistical Software (v4.4.2; R Core Team 2024).