URINE STEROID BIOCHEMISTRY RESULTS

In RADS2, a comprehensive panel of urine steroids were measured at baseline, Week 12 and Week 48: GC precursors, GC metabolites, MC precursors, MC metabolites and androgens were measured by gas chromatography/mass spectrometry (GC/MS) in the laboratory at CEDAM (Birmingham) (see **Figure 1** depicting individual metabolites which constitute grouped panels; 24 hour urinary steroid metabolite excretion in healthy controls is also shown (Arlt, Biehl et al. 2011)).

Urine collections were performed during overnight admission major outcome visits which encompassed the 36-hour 'steroid-free' window. Variability in the time of hospital admission and time of commencement of the urine collection meant the duration of collection was not consistent (range 12-24 hours); results were corrected for collection duration when analysis was performed.
Urine samples (2x20mls) were stored at -80°C before shipping to CEDAM in Birmingham for batch processing and analysis at study completion. All samples were accompanied by local information detailing urine volume, urine creatinine excretion and timing of collection.
No female patients were taking the contraceptive pill during the study window that encompassed urine sample collection.

GC/MS of 32 individual urinary steroids was performed on an Agilent 5973 instrument after free and conjugated steroids were extracted from 1ml of urine by solid-phase extraction. A full description of the methodology has previously been published (Arlt, Biehl et al. 2011) .

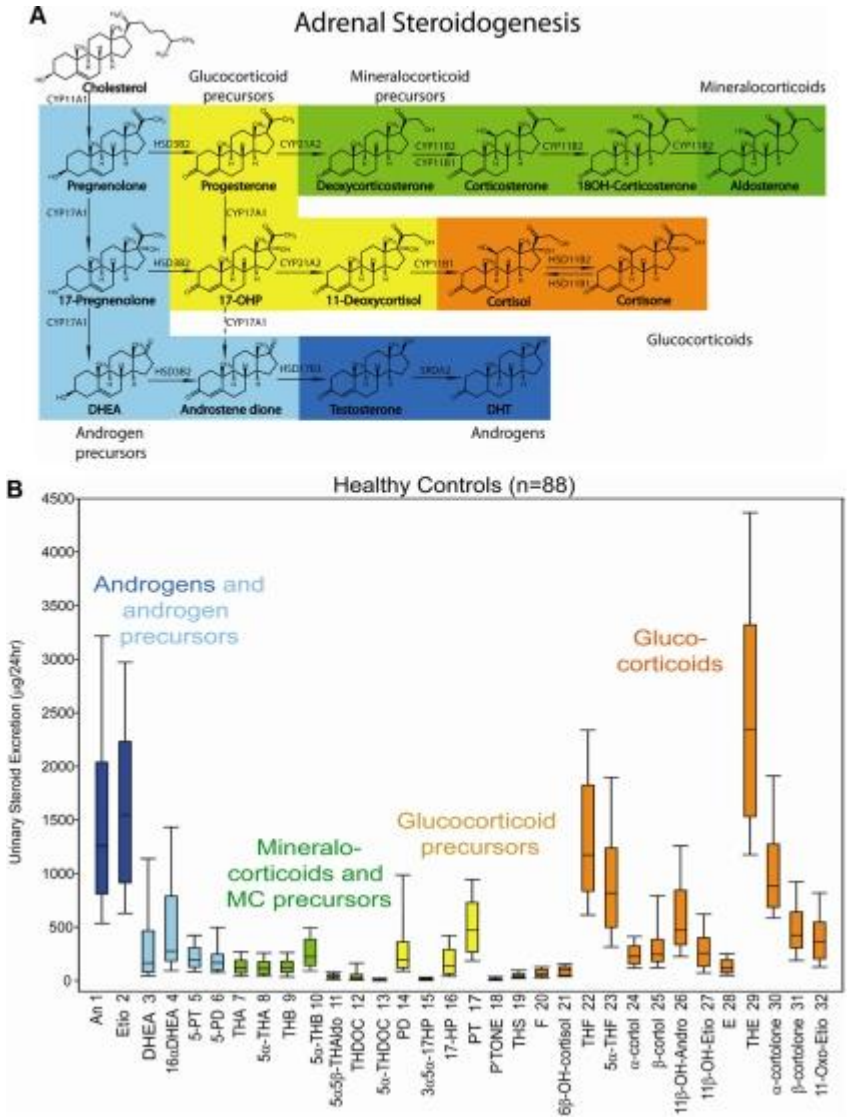


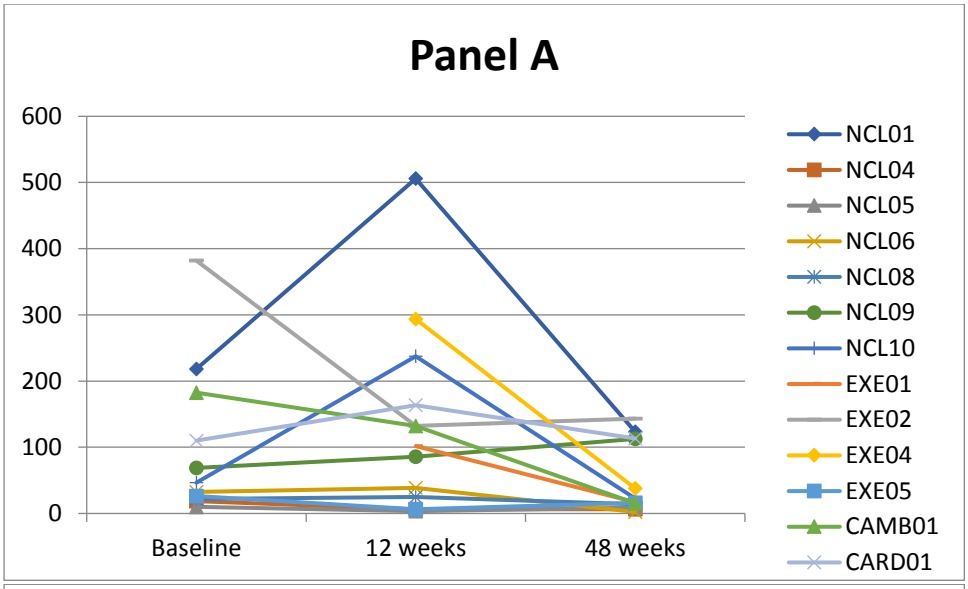
Figure 1 Urinary Steroid Metabolite Excretion (24 hours) **in Healthy Controls** (Arlt, Biehl et al. 2011)

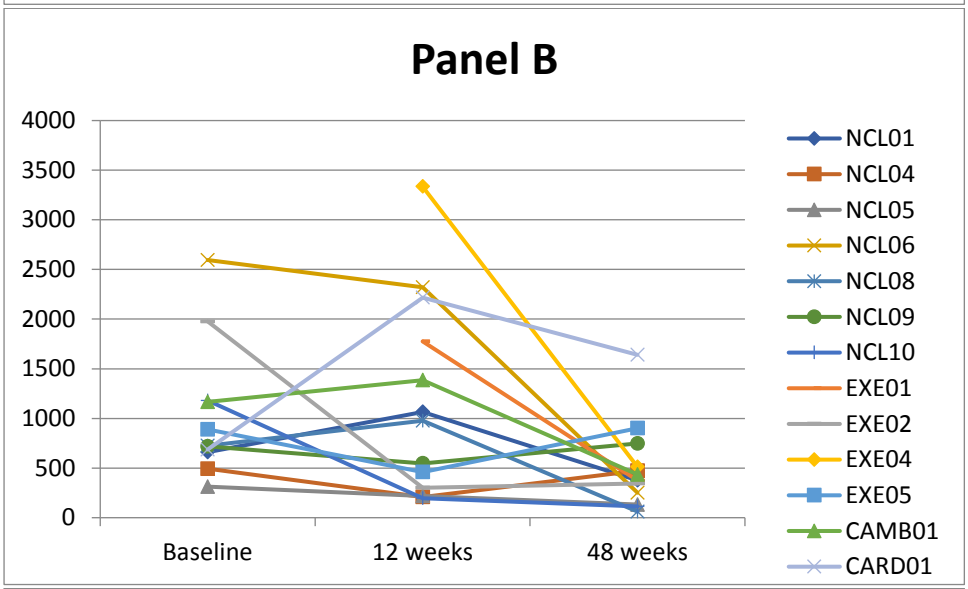
A - Schematic representation of steroidogenesis depicting the major products of adrenocortical steroid synthesis, the mineralocorticoid aldosterone (*dark green*) and its precursors (*light green*), glucocorticoid precursors (*yellow*), the active glucocorticoid cortisol (*orange*) and its metabolite cortisone, and the adrenal androgens and their precursors (*light blue*). Synthesis of active androgens (*dark blue*) mainly takes place in the gonads.

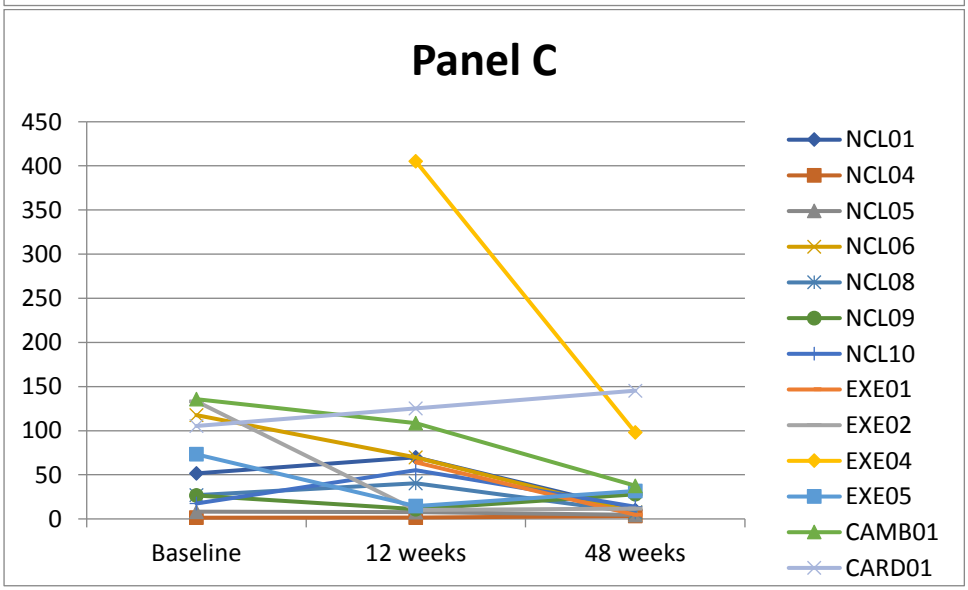
B - The 24-h urinary steroid metabolite excretion in healthy controls (n = 88). Box plots represent median and interquartile ranges; the whiskers represent 5th and 95th percentile, respectively. Colour coding of steroid metabolites mirrors that used for depicting the major adrenal corticosteroid classes in A.

CYP, Cytochrome P450; HSD, hydroxysteroid dehydrogenase; DHT, 5α-dihydrotestosterone.

The following **Figure 2** shows the sum of excretion (ug/estimated excretion in 24 hours) of grouped panels of urine steroid metabolites: glucocorticoid precursors, glucocorticoids, mineralocorticoids, androgen precursors and androgens at baseline *vs.* Weeks 12 and 48 post-intervention. EXE01 and EXE04 Baseline visit urine samples are missing. Panels of individual steroid excretion at these timepoints are included in the Appendix.


Panel A
600
500
400
300
200
100
0
Baseline
12 weeks
48 weeks
NCL01
NCL04
NCL05
NCL06
NCL08
NCL09
NCL10
EXE01
EXE02
EXE04
EXE05
CAMB01
CARD01


Panel B
4000
3500
3000
2500
2000
1500
1000
500
0
Baseline
12 weeks
48 weeks
NCL01
NCL04
NCL05
NCL06
NCL08
NCL09
NCL10
EXE01
EXE02
EXE04
EXE05
CAMB01
CARD01


Panel C
450
400
350
300
250
200
150
100
50
0
Baseline
12 weeks
48 weeks
NCL01
NCL04
NCL05
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NCL08
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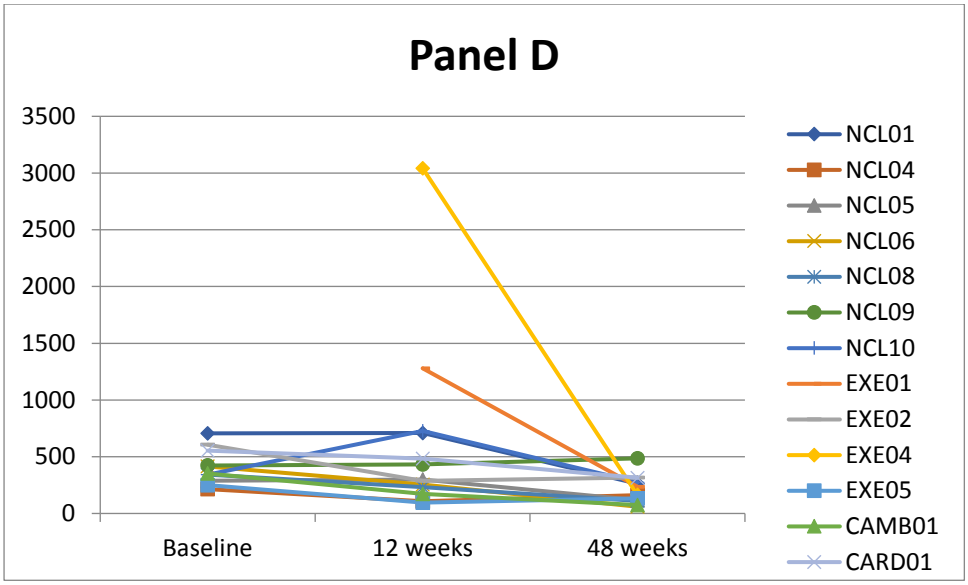
EXE01
EXE02
EXE04
EXE05
CAMB01
CARD01



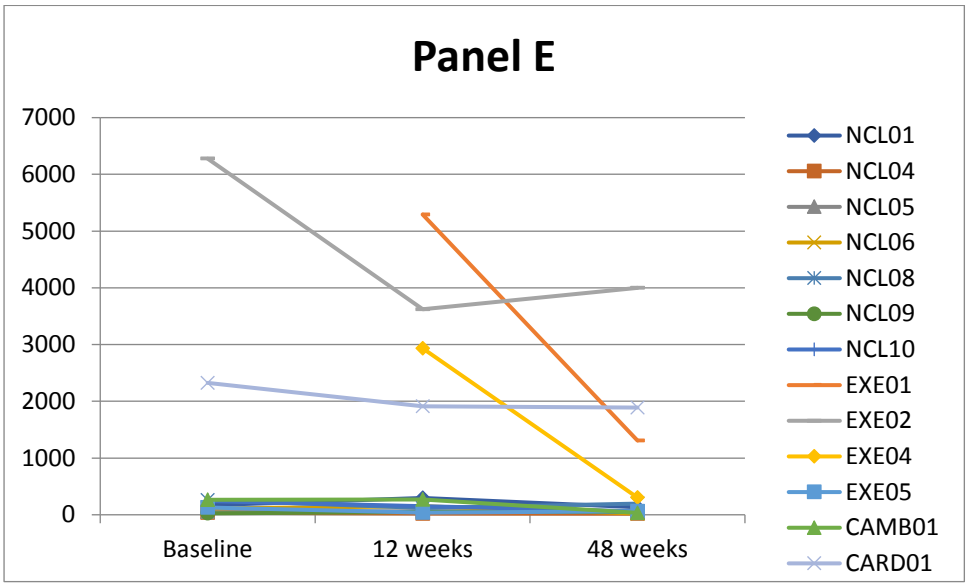


Figure 2 Excretion of Urine Steroid Metabolites (ug/estimated excretion in 24hrs) **in Panels of GC Precursors** (panel A), **GC** (panel B)**, Mineralocorticoids** (panel C), **Androgen Precursors** (panel D) and **Androgens** (panel E) **in All Treated Patients at Baseline, Week 12 and Week 48**.

Y-axis shows estimated total ugs of grouped steroids excreted in 24 hours. Individual metabolite excretion (ug/estimated excretion in 24hrs) is tabulated in Appendix.

In summary, these detailed measurements show that 8 treated patients can be classed as 'responders' in terms of increased urinary excretion of steroid metabolites (summarised in **Table 1** below), indicative of an increase in endogenous adrenal steroidogenesis.

	ANDROGENS			GLUCOCORTICOID PRECURSORS					GLUCOCORTICOIDS	
	An	Etio	11bOHAn	PD	17HP	PT	PTONE	THS	THE	a-cortolone
NCL01	1	1	1	1	1	1	1	1	1	1
NCL06	1	1	0	1	1	1	0	1	0	0
NCL08	0	0	0	0	0	1	0	1	1	1
NCL09	1	1	1	0	1	1	1	1	1	1
NCL10	0	0	1	1	1	1	0	0	0	0
EXE05	0	0	0	1	0	0	1	0	0	0
CARD01	0	0	1	1	1	1	1	1	1	1
CAMB01	0	1	0	0	0	0	0	1	1	1

Table 1 Steroid Metabolite Response in 8/13 Patients with an Increase in Urinary Steroid Excretion

Only patients with a detectable rise in urine steroid excretion included.
1 = increase in measured metabolite excretion; 0 = no increase

Study ID	**Peak increment on random or stimulated cortisol** (nmol/L); **Week achieved**	**Baseline SST peak cortisol** (nmol/L)
NCL01	**30; Week 12**	**40**
NCL04	N/A	undetectable
NCL05	N/A	undetectable
NCL06	**70; Week 18**	**265**
NCL08	**100; Week 12**	**55**
NCL09	**73; Week 48**	**26**
NCL10	**47; Week 48**	**undetectable**
EXE01	N/A	30
EXE02	N/A	40
EXE04	**89; Week 6**	**145**
EXE05	N/A	45
CAMB01	N/A	88
CARD01	**46; Week 48**	**81**

Table 2 Serum and Urine 'Responders'

Serum 'responders' (7/13), as evidenced by baseline cortisol and cortisol increment in vs. urine 'responders' (8/13), depicted as yellow background shading.

bold,

N/A = not applicable

As is evidenced in **Table 1** above, a proportion of patients demonstrated increased excretion of multiple steroid metabolites, whereas others demonstrated only a rise in a small number of examined metabolites (EXE05 = 2). A single patient (NCL01) demonstrated an increase in all 10 examined key metabolites.

On analysis of individual steroid metabolites, there is a pattern of increased pregnenolone and 17OHP under the influence of ACTH stimulation across several patients: this tends to rise by Week 12 and fall again by Week 48. NCL01 can be considered to have the most

significant overall change in urinary steroid excretion, with increases in cortisol and cortisone metabolites and in 17OHP and pregnenolone metabolites; nevertheless, none of these rise back into the normal range. The increase in steroid precursors reflects a genuine steroidogenic response to ACTH stimulation.

In terms of corroboration between serum and urine steroid response, 7/13 had a detectable serum response (any rise in peak stimulated cortisol from baseline) whereas 8/13 had a detectable rise in urine steroid excretion (**Table 2** above). Interestingly, one patient who had a serum response had no urine response (EXE04) and importantly, 2 patients without any evident serum response had a detectable increase in urine steroid excretion (EXE05 and CAMB01).

Interestingly, the number and range of metabolites with increased excretion post-treatment does not reflect serum steroid response. For example, NCL01 had only a small increase in peak stimulated cortisol following intervention (increment of 30nmol/L at Week 12), but demonstrated rising urinary excretion of steroid metabolites across all panels (GC, GC precursors, MC, androgens and androgen precursors).

The mean excretion of grouped panels of urine steroid metabolites and specific individual metabolites has been compared between Baseline visit *vs.* Week 12 and Week 48 in **Tables 3 and 4** below. Overall, no statistically significant increases in urinary steroid excretion were seen pre- *vs.* post-intervention (paired t-tests). This may well reflect the small group of patients examined and while not statistically significant, changes in urinary steroid excretion have clearly been demonstrated pre- *vs.* post-intervention in over 50% of patients. Radar charts below (**Figure 3**) have been included to show a schematic representation of changes in grouped panels of urinary steroids between Baseline visit and Week 48.

	Baseline Mean (SEM) urinary excretion (ug/24hrs)	**Week 48** Mean (SEM) urinary excretion (ug/24hrs)	*P*value (paired t-test)
GLUCOCORTICOID PRECURSORS	102 (35)	53 (17)	0.084
GLUCOCORTICOIDS	1037 (206)	498 (138)	0.075
MINERALOCORTICOIDS	63 (16)	27 (12)	0.047*
ANDROGEN PRECURSORS	408 (47)	211 (40)	0.001***
ANDROGENS	899 (573)	608 (343)	0.252

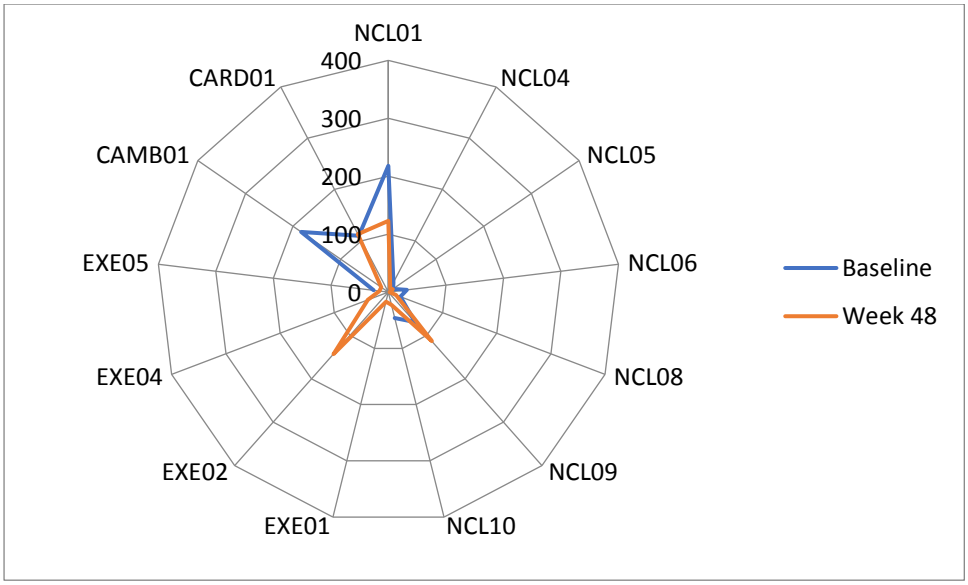
Table 3 Mean Excretion of Urine Steroid Metabolites (ug/estimated excretion in 24hrs) **Grouped into GC precursors, GC, Mineralocorticoids, Androgen Precursors and Androgens in Patients at Baseline and Week 48**

Mean excretion ug/24hrs shown with SEM and *P*value between Baseline *vs.* Week 48 (paired t-test). EXE01 and EXE04 omitted because no Baseline visit urine sample available. 11 patients included in analysis.

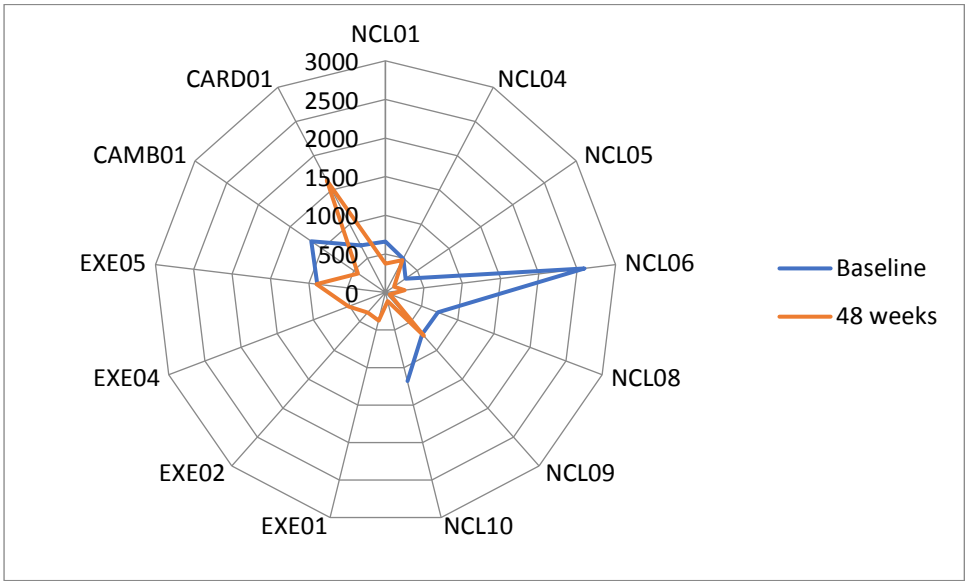
	Baseline Mean (SEM) urinary excretion (ug/24hrs)	**Week 48** Mean (SEM) urinary excretion (ug/24hrs)	*P*value (paired t-test)
Androstenedione	616 (391)	431 (289)	0.125
Etiocholanolone	311 (182)	162 (91)	0.143
11bOHAndro	117 (33)	38 (8)	0.053
PTONE	16 (8)	20 (12)	0.526
THS	20 (8)	9 (3)	0.263
PD	69 (18)	57 (20)	0.666
17HP	82 (28)	45 (16)	0.071
PT	146 (55)	99 (40)	0.13
THE	233 (60)	127 (45)	0.228
a-cortolone	140 (40)	53 (16)	0.045*

Table 4 Excretion of Individual Urine Steroid Metabolites (ug/estimated excretion in 24hrs) **at Baseline and Week 48**

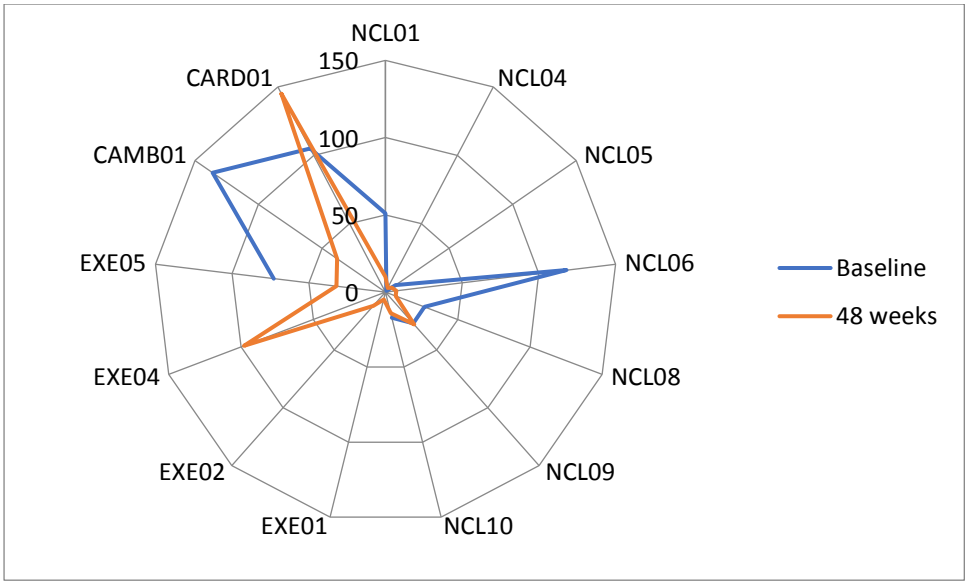
Mean excretion ug/24hrs shown with SEM and *P*value between Baseline visit *vs.* Week 48 (paired t-test). EXE01 and EXE04 omitted because no Baseline visit urine sample available. 11 patients included in analysis.



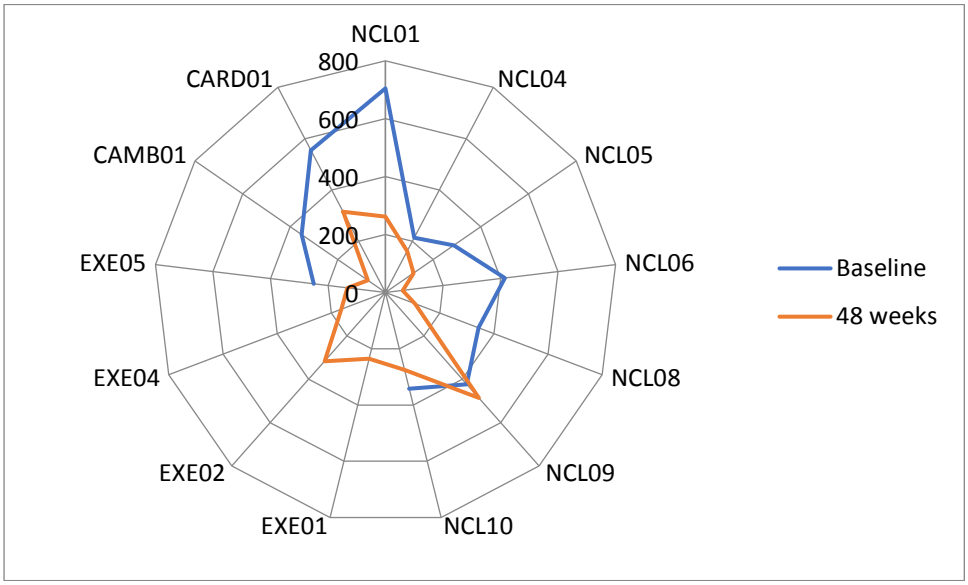
A: GLUCOCORTICOID PRECURSORS



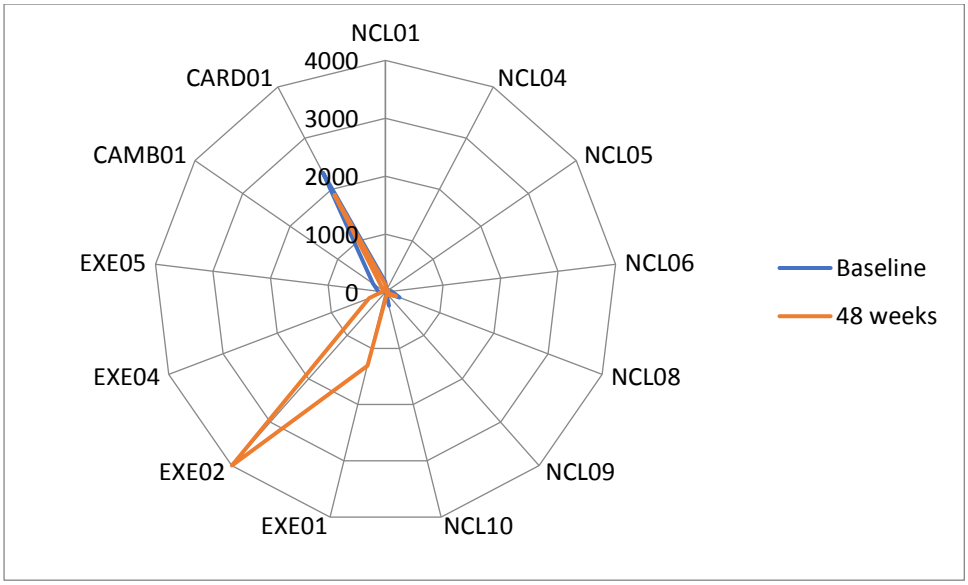
B: GLUCOCORTICOIDS



C: MINERALOCORTICOIDS



D: ANDROGEN PRECURSORS



E: ANDROGENS

Figure 3 Change in Volume of Urinary Steroids Excreted Between Baseline and Week 48 Post-Intervention

Radar charts used to illustrate change in urinary excretion between 2 time points (Baseline and Week 48 visit) for each grouped panel of urinary steroids. Urine steroid excretion is in ug/estimated 24 hours.