Supplemental file

Plasma steroid profiles in subclinical compared to overt adrenal Cushing's syndrome

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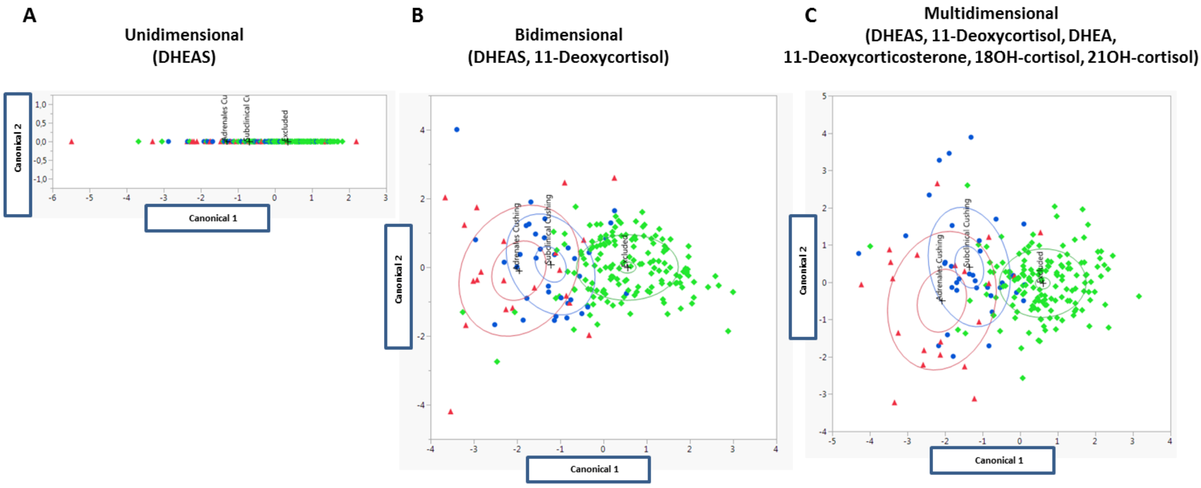
This supplemental file is derived from responses to the reviewers of the manuscript to provide background to the associated concepts and mathematical and statistical methods.

A multidimensional diagnostic approach

Although steroid profiles have been employed for some time for diagnosis of endocrine disorders of childhood the move from traditional unidimensional to multidimensional diagnostic approaches can now take advantage of 21st century computational power for applications utilizing established statistical approaches as well as newer approaches (machine learning and artificial intelligence) for interpreting data. In this way the concepts introduced in our manuscript offer a potential "paradigm shift" in approaching the diagnosis of disorders of adrenal steroidogenesis.

Principal component analysis (PCA) and discriminant analysis are statistical approaches established over 80 years ago that employ orthogonal transformations and vectors to obtain groupings for data. The key difference for discriminant analysis relates to its use when groups are already established. Since data involving more than three features is difficult to visualize, both approaches enable feature reduction and combinations of components to be displayed in two or three dimensions. Display of data in the manuscript utilizes canonical plots reduction multidimensional data into a form that can be visualized. This is achieved in panels B and E of Figure 3 of the manuscript by canonical plots displaying separation of groups in 2 dimensions. ROC curves, a basic concept in diagnostics, shown in panels A and D are derived within the discriminant function by logistic regression. Panels C and F display the "confusion matrices", which are the common outputs of machine learning approaches for categorization according to classification algorithms.

The example below ***(Supplemental figure 1A-C)***, utilizing data from the present study, illustrates how moving from the single dimension (DHEAS) to multiple dimensions improves group discrimination. For the two steroids of DHEAS and 11-deoxycortisol in the middle panel there is further improved discrimination with addition of four further key steroids. This example serves to illustrate how DHEAS used alone is not such a good marker for autonomous steroid production as when combined with other steroids.



Percent Missclassified	28%

Actual Grouping	Predicted Grouping			
	Adrenal Cushing	Subclinical Cushing	Excluded	
Adrenal Cushing	9	9	3	**43%**
Subclinical Cushing	5	17	13	**49%**
Excluded	5	72	75	**49%**

Percent Missclassified	18%

Actual Grouping	Predicted Grouping			
	Adrenal Cushing	Subclinical Cushing	Excluded	
Adrenal Cushing	14	7	0	**66%**
Subclinical Cushing	5	25	5	**71%**
Excluded	3	18	131	**86%**

Percent Missclassified	14%

Actual Grouping	Predicted Grouping			
	Adrenal Cushing	Subclinical Cushing	Excluded	
Adrenal Cushing	18	3	0	**86%**
Subclinical Cushing	5	25	5	**71%**
Excluded	4	12	136	**90%**

Supplemental figure 1. Result of a unidimensional approach for diagnosis (DHEA-S) (A) compared to bidimensional (DHEA-S and 11-Deoxycortisol) (B) and multidimensional (DHEA-S, 11-deoxycortisol, approaches (DHEA-S, 11-deoxycortisol, DHEA, 11-deoxycorticosterone, 18OH-cortisol and 21OH-cortisol) (C). Predicted versus actual groupings according to discriminant analyses are shown in the confusion matrices below each designated panel.

Stepwise analysis

The tables derived from the stepwise analysis illustrate F-ratios that reflect the relative value of each steroid used alone for discrimination ***(Supplemental table 1A-C).*** With consideration of all steroids 11-deoxycortisol has the highest F-ratio, with 11-deoxycorticosterone and DHEAS in 2nd and 3rd places, while cortisol is relegated to 10th place. F values do change with omission of steroids or different combinations, which is considered in the selection process (***Supplemental figure 2A-C***). Note that 11-deoxycortisol remains in first place, illustrating the importance of this steroid.

A

Steroid	F-ratio	Rank
11-Deoxycortisol	41.77	1
11-Deoxycorticosterone	35.684	2
DHEA-SO4	34.809	3
DHEA	28.571	4
Androstenedione	9.355	5
Progesterone	8.685	6
Pregnenolone	4.356	7
18-OH Cortisol	2.736	8
Corticosterone	1.878	9
Cortisol	1.592	10
Aldosterone	1.587	11
18-oxo-cortisol	1.239	12
21-Deoxycortisol	0.785	13
Cortisone	0.341	14
17-Hydroxyprogesterone	0.077	15

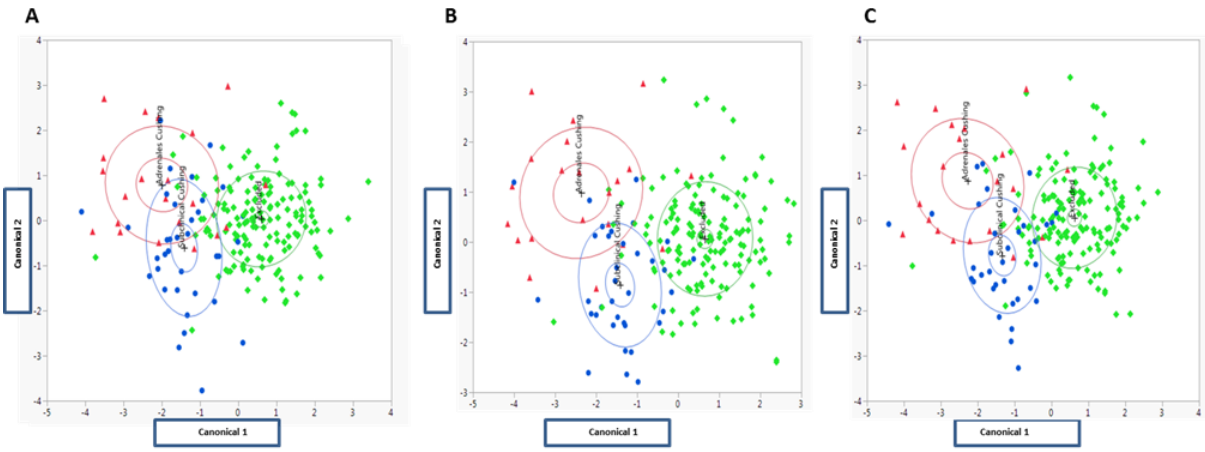
B

Steroid	F-ratio	Rank
11-Deoxycortisol	15.989	1
11-Deoxycorticosterone	2.297	10
DHEA-SO4	8.074	3
DHEA	5.479	5
Androstenedione	1.328	13
Progesterone	5.296	6
Pregnenolone	2.817	9
18-OH Cortisol	0.564	14
Corticosterone	2.005	11
Cortisol	9.244	2
Aldosterone	1.356	12
18-oxo-cortisol	3.473	8
21-Deoxycortisol	4.099	7
Cortisone	7.286	4
17-Hydroxyprogesterone	0.060	15

C

Steroid	F-ratio	Rank
11-Deoxycortisol	44.652	1
11-Deoxycorticosterone	1.260	13
DHEA-SO4	7.790	3
DHEA	4.584	7
Androstenedione	1.558	11
Progesterone	4.846	6
Pregnenolone	3.032	8
18-OH Cortisol	0.169	14
Corticosterone	1.566	10
Cortisol	7.785	4
Aldosterone	1.415	12
18-oxo-cortisol	2.814	9
21-Deoxycortisol	6.819	5
Cortisone	9.523	2
17-Hydroxyprogesterone	0.036	15

Supplemental table 1. F-ratios of steroids in a stepwise analysis by inclusion of 15 steroids (A), by omission of androstenedione, 17OH-progesterone and 18OH-cortisol (B) and by omission of androstenedione, 17OH-progesterone, 18OH-cortisol, aldosterone, corticosterone and 18-oxo-cortisol of the steroid panel (C).



A

Actual Grouping	Predicted Grouping			
	Adrenal Cushing	Subclinical Cushing	Excluded	
Adrenal Cushing	20	0	1	**95%**
Subclinical Cushing	0	34	1	**97%**
Excluded	2	5	145	**95%**

B

Actual Grouping	Predicted Grouping			
	Adrenal Cushing	Subclinical Cushing	Excluded	
Adrenal Cushing	19	1	1	**90%**
Subclinical Cushing	1	32	2	**91%**
Excluded	2	9	141	**93%**

C

Actual Grouping	Predicted Grouping			
	Adrenal Cushing	Subclinical Cushing	Excluded	
Adrenal Cushing	19	1	1	**90%**
Subclinical Cushing	5	27	3	**77%**
Excluded	5	8	139	**91%**

Supplemental figure 2. Results of discriminant analyses for use of all 15 steroids (A), by omission of androstenedione, 17OH-progesterone and 18OH-cortisol (B) and by omission of androstenedione, 17OH-progesterone, 18OH-cortisol, aldosterone, corticosterone and 18-oxo-cortisol (C) of the steroid panel in a stepwise analysis that provided discrimination of the 3 patient groups (adrenal Cushing ▲, subclinical hypercortisolism ●, excluded ◆). Predicted versus actual groupings according to discriminant analyses are shown in the designated confusion matrices.

Supplemental tables 2 and 3 illustrate how steroids in the panel were identified for optimal discrimination according to misclassification rates and discriminant scores with different combinations of steroids. Although omission of either 18OH-cortisol or 18-oxo-cortisol ***(Supplemental table 3E-F)*** resulted in similar misclassification rates, the deletion of 18-oxo-cortisol from the panel of steroids led to a slightly less number of EX cases being misclassified as SC patients (5 vs 6 cases).

A

Steroid
11-Deoxycortisol
DHEA-SO4
DHEA
Progesterone
Pregnenolone
21-Deoxyc ortisol

Percent Missclassified	12.9808%

Actual Grouping	Predicted Grouping			
	Adrenal Cushing	Subclinical Cushing	Excluded	
Adrenal Cushing	19	2	0	**90%**
Subclinical Cushing	7	25	3	**71%**
Excluded	7	8	137	**90%**

B

Steroid
11-Deoxycortisol
11-Deoxycorticosterone
DHEA-SO4
DHEA
Progesterone
18-oxo-cortisol
21-Deoxycortisol
Pregnenolone

Percent Missclassified	9.61538%

Actual Grouping	Predicted Grouping			
	Adrenal Cushing	Subclinical Cushing	Excluded	
Adrenal Cushing	19	2	0	**90%**
Subclinical Cushing	3	29	3	**83%**
Excluded	3	9	140	**92%**

C

Steroid
11-Deoxycortisol
11-Deoxycorticosterone
DHEA-SO4
DHEA
Progesterone
18-OH Cortisol
Corticosterone
Aldosterone
21-Deoxycortisol
18-oxo-cortisol
Pregnenolone

Percent Missclassified	6.73077%

Actual Grouping	Predicted Grouping			
	Adrenal Cushing	Subclinical Cushing	Excluded	
Adrenal Cushing	20	1	0	**95%**
Subclinical Cushing	2	31	2	**88%**
Excluded	2	7	143	**94%**

Supplemental table 2. Misclassification and results of discriminant analyses for use of a combination of 6 (A), 8 (B) and 11 (C) steroids.

D

Steroid
11-Deoxycortisol
11-Deoxycorticosterone
DHEA-SO4
DHEA
Androstenedione
Progesterone
18-OH Cortisol
Corticosterone
18-oxo-cortisol
Aldosterone
21-Deoxycortisol
17-Hydroxyprogesterone
Pregnenolone

Percent Missclassified	5.76923%

Actual Grouping	Predicted Grouping			
	Adrenal Cushing	Subclinical Cushing	Excluded	
Adrenal Cushing	20	1	0	95%
Subclinical Cushing	1	32	2	91%
Excluded	2	6	144	95%

E

Steroid
11-Deoxycortisol
11-Deoxycorticosterone
DHEA-SO4
DHEA
Androstenedione
Progesterone
Pregnenolone
18-OH Cortisol
Corticosterone
Cortisol
Aldosterone
21-Deoxycortisol
Cortisone
17-Hydroxyprogesterone

Percent Missclassified	4.80769%

Actual Grouping	Predicted Grouping			
	Adrenal Cushing	Subclinical Cushing	Excluded	
Adrenal Cushing	20	1	0	95%
Subclinical Cushing	0	34	1	97%
Excluded	3	5	144	95%

F

Steroid
11-Deoxycortisol
11-Deoxycorticosterone
DHEA-SO4
DHEA
Androstenedione
Progesterone
Pregnenolone
18-oxo-cortisol
Corticosterone
Cortisol
Aldosterone
21-Deoxycortisol
Cortisone
17-Hydroxyprogesterone

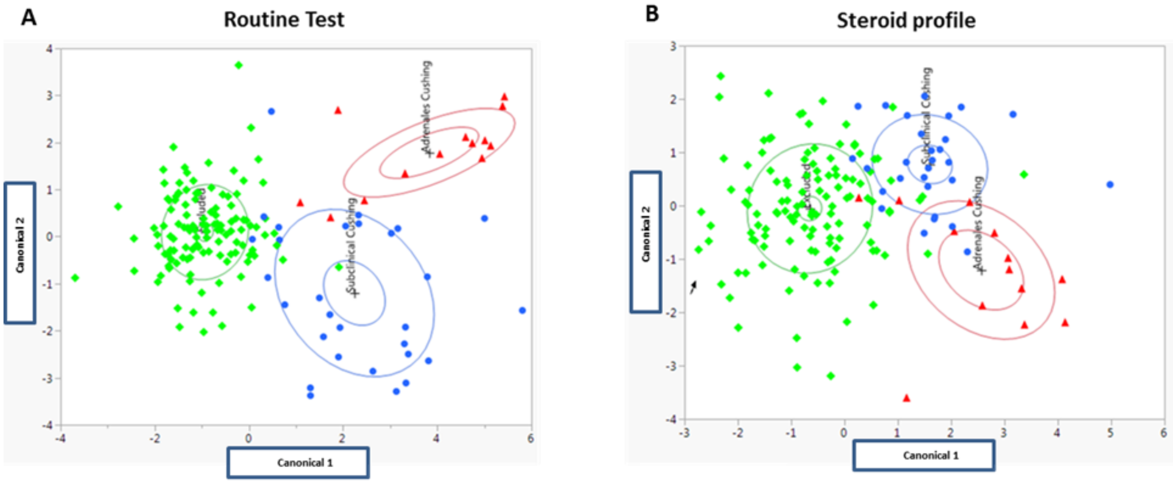
Percent Missclassified	4.80769%

Actual Grouping	Predicted Grouping			
	Adrenal Cushing	Subclinical Cushing	Excluded	
Adrenal Cushing	20	1	0	95%
Subclinical Cushing	0	34	1	97%
Excluded	1	6	145	95%

Supplemental table 3. Misclassification and results of discriminant analyses for use of a combination 13 (D), 14 (E) and 14 (F) steroids.

Model comparison

Areas under ROC curves and model comparisons indicated equivalent diagnostic performance of the steroid profile and the dexamethasone-test and superiority of the steroid profile over other routine diagnostic tests. Nevertheless, in terms of significance of differences in areas between ROC curves (Figure 2) the model comparisons only involved paired data. Thus the unequal numbers of patients in whom routine versus steroid profile tests was irrelevant. With regard to figure 3c and 3f we provide both the canonical plots and the confusion matrices with steroid profiles restricted to the same patients in who routine test results were available ***(Supplemental figure 3A-B).***



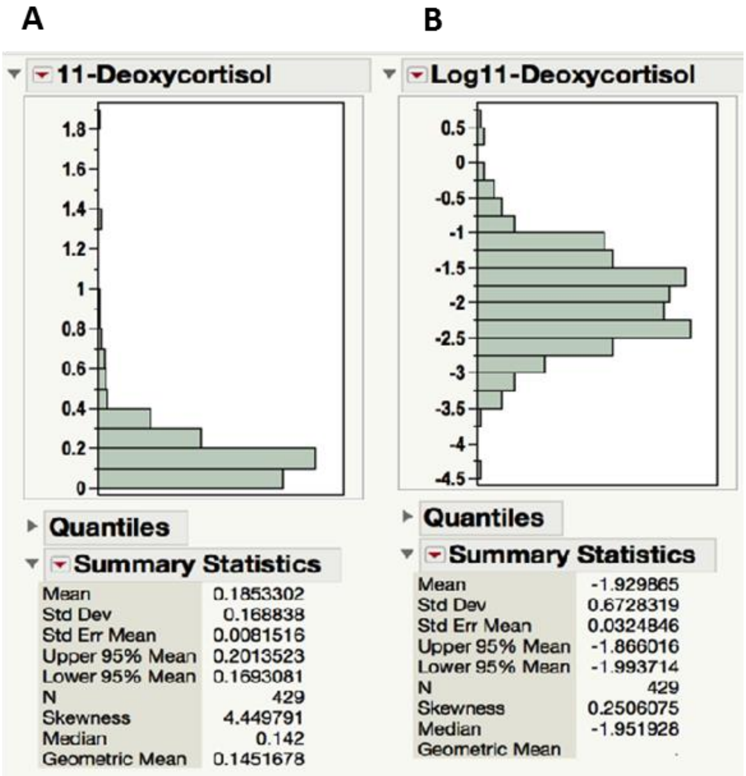
Actual Grouping	Predicted Grouping			
	Adrenal Cushing	Subclinical Cushing	Excluded	
Adrenal Cushing	13	0	0	100%
Subclinical Cushing	2	23	4	79%
Excluded	1	2	120	97%

Actual Grouping	Predicted Grouping			
	Adrenal Cushing	Subclinical Cushing	Excluded	
Adrenal Cushing	12	1	0	92%
Subclinical Cushing	1	27	1	93%
Excluded	1	3	119	97%

Supplemental figure 3. Results of discriminant analyses for use of routine diagnostic tests (A) compared to 14 steroids of the steroid panel (B) that provided optimal discrimination of the 3 patient groups (adrenal Cushing ▲, subclinical hypercortisolism ●, excluded ◆). Two-dimensional canonical plots are shown in upper panels, whereas predicted versus actual groupings according to discriminant analyses are shown in the confusion matrices.

Logarithmic transformation

A non-parametric post-hoc steel Dwass test was applied to the data of table 1 in the manuscript. However, for Figure 1, where data are depicted after parametric least square multivariate analyses, statistical tests were carried out after normalization of the data by natural logarithmic transformation. As outlined in the statistics section of the manuscript the post-hoc tests for the comparisons of the four groups utilized the Tukey HSD test. The importance of logarithmic transformation is shown using 11-deoxycortisol as an example before and after logarithmic transformation ***(Supplemental figure 4A-B).***



Supplemental figure 4. Result of logarithmic transformation with 11-deoxycortisol as an example before (A) and after transformation (B). Associated data include means, standard deviations, standard errors of means, upper 95% confidence intervals, lower 95% confidence intervals, skewness, medians and geometric means.

For such skewed data it is also inappropriate to display the data as means±SEM without first normalizing the data. Data in tables may be shown as medians with interquartiles or ranges, but such displays of data are inappropriate for figures. Rather for figures geometric means may be used. This in ***Supplemental figure 4A*** for non-transformed data the geometric mean is 0.145, which is close to the median (0.142), but lower than the actual mean (0.185), the latter skewed to higher values. The geometric mean in this case can also be calculated from the exponent of the log transformed mean (EXP -1.9298), which of course equals 0.145. For display of standard errors, exponents again have to be used according to the decrease and increase of the log-transformed standard error (0.03248) above the log-transformed mean. In this case the minus SE is 0.0046 and the plus SE is 0.0048.

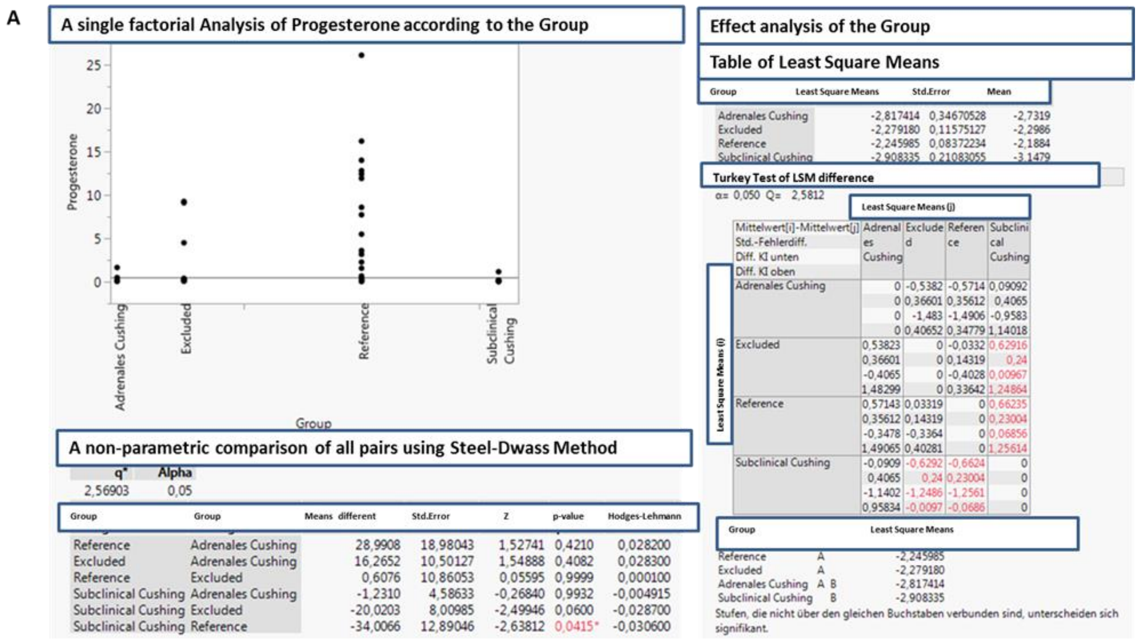
Use of least square means on the other hand is a commonly applied method to correct data where there are influential covariates, which in the case of our data included age and gender. Thus, for figure 1 the data are not only appropriately presented as geometric means with standard errors but there are also slight corrections according to the least square means multivariate analyses.

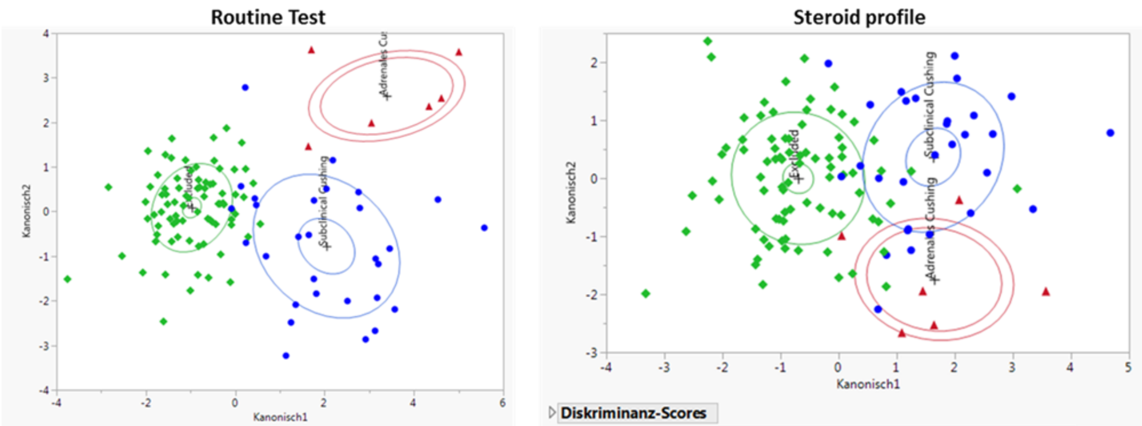
Phase of the menstrual cycle and progesterone concentration

There was no record of whether women were in the luteal phase of the menstrual cycle, which is associated with substantially increased progesterone; however, our observations indicated significantly decreased plasma concentrations of progesterone in both SC and AC patients compared to the reference group. Nevertheless, given the fact that the SC group consists of equal numbers of males and females and that the youngest age in the SC group was 45 it does seem possible that younger patients in the excluded and reference groups might contribute to the lower progesterone concentrations in the SC group. We explored this possible impact by exclusion of premenopausal women from all groups. Both the non-parametric and parametric comparisons showed that progesterone remains significantly lower in the SC group than either the reference (non-parametric) or both reference and excluded groups (parametric). This indicates that at least for the SC group the lowered progesterone is in fact a feature of the disorder and not some artifact related to phase of the menstrual cycle. Thus, in the discussion of the manuscript we make note that the low levels of progesterone can be explained by the following mechanism. Continuously elevated cortisol levels may inhibit the pituitary-gonadal axis leading to anovulatory cycles, prohibiting the action of progesterone in the uterus as well as the secretion of GnRH from the thalamus. We conclude that such effects may be responsible for the low progesterone in our mainly female premenopausal cohort with florid AC.

In addition to the above analyses in which premenopausal women were excluded we also show with additional discriminant analyses no notable impact on the diagnostic performance of the panel of steroids for detection of SC patients (83% of Routine Test vs 93 % of Steroid Profile). With omission of menopausal women there were similar patterns of discriminatory performance (92% of routine test vs 100% of steroid profile) ***(Supplemental Figure 5A-B).***

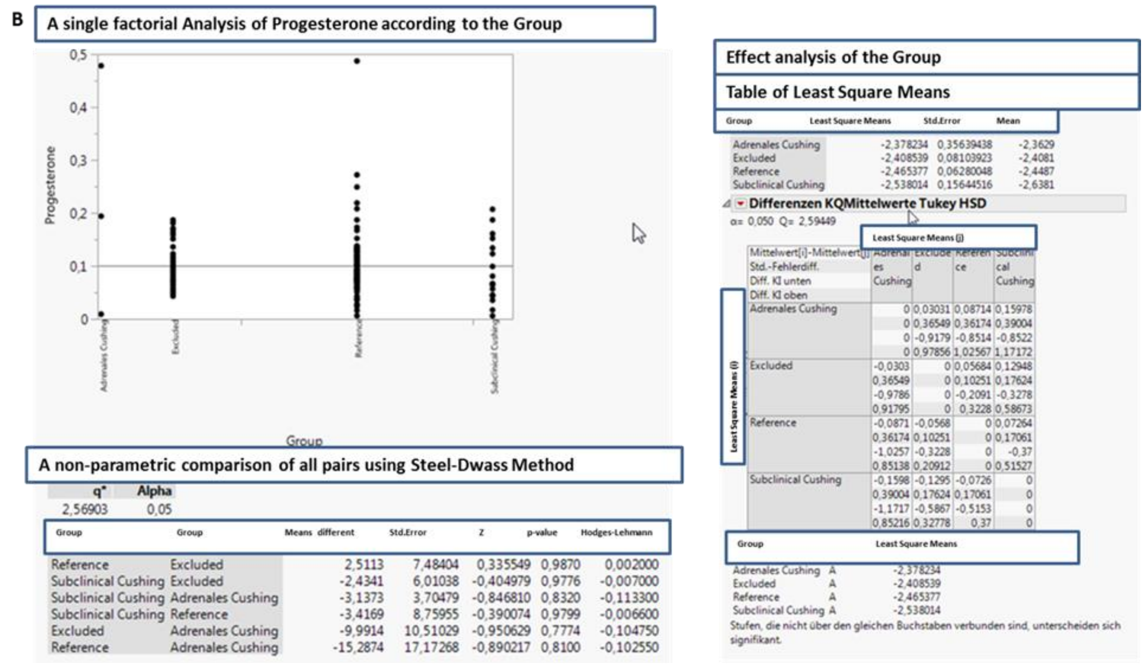
A

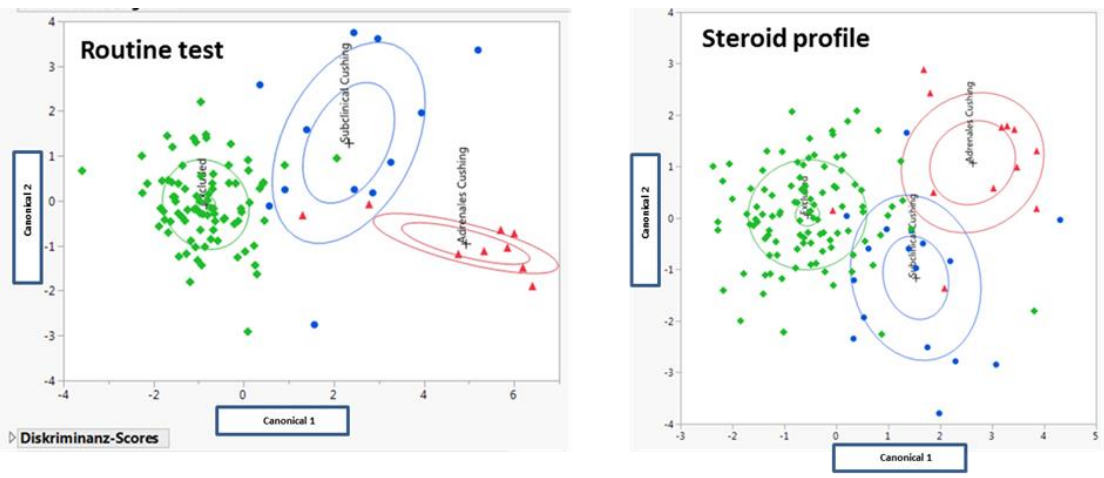




Actual Grouping	Predicted Grouping			
	Adrenal Cushing	Subclinical Cushing	Excluded	
Adrenal Cushing	6	0	0	**100%**
Subclinical Cushing	1	24	4	**83%**
Excluded	0	1	81	**99%**

Actual Grouping	Predicted Grouping			
	Adrenal Cushing	Subclinical Cushing	Excluded	
Adrenal Cushing	5	1	0	**83%**
Subclinical Cushing	0	27	2	**93%**
Excluded	5	1	76	**93%**





Actual Grouping	Predicted Grouping			
	Adrenal Cushing	Subclinical Cushing	Excluded	
Adrenal Cushing	9	0	0	**100%**
Subclinical Cushing	0	11	1	**92%**
Excluded	0	2	84	**98%**

Actual Grouping	Predicted Grouping			
	Adrenal Cushing	Subclinical Cushing	Excluded	
Adrenal Cushing	12	0	0	**100%**
Subclinical Cushing	0	17	0	**100%**
Excluded	0	1	102	**99%**

Supplemental figure 5. Results of non-parametric and parametric comparisons of plasma concentrations of progesterone in both SC and AC patients compared to the reference group after exclusion of premenopausal women (A) and exclusion of menopausal women from all groups. Two-dimensional canonical plots as well as predicted versus actual groupings according to discriminant analyses are shown in the designated panels.

Despite the additional analyses outlined below we certainly agree that ideally blood samples from premenopausal women who are not taking oral contraceptives should be taken in the follicular phase of the menstrual cycle.