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1 **A proof of concept of a machine learning algorithm to predict late-onset 21-**
2 **hydroxylase deficiency in children with premature pubic hair.**

3

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15 * The two authors equally contribute to this work

16 The authors have nothing to disclose

17

18 **HIGHLIGHTS**

- 19 • Steroid levels assayed by mass spectrometry at baseline combined with
20 anthropometrics and bone ages were used to establish a diagnosis score in children
21 with premature pubic hair.
- 22 • Machine learning models fully distinguished children with premature pubarche from
23 those with non-classical congenital adrenal hyperplasia, without the need for
24 adrenocorticotrophic hormone testing.
- 25 • The most significant variables were levels of 21-deoxycorticosterone, 17-
26 hydroxyprogesterone, and 21-deoxycortisol steroids.

27

28 **ABSTRACT**

29 In children with premature pubarche (PP), late onset 21-hydroxylase deficiency (21-OHD),
30 also known as non-classical congenital adrenal hyperplasia (NCCAH), can be routinely ruled
31 out by an adrenocorticotrophic hormone (ACTH) test. Using liquid chromatography–tandem
32 mass spectrometry (LC-MS/MS), a quantitative assay of the circulating steroidome can be
33 obtained from a single blood sample.

34 We hypothesized that, by applying multivariate machine learning (ML) models to basal
35 steroid profiles and clinical parameters of 97 patients, we could distinguish children with PP
36 from those with NCCAH, without the need for ACTH testing.

37 Every child presenting with PP at the Trousseau Pediatric Endocrinology Unit between 2016
38 and 2018 had a basal and stimulated steroidome. Patients with central precocious puberty
39 were excluded. The first set of patients (year 1, training set, n=58), including 8 children with
40 NCCAH verified by ACTH test and genetic analysis, was used to train the model.
41 Subsequently, a validation set of an additional set of patients (year 2, n=39 with 5 NCCAH)
42 was obtained to validate our model. We designed a score based on an ML approach

43 (orthogonal partial least squares discriminant analysis). A metabolic footprint was assigned
44 for each patient using clinical data, bone age, and adrenal steroid levels recorded by LC-
45 MS/MS.

46 Supervised multivariate analysis of the training set (year 1) and validation set (year 2) was
47 used to validate our score. Based on selected variables, the prediction score was accurate
48 (100%) at differentiating premature pubarche from late onset 21-OHD patients. The most
49 significant variables were 21-deoxycorticosterone, 17-hydroxyprogesterone, and 21-
50 deoxycortisol steroids.

51 We proposed a new test that has excellent sensitivity and specificity for the diagnosis of
52 NCCAH, due to an ML approach.

53

54 **ABBREVIATIONS**

55 11OHA4: 11 β -hydroxyandrostenedione

56 11-DF: 11-deoxycortisol

57 17-OHP: 17 α -hydroxyprogesterone

58 17-OHPreg: 17 α -hydroxypregnenolone

59 21-DB: 21-deoxycorticosterone

60 21-DF: 21-deoxycortisol

61 ACTH: Adreno corticotropic hormone

62 AI: Adrenal insufficiency

63 ALDO: Aldosterone

64 B: Corticosterone

65 BA: Bone age

66 BMI: Body mass index

67 CAH: Congenital adrenal hyperplasia

68 A4: Androstenedione

69 DHEA: Dehydroepiandrosterone

70 DOC: 11-deoxycorticosterone

71 E: Cortisone

72 F: Cortisol

73 GV: Growth velocity

74 LC-MS/MS: Liquid chromatography tandem mass spectrometry

75 NCCAH: Late-onset 21-hydroxylase deficiency

76 P: Progesterone

77 PP: Premature pubarche

78 Preg: Pregnenolone

79 RIA: Radioimmunoassay

80 T: Testosterone

81 ML : Machine Learning

82

83 **INTRODUCTION**

84 Premature pubarche (PP), defined as the development of pubic hair before the age of 8 for
85 girls and 9 for boys, is a common feature in children and is usually due to physiological
86 adrenarche [1]. In individuals with PP, it is necessary, however, to rule out an adrenal
87 pathology, especially late onset congenital adrenal hyperplasia (NCCAH), which is due to
88 steroid 21-hydroxylase deficiency (21-OHD).

89 NCCAH, one of the most common genetic autosomal recessive disorders, results from
90 molecular abnormalities of the *CYP21A2* gene. It occurs in 0.1% of the general population [2],
91 with some variation between ethnic groups [3]. In NCCAH, residual enzyme activity of about
92 50–70% [4] leads to less severe features than classical neonatal congenital adrenal hyperplasia
93 (CAH). The accumulation of androgens upstream of the enzyme defect [5] is responsible for
94 hirsutism, acne [6], accelerated bone maturation [7], and short final height [8]. The phenotype
95 of NCCAH is determined by the less severe mutation of *CYP21A2* [9]. However, molecular
96 diagnosis is mandatory for familial planning [10].

97 Based on the current consensus [11], adrenocorticotrophic hormone (ACTH) testing is
98 performed on a routine basis for all children with PP. NCCAH is currently defined by a post-
99 ACTH test level of 17-hydroxyprogesterone (17-OHP) in the serum greater than 10 ng/mL
100 [12]. After parental consent (according to the Declaration of Helsinki), these results must be
101 confirmed by a genetic test of the *CYP21A2* gene. The use of mass spectrometry for
102 multidimensional steroid profiling in 21-OHD is well established, starting with use of gas
103 chromatography–mass spectrometry in the 1980s where urinary steroid profiles were useful,

104 especially in newborns [13,14]. Furthermore, with the development of liquid
105 chromatography–tandem mass spectrometry methods (LC-MS/MS), the diagnosis of adrenal
106 diseases [15,16] and especially of CAH [17,18] has improved specificity for steroid analysis
107 when compared with immunoassays [19], because these methods are free from cross reactions
108 of 17-OHP with others steroids [20] and can reduce false positive results. Spectrometry
109 methods can also reduce the number of unnecessary tests and ease the anxiety of the patients
110 [21]. With LC-MS/MS, a circulating steroidome quantitative assay [22], including more than
111 16 molecular species, is now available in a single analysis.

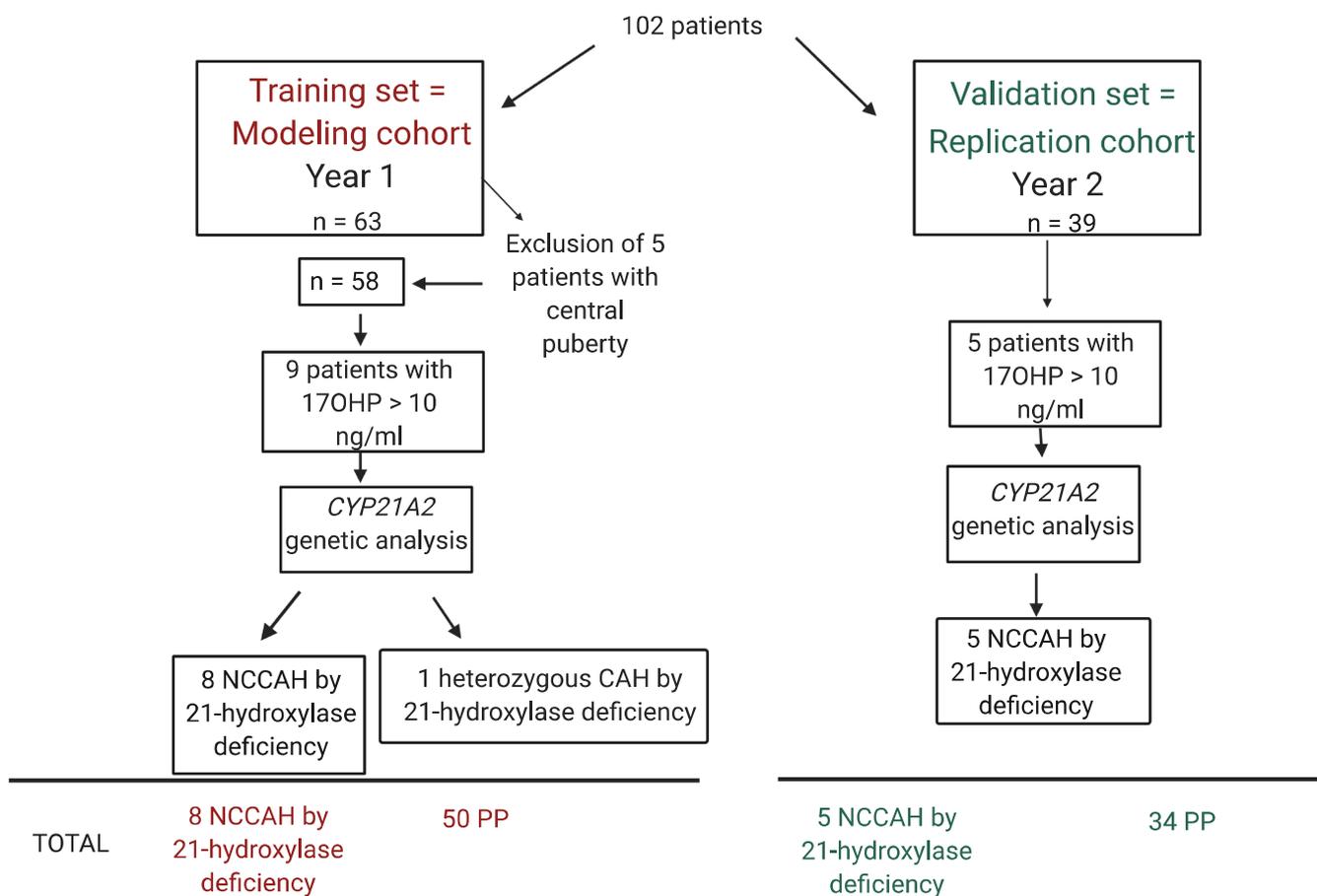
112 We hypothesized that combining patient anthropometric data with the analytical performances
113 of mass spectrometry and the use of multivariate statistical approaches, such as machine
114 learning (ML) algorithms, could lead to the development of a new practical diagnostic tool for
115 clinical routine analysis. The aim of the present study was to generate a score based on
116 multivariate training to easily and accurately distinguish children with PP from those with
117 NCCAH. Routine ACTH tests would therefore be unnecessary and limited to ruling out
118 adrenal insufficiency (AI).

119 **PATIENTS AND METHODS**

120 *Patients*

121 The exploration of leftover samples of serum of all children who had had routine ACTH tests
122 to screen for NCCAH at the Trousseau Pediatric Endocrinology Unit from June 2016 to July
123 2018 was extended to 16 steroid profile analyses. All patients came to the endocrine unit
124 because of premature pubic hair, to rule out either adrenal disease or precocious puberty. Only
125 children with early pubic hair (before 8 years in girls, and before 9 years in boys) were
126 included in the study. Children with central precocious puberty (CPP) (> Tanner stage 1
127 associated with an increase in luteizing hormone LH > 5 after gonadotropin-releasing
128 hormone (GnRH) testing) were excluded. One younger patient (1 year and 3 months old)

129 without premature pubic hair was included because of high basal 17-OHP levels in an
 130 unrelated context of cryptorchidism; one patient was older than the others (12 years and 10
 131 months old) and had been tested because of Prader-Willi syndrome-associated PP [23].
 132 The first set of patients (year 1, n=58), recruited from June 2016 to July 2017, was used to
 133 train the model. Subsequently, a validation set (year 2) of an additional group of patients (n=
 134 39) was obtained to validate our model (Figure 1). The latter had the same inclusion and
 135 exclusion criteria as patients in the training set. The inclusion of patients in the validation set
 136 started in November 2017 and ended in July 2018. The study was conducted in accordance
 137 with the Declaration of Helsinki.



138
 139 *Figure 1: Data flow diagram for the study (17-OHP > 10ng/ml corresponds to the 17-OHP*
 140 *stimulated concentration)*

141

142 The model was first trained and tuned (feature selection) on a training set of children (n=63
143 with exclusion of 5 boys with CPP) from a pediatric endocrinology unit (year 1) and
144 internally evaluated using cross-validation. A validation set of children (year 2), recruited
145 consecutively to the training set from the same clinical institution, was used for external
146 evaluation (n=39). The results were matched and labeled according to the expected diagnosis
147 output.

148

149 *ACTH test*

150 All patients underwent ACTH testing at 8:30 AM, after a night of fasting. Physical
151 examination was performed by a pediatric endocrinologist. The data collected included height
152 in standard deviation score (SDS), growth velocity in SDS, body mass index (BMI) in Z-score,
153 pubertal status as defined by Tanner, and bone age (BA) increment (BA–chronological age).
154 Growth curves were standardized according to Sempé [24]. BMI was analyzed according to
155 Rolland-Cachera standards [25]. The BA was determined according to the method of Greulich
156 and Pyle [26]. When the patients had breast development or increased testicular volumes, they
157 also had a GnRH test. Steroids were assayed at baseline, then stimulated by ACTH and
158 assayed by LC-MS/MS as previously described [22]. Informed consent for *CYP21A2* gene
159 analysis was obtained from the patients themselves and their parents when 17-OHP levels
160 were above 10 ng/ml, as approved by our local ethics committee. Following the criteria
161 defined by Kuttann et al. [12], patients with 17-OHP concentrations above 10 ng/ml after
162 ACTH test and bearing abnormalities in *CYP21A2* gene on different alleles were defined as
163 NCCAH. In our cohort, all NCCAH patients presented a 17-OHP concentration above 22
164 ng/ml after ACTH testing. All PP patients exhibited a 17-OHP concentration below 5 ng/ml
165 after ACTH test (except one heterozygous patient with a 9 ng/ml peak 17-OHP).

166

167

168 *LC-MS/MS steroid profiling*

169 A panel of 16 steroids was analyzed before ACTH test (basal) and 60 minutes after 250 µg
170 ACTH injection (peak) in the Department of Clinical Metabolomics at the Saint Antoine
171 Hospital [22]. The 16 steroids were 11-deoxycortisol (11-DF), 21-deoxycortisol (21-DF),
172 11β-hydroxyandrostenedione (11OHA4), androstenedione (A4), testosterone (T), 17α-
173 hydroxyprogesterone (17-OHP), 17α-hydroxypregnenolone (17-OHPreg),
174 dehydroepiandrosterone (DHEA), aldosterone (ALDO), cortisone (E), cortisol (F),
175 corticosterone (B), 11-deoxycorticosterone (DOC), 21-deoxycorticosterone (21-DB),
176 progesterone (P) and pregnenolone (Preg). Steroids were measured in serum by LC-
177 MS/MS as described elsewhere [22].

178 Briefly, a mixture of the deuterated internal standard (150 µL) was added to 50–100 µL of
179 serum. The solution was mixed and left standing for 5 minutes, then loaded into
180 an Isolute SLE + 0.4 mL cartridge (Biotage, Uppsala, Sweden). The samples were allowed to
181 adsorb for 5 minutes before elution of the steroids through the addition of 2 × 0.9 mL
182 methylene chloride. The eluates, which contained the non-conjugated steroids, were
183 evaporated until dry and reconstituted to 150 µL in methanol/water (50/50, volume-to-volume
184 ratio). Steroids were chromatographically separated by high-performance liquid
185 chromatography using a Shimadzu Nexera XR system (Shimadzu France, Marne la Vallee,
186 France) and a Coreshell C18 column (Kinetex, 2.6 µm 100 Å 100 × 2.1 mm; Phenomenex,
187 Le Pecq, France). Detection was performed using a triple quadrupole mass spectrometer
188 (Triple Quad 6500, ABSciex, Foster City, CA). Upon collection, the LC-MS/MS data were
189 analyzed using MultiQuant software (ABSciex, Foster City, CA, version 3.0) with built-in
190 queries and quality control rules that allowed for compound-specific criteria for flagging
191 outlier results. These flagging criteria included accuracies for standards and quality control,

192 quantifier ion/qualifier ion ratios, and lower/upper calculated concentration limits. For each
193 calibration curve, the regression line used for quantification was calculated using least-
194 squares weighting (1/x).

195

196 *Data processing and statistical analysis*

197 Biological and clinical data were combined and subjected to multivariate analyses and were
198 compared between the NCCAH children and the other children (referred to as the PP group)
199 from the training set. Univariate analysis was first performed using Mann–Whitney testing
200 according to the number of observations (statistical significance threshold $p < 0.05$).

201 The raw data were then loaded into SIMCA 15 software (version 15, Umetrics, Västerbotten,
202 Sweden), using principal component analysis (PCA) and orthogonal partial least square
203 discriminant analysis (OPLS-DA) after standardization (removing the mean and scaling to
204 unit variance). A metabolic footprint was assigned for each patient using clinical data and the
205 baseline adrenal steroid levels were recorded by the LC-MS/MS. The goal was to determine
206 whether basal levels can be as accurate as an ACTH test in predicting NCCAH.

207

208 *Unsupervised multivariate analysis of the training set*

209 The purpose of this first step was descriptive; the goal was to provide an overview of the
210 distribution of the two groups of patients and the variability of the system. PCA, an
211 unsupervised method (dimension reduction), was applied to the data to observe the possible
212 presence of trends and groupings, which were previously not obvious by observing the raw
213 data. PCA allowed the detection of potential outliers as well. PCA is used to preprocess and
214 reduce the dimensionality of datasets, while preserving the original structure and relationships
215 inherent to the dataset. The resulting data were displayed as score plots representing the
216 distribution of the samples in multivariate space.

217

218 *Supervised multivariate analysis of the training set*

219 The objective of this next step was to determine if the distribution was significantly different
220 between the NCCAH patients and the PP patients of the training set. A discriminant analysis
221 of variables was performed. The assignment of patients in each group (NCCAH and PP) was
222 made *a priori* for the construction of the model. OPLS-DA, as a supervised and useful
223 multivariate ML algorithm [27], was applied to the training set. Based on recent studies, the
224 OPLS approach was considered particularly appropriate, given the input data type
225 (multicollinearity) and size of the present study [28].

226 The developed model can be evaluated using several parameters, such as goodness of fit (R^2),
227 a test of permutation, and the capability to predict (Q^2). Eventually, owing to the validation
228 set, we were able to build a predictive classification score. In order to determine the relevance
229 of the variables and to rank their predictive capacity in the model, we used variable
230 importance in projection (VIP) [29].

231

232 **RESULTS**

233 *Patient's characteristics from the training and validation set*

234 All patients visited the endocrine unit because of precocious pubic hair to exclude adrenal
235 disease or precocious puberty. A total of 97 patients (81 girls and 16 boys) were included in
236 our study – 58 patients in the training set and 39 in the validation set (figure 1).

	Training set		Validation set	
	NCCAH	PP	NCCAH	PP
Age (years)	8.2 ± 3.2	7 ± 2.2	7.3 ± 1.7	7.5 ± 1.5
% of girls	50	82	100	91
Body Mass Index (SD)	1.3 ± 1.2	1.6 ± 2.2	2 ± 0.8	1.4 ± 2.1
Bone Age (delta)	3 ± 1.5	1.3 ± 1	2 ± 0.9	1.4 ± 0.9
Growth Velocity (SD)	0.8 ± 1.6	2 ± 2.1	4.8 ± 0.4	1.3 ± 1.5

237

238 *Table I: Clinical characteristics (means ± SD) of patients from both training and validation*
 239 *sets.*

240

241 All patients were in Tanner stage 1 (prepubertal stage) (Table I). Thirteen patients were
 242 diagnosed with NCCAH by 21-OHD, confirmed by *CYP21A2* genetic analysis: 8 patients
 243 from the training set and 5 patients from the validation set (Tables II). All these patients
 244 presented with a V281L mutation; 54% had an additional severe mutation or deletion. 12 of
 245 the 13 had partial AI, defined as a post-ACTH cortisol peak < 180 ng/ml. Basal levels of 17-
 246 OHP ranged from 3.62 ng/ml to 87.94 ng/ml. Peak 17-OHP levels after ACTH testing ranged
 247 from 22.73 ng/ml to 92.57 ng/ml (Table II).

248

	NCCAH patients	Gender	Basal 17 α -hydroxy progesterone	Basal 21-deoxycortisol	Basal cortisol	Peak 17 α -hydroxy progesterone	Peak 21-deoxycortisol	Peak cortisol	Genotypes
Training set (year 1)	1	M	16.9	4.058	170.00	35.14	7.308	206.00	V281L/V281L
	2	F	5.16	0.54	86.79	27.41	5.65	128.36	V281L/P453S
	3	F	23.74	3.18	149.34	54.37	12.15	169.49	V281L/IVS2-13C>G
	4	F	28.50	2.17	137.31	59.80	9.70	152.28	V281L/IVS2-13C>G
	5	F	5.13	0.81	61.53	38.5	11.57	97.82	V281L/IVS2-13C>G
	6	M	3.62	0.04	47.88	22.73	3.72	122.81	V281L/V281L
	7	M	87.937	15.385	127.156	92.567	15.069	138.569	V281L/deletion
	8	M	18.762	2.025	83.779	52.754	9.593	113.229	V281L/deletion
Validation set (year 2)	9	F	11.73	2.56	112.20	39.80	9.93	131.63	V281L/[Q318X;A265V]
	10	F	3.91	0.09	57.36	45.11	0.04	161.86	V281L/M383V
	11	F	34.16	7.37	152.67	58.2	15.66	169.29	V281L/P30L
	12	F	8.44	0.81	89.11	51.4	13.77	170.63	V281L/V281L
	13	F	12.42	1.66	79.01	73.36	17.59	135.8	V281L/R356W

249

250 *Table II: Basal and peak (after ACTH test) steroid concentrations (in ng/mL) of 17-OHP, 21-*
251 *DF, F, and genotypes of NCCAH patients. Patients 1–8 belonged to the training set and*
252 *patients 9–13 to the validation set.*

253

254 A total of 58 patients (45 girls and 13 boys) with a mean age of 7.14 years (+/- 2.32) were
255 included in our training set (Figure 1). Five boys had an increase in testicular volume (> G1)
256 with peak LH > 5 after GnRH test and were not included in the study. Nine patients after
257 ACTH testing had a peak 17-OHP level > 9 ng/ml. *CYP21A2* gene analysis was performed on
258 these patients. A total of eight patients were diagnosed with homozygosity for NCCAH and a
259 diagnosis of heterozygosity for 21-hydroxylase was made in one girl, an 8-year-old with
260 pubic hair appearance at the age of 7 years and 3 months. she had an accelerated growth
261 velocity (10 cm in one year, 5.4 SDS) and a two-year BA advance; 17-OHP basal level was
262 0.57 ng/ml with a peak at 9.06 ng/ml. Sequencing of the *CYP21A2* gene found a moderate
263 mutation p.H38L in a heterozygous state.

264 Table III compares the basal biological characteristics of the children in the training set. The
265 difference between NCCAH and PP children was significant ($p < 0.05$) for A4, T, 17-OHP,
266 21-DB, 21-DF, PREG, 11OHA4, and P.

Steroids	NCCAH	PP	P values
	n= 8	n=50	
	Mean (range)	Mean (range)	
Aldosterone	164.52 (49-311.05)	114.19 (10-712)	NS
Dehydroepiandrosterone	2.85 (0.1-6.36)	2.15 (0.1-13.83)	NS
Androstenedione	0.84 (0.11-1.28)	0.28 (0.04-0.81)	***
Testosterone	0.2 (0.07-0.44)	0.07 (0.01-0.32)	***
Pregnenolone	1.36 (0.77-2.23)	0.82 (0.04-3.19)	**
11β-hydroxyandrostenedione	2.05 (0.72-3.73)	0.59 (0.1-1.89)	***
17α-hydroxypregnenolone	3.26 (0.96-5.71)	1.8 (0.01-15.04)	**
Progesterone	0.88 (0.15-3.4)	0.09 (0.001-0.99)	***
21-deoxycorticosterone	20.11 (12.42-31.34)	5 (5-5)	***
11-deoxycorticosterone	31.79 (8.99-71)	54.33 (6.77-227.13)	NS
17α-hydroxyprogesterone	14.19 (3.62-28.5)	0.28 (0.05-1.03)	***
21-deoxycortisol	1.92 (0.04-4.06)	0.03 (0.001-0.51)	***
Corticosterone	0.67 (0.24-1.01)	2.74 (0.37-26.95)	**
11-deoxycortisol	0.55 (0.23-1.04)	0.46 (0.006-3.07)	NS
Cortisone	26.58 (18.01-35.56)	21.99 (9.48-32.22)	NS
Cortisol	106.1 (47.88-170)	79.97 (26.81-186.11)	NS

NS : p-value>0.05 ; **: p-value{0.001-0.05} ; *** : p-value<0.001

267

268 *Table III: Steroid concentrations of the training set (mean and range), n=8 for NCCAH and*

269 *n=50 for PP. All variables are in basal values. All steroid concentrations are expressed in*

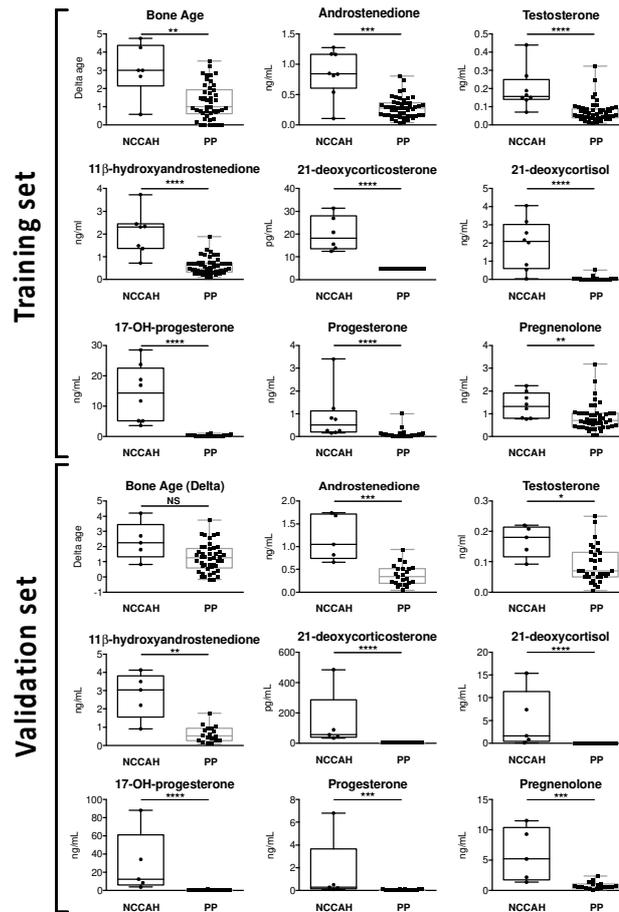
270 *ng/mL except for DOC and ALDO in pg/mL.*

271

272 There was no overlap for the 17-OHP and 21-DB values between the two groups in both sets,

273 with significantly increased 17-OHP, 21-DF, and 21-DB levels in the NCCAH children

274 (Figure 2).



275

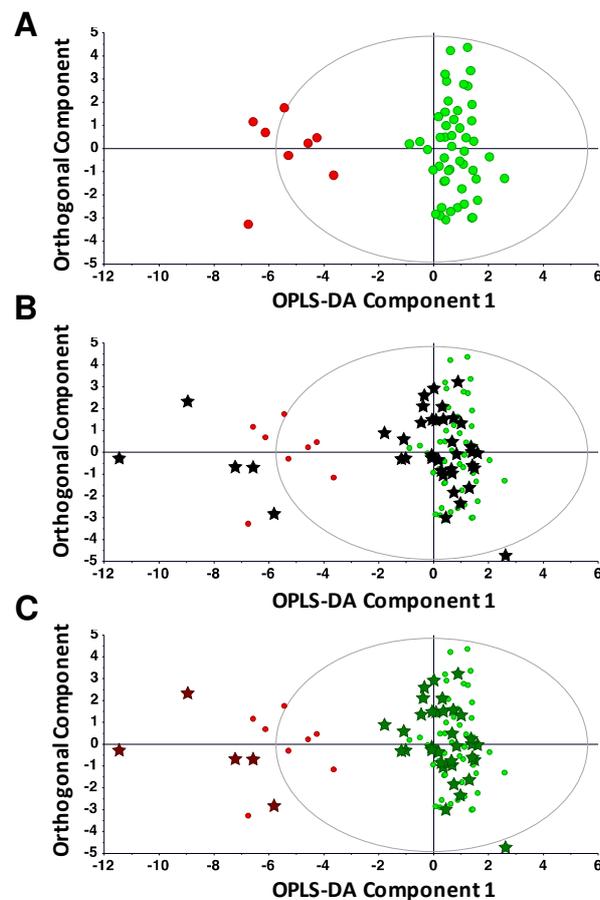
276 Figure 2: Box plots of bone age and some circulating steroid concentrations at their basal
 277 state from the two groups of patients from the training and the validation set: late onset
 278 NCCAH children (n=8 training set and n=5 validation set) and the PP children (n=50
 279 training set and n=34 validation set). All steroids are expressed in ng/mL except for 21-DB in
 280 pg/mL. Bone age is expressed as a difference in years (BA–chronological age).

281 Unsupervised multivariate analysis of the training set was first completed. The descriptive
 282 analysis showed a good separation between the NCCAH group and the PP group (R2X =
 283 0.350). The variables with poor discrimination were removed from the study, which were
 284 growth velocity and BMI.

285

286 *Model construction*

287 Secondly, a score plot was obtained from a supervised multivariate analysis of the training set
288 using OPLS-DA (Figure 3A). The observations were projected in the system's maximum
289 variability plan ($R^2X = 0.900$ and $Q^2X = 0.868$) on two components. NCCAH patients were
290 segregated on the left side of the score plot (red dots) while PP patients were segregated on
291 the right quadrants (green dots). There was a good separation of the groups without any
292 overlap. The heterozygous patient was segregated in the PP group.

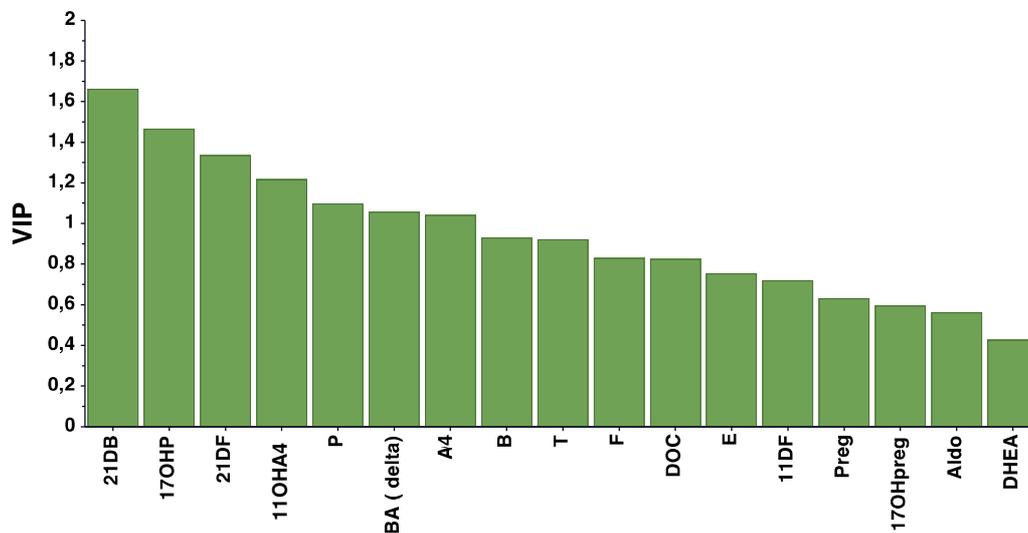


293
294 *Figure 3: Score plots from orthogonal partial least squares discriminant analysis*
295 *(incremental process with model training and external evaluation) of late onset NCCAH and*
296 *PP children as assessed by their basal circulating signature (16 steroids and BA). Panel A:*
297 *Score scatter plot plots of the training set generated with a supervised approach from known*
298 *NCCAH (n=8, red dots) and PP (n=50, green dots) children. R^2 and Q^2 were 0.900 and*
299 *0.868 respectively. Panel B: Score plot of the initial training set (NCCAH (n=8, red dots) and*

300 *PP (n=50, green dots)) superimposed with new children from the validation set (n=39*
301 *children) labelled with black dots, indicating no a priori class attribution. Panel C: Score plot*
302 *of the initial training set (NCCAH (n=8, red dots) and PP (n=50, green dots)) superimposed*
303 *with the new children from the validation set labeled according to their retrospective final*
304 *diagnosis: NCCAH (n=5, red stars) and PP (n=34 green stars). Validation accuracy = 1.*

305

306 To build a prediction score, the contribution of each variable of our system was calculated
307 (Supplementary Figure 1). The higher the absolute value of the coefficient of a variable, the
308 more discriminatory and important the variable was for prediction and group separation. The
309 coefficients were calculated using the algorithm established by the OPLS-DA regression. As
310 shown in Supplementary Figure 1, the variables 21-DB, 17-OHP, 21-DF, 11OHA4, and P
311 were the top 5 highest contribution coefficients for NCCAH group discrimination followed by
312 BA, the only discriminating clinical factor with a VIP score > 1.



313

314 Supplementary Figure 1: VIP plots to classify in descending order with confidence intervals
315 the importance of each of the 17 variables (11-DF, 21-DF, 11- β OHA, A4, T, 17-OHP, 17-
316 OHPreg, DHEA, ALDO, E, F, B, DOC, 21-DB, P, Preg and BA) for the construction of the
317 score.

318

319 *Validation of the model with the replication cohort*

320 The capability to predict the model was good according to internal cross validation $QX2 =$
 321 0.868. A validation set of 39 patients (36 girls and 3 boys) was used to assess a second step
 322 validation. The average age was 7.50 years (± 1.46), and no patient was excluded. Five
 323 patients had a peak 17-OHP level > 10 ng/ml and were diagnosed as NCCAH after *CYP21A2*
 324 gene analysis (see Table I, patients 9–13).

325 To note, by definition, the data from the validation set were not used to refit the model; they
 326 were only used to test its accuracy. To mimic future prospective routine diagnosis,
 327 observations from the validation set were first labelled with black stars, as no *a priori* class
 328 attribution was achieved (Figure 3B). Eventually, as seen in Figure 3C, to evaluate the true
 329 model performances, the black stars corresponding to children from the validation set were
 330 labeled according to their retrospective final diagnosis: NCCAH (n=5, red stars) and PP (n=34
 331 green stars). Five patients of the validation set were segregated in the same area of the score
 332 plot as the NCCAH patients based on the training set. These 5 patients were eventually *a*
 333 *posteriori* genotyped as NCCAH. The misclassification table (Table IV) summarizes the
 334 prediction results of the model.

		Number of Patients	Correct classification rate	NCCAH predicted	PP predicted
Training set	NCCAH	8	100%	8	0
	PP	50	100%	0	50
Validation set	NCCAH*	5	100%	5	0
	PP*	34	100%	0	34
	Total	97	100%	13	84
	Fisher's prob	5.2e-10			

335 *: diagnosis made with post-hoc confirmation by *CYP21A2* analysis

336 *Table IV: Model prediction results. Assignment in each class (NCCAH or PP) of the patients*
 337 *from the training set and the validation set. The predictive results obtained on the training set*
 338 *were obtained using cross-validation.*

339

340 The first column displays the total number of observations in the training set and the
341 validation set, the average percentage correctly classified, and the number of observations
342 classified to each class. As mentioned in the No class column, the model never classified any
343 of the patients to other classes than those available (i.e., YPred score value <0). Fisher's
344 probability is the probability of the table occurring by chance and is satisfied when $p < 0.05$
345 for 95% confidence. Here, the success rate for the prediction of the membership class was
346 100% with either no false positive or false negative scores. The five NCCAH patients in the
347 validation set (post-hoc confirmation by *CYP21A2* analysis) were all assigned to the NCCAH
348 group in our model. Therefore, thanks to the OPLS model integrating steroid fingerprint and
349 clinical data, the classification of NCCAH patients and PP patients was optimum with a
350 validation accuracy of 100%.

351

352 **DISCUSSION**

353 The objective of the present study was to develop, based on a mathematical ML model, a
354 score including basal circulating steroid profile and clinical data to avoid the use of an ACTH
355 testing in the differential diagnosis of NCCAH in a pediatric population.

356 Indeed, ACTH tests are useful in detecting AI in children with NCCAH. In our study, similar
357 to other pediatric studies [30–32], a high number of NCCAH patients presented a cortisol
358 peak below 180 ng/ml (12 NCCAH patients out of 13). These data underlined the importance
359 of glucocorticoid stress dose in children undergoing stressful situations like surgery or illness.

360 Our findings uncovered clinical and biological differences between children with premature
361 pubic hair and NCCAH with significant results for BA and basal levels of 21-DB, 17-OHP,
362 21-DF, A4, T, Preg, and 11OHA4. There were no overlaps for 21-DB and 21-DF
363 (concentration threshold = 12.42 pg/mL), or for 17-OHP (concentration threshold = 3.62
364 ng/mL). It was consistent with previous studies, in particular for 21-deoxysteroids [19,20,22].

365 Indeed, 21-DF and 21-DB are strictly adrenal metabolites and therefore represent potentially
366 more specific biomarkers for NCCAH diagnoses than 17-OHP, which has a dual ovarian and
367 adrenal origin [22,43,44]. Some authors have already proposed a threshold of basal 17-OHP
368 [34], but this remains controversial [32,33]. With the validated mass spectrometry approach
369 (LC-MS/MS), we were able to routinely quantify simultaneously in 10 minutes 16 circulating
370 steroids via a direct measurement method with excellent sensitivity and specificity (certified
371 by the French national quality control organization). We can thereby attribute a specific
372 steroidome, hormonal signature, or “fingerprint” to each patient [22].

373 In our patients, basal measurements of 17-OHP were surprisingly elevated, and could already
374 distinguish NCCAH patients from PP patients in univariate analysis. This was not always the
375 case for our patients, for whom a threshold value of 17-OHP was always discussed,
376 presumably because our pediatric patients had more severe features than late onset adrenal
377 hyperplasia diagnosed in adulthood and because they were bearing a severe mutation in 54%
378 of the cases. However, the use of 17-OHP which characterizes 21-OHD is not without
379 criticism [35]. False negative rates of up to 22% have been reported in infant screening [36],
380 particularly when mothers were exposed to glucocorticoids prenatally [37]. False positives in
381 some premature infants or in other forms of CAH including 11-hydroxylase deficiency were
382 also reported [38]. Moreover a single basal 17-OHP concentration is often insufficient to
383 diagnose non-classic 21-OHD carriers [39].

384 The use of mass spectrometry gives a more specific view of adrenal steroid levels in 21-OHD
385 compared with immunoassays [40]. Furthermore, in terms of cost, according to our own
386 experience in our clinical laboratory, a panel of about 16 steroids can be more advantageous
387 than relying on specific expensive immunoassay kits. This method is more specific and more
388 sensitive than radioimmunoassay (RIA) because it allows the quantifying of steroid profiles
389 including 21-DF within 150 µl of serum. It also allows the measurement of other steroids such

390 as 21-DB, which is particularly informative for CAH. To our knowledge, 21-DB has not been
391 extensively studied until now. According to our previous data, plasma basal 21-DB
392 concentrations measured by RIA [41], and more recently in LC-MS/MS [22], could represent
393 an interesting additional biomarker to identify patients with NCCAH. Although 17-OHP is
394 usually quantified on its own, the addition and combination of new strictly adrenal steroids,
395 such as 21-DF and 21-DB, could enhance the specificity for the diagnosis of NCCAH. Miller
396 et al. even suggest replacing 17-OHP with 21-DF in the newborn screening program [35].
397 With the ability to routinely and simultaneously quantify 16 circulating steroids, we
398 highlighted the use of some steroids not often used in practice (such as 21-DB with no overlap)
399 and other better-known steroids (like 21-DF). 21-DF has already been evaluated in children
400 with 21-OHD, and was deemed an excellent specific marker of this disease [20]. 21-DF is
401 useful in the diagnosis and the follow-up of NCCAH and for the detection of heterozygotes
402 [42].
403 Determining the status of heterozygotes and NCCAH is very important, especially in those
404 carrying severe alleles, for genetic counseling to anticipate the risk of AI and genital
405 ambiguity at birth [43]. In our study, 54% of NCCAH children had a severe mutation. It is
406 further essential to explain the screening to the family's proband and the future partner in
407 family planning [44,45].
408 A non-classical form of mutation (M283V of the NCCAH patient no. 10, Table I) has been
409 described in just one case of a patient with NCCAH [46]. Notably, all of our NCCAH patients
410 had the V281L mutation, the most common mutation in patients with NCCAH [47]. Three
411 had the IVS2-13C>G allele, the most frequent mutation in classical forms [9].
412 Our findings demonstrated a difference in clinical features and advanced BA, as is already
413 known in the literature [7]. Even using subjective methods, it is easy to perform routinely in
414 clinical settings [26]. In our study, all children with NCCAH demonstrated advanced BA and

415 six had advanced BA of more than two years. Some reports propose glucocorticoid therapy
416 for these patients to avoid a short final stature [11]. In combination with biological
417 measurements, BA was an important part of our design score for the diagnosis of NCCAH,
418 suggesting that an advanced BA may be the only difference between clinical features of
419 NCCAH patients and PP [34]. We limited our study to prepubertal children from 7 to 9 years
420 old (mean age 7.14 years (+/- 2.32) to focus on adrenal steroids responsible for adrenarche
421 and to free ourselves from sex variations induced by gonadal puberty. Analysis of steroids
422 only secreted by the adrenal gland could be a further area of study [47].

423 The model we developed predicted with 100% sensitivity and specificity the diagnoses of
424 NCCAH in a population of children with premature pubic hair (n=97), and could probably be
425 extended to older populations with mild features. The constitutions of the training set (first
426 year) and the validation set (second year) led us to evaluate the robustness of our model more
427 accurately than with a simple internal cross-validation. Multivariate data analysis [48] and
428 ML methods have already been evaluated in adrenal diseases and can shorten diagnosis in
429 some cases [49,50]. In this study, OPLS-DA analysis was chosen as the ML algorithm since
430 our dataset included many steroids with potentially high collinearity. We trained and
431 evaluated 5 other ML models (support vector machine, nearest neighbors classifier, decision
432 tree, random forest, and Gaussian Naive Bayes) which confirmed the discriminative power of
433 the data, independently of the chosen method (not shown). However, their performances were
434 either worse or equivalent with OPLS-DA. Moreover, this approach gave us the opportunity
435 to present the data on 2D score plots, creating a bird's eye view to summarize and study the
436 degree of similarity (or dissimilarity) of the patient's bio-clinical signatures.

437 In future, multivariate mathematical approaches such as these, which can integrate
438 anthropometric data (gender, age, BMI, etc.), biological measurements, and even radiological
439 images, could generate a unique fingerprint for each patient and offer new potentialities in the

440 future diagnosis toolbox. Model training iteration processes could eventually be performed.
441 Indeed, with the selected features, additional patients could be included to build refined
442 models over time. The proposed model could therefore find its place in the care management
443 pathway of children with premature pubarche and late onset 21-OHD. The method may be
444 extended to higher ages; this work is in progress with adult patients from non-pediatric
445 endocrinology units, considering age and sex references for adult patients. Prospective
446 interventional studies will be needed to validate this model for clinical use [50].

447

448 **CONCLUSION**

449 To conclude, LC-MS/MS enabled assignment of a metabolic fingerprint to each patient.
450 Combining this with a statistical model allowed the construction of a NCCAH diagnostic
451 score with 100% sensitivity and specificity in our cohort. The most significant variables were
452 21-DB, 17-OHP, and 21-DF. If this score was routinely implemented, use of the ACTH test
453 could be restricted to screening patients for AI, which, based on the prevalence of NCCAH,
454 would be nearly one in ten children. Genetic analysis of *CYP21A2* would remain essential in
455 NCCAH patients to identify severe mutations. Further studies are needed for the validation of
456 this score, particularly with the use of a larger multicenter prospective cohort.

457

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461

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463

464 **AUTHOR STATEMENT**

465 **Héléna Agnani** : Conceptualization, Methodology, Writing- Reviewing ; **Guillaume**

466 **Bachelot** : Data curation, Data analysis ; **Thibaut Eguether** : Visualization, Investigation ;

467 **Bettina Ribault** : mass spectrometry analysis ; **Jean Fiet** : Methodology, Writing- Reviewing

468 ; **Yves Le Bouc** : Methodology, Writing- Reviewing ; **Irène Netchine** : Methodology,

469 Writing ; **Muriel Houang** : Conceptualization, methodology, writing-original draft

470 preparation-Reviewing ; **Antonin Lamazière** : Conceptualization, methodology, Data

471 analysis, Writing- Reviewing and Editing.

472

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