Differential Methylation of 11-Oxyandrogen Biosynthetic Pathway Genes in Girls with High DHEAS Levels

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Abstract

Context: Premature adrenarche in girls is defined biochemically by an increase in adrenal androgen (DHEA and DHEAS) levels above the age-specific reference range before age 8 years. Recently, increased levels of 11-oxyandrogens have also been observed in girls with premature adrenarche and it is hypothesized that epigenetic modifications, specifically CpG methylation, may affect gene expression and/or activity of steroidogenic enzymes.

Objective: To determine whether circulating DHEAS levels in pre-pubertal girls are associated with methylation status of genes involved in DHEAS and 11-oxyandrogen steroidogenesis.

Design and Methods: Ninety-seven healthy girls followed since the age of 3 years were classified, according to DHEAS serum concentration at age 6-7 years, as normal DHEAS (< 42 µg/dL [75th percentile for population]) or high DHEAS (≥ 42 µg/dL). At Tanner stage 2, methylation status of CpG sites located in genes *SULT2A1*, *HSD11B1*, *HSD11B2*, *CYP11B1*, *HSD17B2* and *HSD17B5* were analyzed in genomic DNA from peripheral blood leukocytes either by Methylation-Sensitive Restriction Enzymes Quantitative PCR assay or by Melting Curve Analysis Methylation assay.

Results: Significantly lower methylation levels were detected in the *CYP11B1* gene in girls with high versus normal serum DHEAS, with no differences found in the other genes. In addition, we found a significant inverse correlation between *CYP11B1* methylation and insulin level at Tanner 1 and BMI at Tanner 1 and 2 in the whole cohort.

Conclusion: These results suggest that a lower methylation of *CYP11B1* could be a mechanism contributing to increased concentrations of 11-oxyandrogens in premature adrenarche and to the associated metabolic risk.

26 **Introduction**

27 Adrenarche denotes the maturation of the adrenal gland zona reticularis, the principal site where
28 adrenal androgens are synthesized (1). This developmental process precedes and is independent
29 from gonadarche. Premature adrenarche (PA), described by Silverman and co-workers (2), is
30 defined by the precocious appearance of adrenarche's clinical signs, such as axillary or pubic hair
31 or adult body odor, before age 8 years in girls and age 9 years in boys (3)—accompanied by
32 elevated age and sex-adjusted DHEA and DHEAS levels (DHEAS ≥~40 μg/dL) (4, 5). Premature
33 adrenarche is more frequent in girls than in boys (6).

34 Although PA is a diagnosis of exclusion, studies in the last two decades suggest that this condition
35 is associated with higher BMI (7-10) and cardiometabolic risk (11-17). However, the association of
36 prepubertal obesity with increased androgen may be transient and mediated mainly by increased
37 BMI (18). Also, previously described associations may depend on the prevalence of low birth
38 weight and ethnic background of the study population (19, 13). Although different molecular
39 etiologies have been postulated for PA (20-23), its molecular basis remains unresolved.

40 Dehydroepiandrosterone (DHEA) and DHEA-sulphate (DHEAS) have been the "traditional" markers
41 of adrenarche onset (24, 25), but recently the adrenal gland has also been shown to produce 11-
42 oxyandrogens in addition to the classical androgenic steroids (Supplementary Figure 1) (26, 27).
43 11-Oxyandrogens are fully active androgens, with androgen activity comparable to their 11-deoxy
44 analogs, and are increased in girls with PA (28). Moreover, it was recently shown that the activity
45 of 17β-Hydroxysteroid dehydrogenase (HSD17B), which is involved in 11-oxyandrogen synthesis, is
46 higher in girls with PA than in age-matched controls (29).

47 Epigenetic modifications, among them cytosine methylation at dinucleotide CpG, are involved in
48 gene expression control according to a tightly regulated epigenetic program that is also affected
49 by the environment (30). Epigenetic control of some genes that code for enzymes that participate

50 in the synthetic pathway to 11-oxyandrogens (e.g., *CYP11B1*, *HSD11B2*, *HSD17B2* and *SULT2A1*)
51 has also been described (31-35). Consequently, we sought to determine whether there are
52 differences in the methylation status of CpG sites associated with DHEAS and 11-oxyandrogen
53 steroidogenic genes between pre-pubertal girls with normal (<42.0 μg/dL [75th percentile for
54 population]) and those with high (≥42.0 μg/dL) DHEAS levels as a marker of earlier adrenarche.

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57 **Subjects, Materials and Methods**

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59 ***Study population***

60 Study subjects were a subset of the longitudinal Growth and Obesity Cohort Study (GOCS) in
61 Santiago, Chile. This cohort study was initiated in 2006 with recruitment of children from ages 2.6
62 to 4.0 years who fulfilled these inclusion criteria: 1) singletons born in 2002-2003, 2) with birth
63 weight between 2,500 and 4,500 g, 3) gestational age 37 to 42 weeks, and 4) absence of physical
64 conditions known to affect normal development. The GOCS children are representative of the low
65 to middle-income families served by the public nursery schools (5, 9). Of the total cohort, 602 are
66 girls. Among the 166 girls with a peripheral blood specimen collected at Tanner breast stage 2 and
67 a prepubertal serum DHEAS determination at age ~7 years, we selected 97 girls with normal BMI-
68 SDS (-2 to +2) for the current study. Informed consent was obtained from all parents or guardians
69 of children before data and sample collection. The Ethics Committee of the Institute of Nutrition
70 and Food Technology, University of Chile, approved the study protocol.

71 At age ~7 years, a single pediatric endocrinologist (VM) had assessed breast development by
72 palpation and classified girls according to Tanner stages (36). Clinical signs of androgen action
73 were also evaluated (e.g., pubarche and subsequent pubic hair stages). Thereafter, every 6

74 months, a single dietitian (same sex), trained specially for this purpose and with permanent
75 supervision of a single pediatric endocrinologist (V.M.), evaluated secondary sex characteristics
76 (Kappa = 0.9). Anthropometric measures (weight and height) were collected using standardized
77 protocols by a single dietitian (same sex) (37). Body mass index (BMI) was estimated by dividing
78 weight (kg) by height squared (m^2). Height-for-age, weight-for-age and BMI-for-age standard
79 deviation scores (SDS) were determined based on the World Health Organization growth
80 reference (38). Plasma levels of insulin (IU/mL) were measured by colorimetric methods (39) and
81 serum glucose concentrations were assessed by enzymatic colorimetric techniques (HUMAN,
82 Gesellschaft fur Biochemica und Diagnostica, Wiesbaden, Germany).

83 At age ~7 years, a fasting venous sample was collected (8 to 12 am) in girls with confirmed
84 absence of fever (<37.5ºC) and other symptoms of acute infection. Serum DHEAS was determined
85 by competitive specific binding RIA supplied by DIAsource ImmunoAssays (Ottignies-Louvain-la-
86 Neuve, Belgium). Next, a biochemical cut-off for early adrenarche was defined by circulating
87 DHEAS concentration ≥42.0 µg/dl (75th percentile for the age ~7 COGS population) as previously
88 described (9). Girls with serum DHEAS ≥42.0 µg/dL were designated as high DHEAS (HD), and those
89 with DHEAS <42.0 µg/dL were designated as normal DHEAS (ND). During Tanner breast stage 2
90 (T2), genomic DNA was obtained from a buffy coat of a peripheral blood sample for DNA
91 methylation analysis.

92 ***DNA methylation measurements***

93 We evaluated the methylation status of CpG sites within or close to genes involved in 11-
94 oxyandrogen biosynthesis (*CYP11B1*, *HSD11B2*, *HSD11B1*, *HSD17B2,* and *HSD17B5*)—and in gene
95 *SULT2A1,* which is involved in DHEAS synthesis (Table 1, Supplementary Figure 1). The different
96 CpG sites were selected from a previous DNA methylation microarray data analysis

97 (MethylationEPIC 850k array) performed in 85 girls from the same cohort (68 ND and 17 HD)
98 (Diana Ponce et al., unpublished observations). For *CYP11B1* and *HSD11B2,* Methylation-Sensitive
99 High-Resolution Melting Analysis (MS-HRMA) was applied (40). For this analysis, 100 ng of
100 genomic DNA (obtained at Tanner 2) was sodium bisulfate-treated and purified using the
101 Methylamp one-step DNA modification kit (Epigentek Group Inc., USA) according to the
102 manufacturer's protocol. Next, 2 µL of bisulfite-modified DNA was incubated with 1X HOT FirePol®
103 EvaGreen® HRM (Solis BioDyne, Estonia) and 0.5 µM of each primer in a total volume of 10 µL.
104 Primer sets were designed with the open access MethPrimer software, available at
105 http://www.urogene.org/methprimer/index1.html (Table 1). A Polymerase chain reaction was
106 performed in CFX96 Real-Time PCR equipment (Bio-Rad) as follows: pre-incubation for 15 min at
107 95ºC and 40 cycles of denaturation for 15 sec at 95ºC, annealing for 20 sec at 60ºC and extension
108 for 20 sec at 72ºC. A High-resolution melting curve was obtained in a 60ºC to 90ºC interval with a
109 rise of 0.1ºC for each 5 seconds. All reactions were run in triplicates. The Methylation status was
110 determined by the quantitative method described by Smith and co-workers (41) where the *T50*
111 (the temperature at which 50% of the molecules in the PCR product are melted) of each sample
112 was compared to a standard curve generated with a different admixture of completely methylated
113 and unmethylated bisulfite converted human DNA (EpiTect PCR Control DNA Set, Qiagen,
114 Germany).

115 For *SULT2A1*, *HSD11B1*, *HSD17B2* and *HSD17B5* a *M*ethylation-*S*ensitive *R*estriction *E*nzymes–
116 *q*uantitative PCR assay (MSRE-qPCR) was applied (42, 43). Briefly, 70 ng of genomic DNA (obtained
117 at Tanner 2) was incubated in the presence of the appropriated methylation-sensitive restriction
118 enzyme (Thermo Scientific, Lithuania) (Table 1) according to the manufacturer's protocol (digested
119 DNA), or 50% v/v glycerol (non-digested DNA). Next, 14 ng (2 µL) of digested and non-digested
120 DNA was used as template in a qPCR with 1X HOT FirePol® EvaGreen® HRM (Solis BioDyne,

121 Estonia) and 0.5 µM each primer (Table 1) in a total volume of 10 µL. A Polymerase chain reaction
122 was performed in a CFX96 Real-Time PCR equipment (Bio-Rad) as previously described, but
123 without a melting curve. All reactions were run in triplicate. The equation $2^{-\Delta Ct}$ x 100, where ΔC_t
124 corresponds to the C_t difference between digested and non-digested DNA, was used to determine
125 methylation status.

126 ***Statistical Analysis***

127 Statistical analysis of the data was performed using SPSS software version 21 (IBM Corp). Normal
128 data distribution was determined using the Kolmogorov-Smirnov test. Data with normal
129 distribution were expressed as mean ± standard error, while data with non-normal distribution
130 were expressed as median and interquartile range. The T-test and Mann-Whitney test were used
131 to analyze differences between groups for data with normal and non-normal distribution,
132 respectively. Correlations were assessed by the Spearman's correlation coefficient. Differences
133 with a p-value <0.05 were considered statistically significant.

134

135 **Results**

136 Ninety-seven girls were classified into two groups according to the DHEAS concentration at age ~7
137 (Tanner 1). Forty-nine girls belonged to the normal DHEAS group (ND) and 48 girls to the high
138 DHEAS group (HD). The clinical characteristics of the studied population at the age of recruitment
139 (Tanner 1) are presented in Table 2. Girls from the ND group were slightly younger than those of
140 the HD group, but no other anthropometric differences were observed. At the age of obtaining
141 the DNA sample for methylation study (Tanner 2), only the DHEAS concentration difference
142 persisted between groups (Table 2).

143 For the 6 studied steroidogenesis genes, CpG methylation site information and detailed
144 methylation status methodology are provided in Table 1. The sole statistically significant
145 methylation difference between ND versus HD girls was observed within the *CYP11B1* CpG island
146 (Table 3, Figure 1), with HD girls exhibiting decreased *CYP11B1* methylation level compared to ND
147 girls ($p = 0.02$).

148 We next performed correlation analysis between *CYP11B1* methylation status (obtained at Tanner
149 stage 2) and metabolic, anthropometric, and hormonal measures obtained both at Tanner stages 1
150 and 2. The *CYP11B1* methylation status correlated inversely with insulin concentration ($\rho = -0.368$;
151 $p = 0.001$) and BMI SDS ($\rho = -0.246$; $p = 0.028$) at T1 (Figure 2). In addition, the significant inverse
152 correlation with BMI SDS persisted at T2 ($\rho = -0.226$; $p = 0.043$).

153 Some significant correlations were observed among the methylation for the 6 studied enzymes
154 (Supplementary Table 1). Among these, the most relevant was methylation status between
155 *HSD17B5* and *HSD17B2* ($\rho = 0.402$; $p = 0.005$) (Figure 3), the enzymes involved in the
156 interconversion of androstenedione and testosterone, and of their 11β-hydroxy and 11-keto
157 analogs (Supplementary Figure 1).

158 Finally, we searched for possible transcription factor recognition sites that could be affected by
159 the methylation status of CpG sites evaluated for *CYP11B1*. An *in silico* analysis was performed
160 with AliBaba2.1, a program that detects transcription factor-binding sites based on sites collected
161 in TRANSFAC (44). This analysis showed that CpG clusters formed by CpGs +96 and +100; and CpGs
162 +139 and +142 in *CYP11B1* promoter match with binding sites of SP1 (Figure 4).

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166 **Discussion**

167 During the first stage puberty in girls enrolled in the Chilean Growth and Obesity Cohort Study
168 (GOCS), the DNA methylation status of 6 genes involved in DHEAS and 11-oxyandrogen
169 biosynthesis was compared between girls who at age 7 years had normal serum DHEAS ($<75^{th}$
170 percentile for COGS population) versus those who had high DHEAS ($\geq75^{th}$ percentile). We observed
171 that a cluster of CpGs in the *CYP11B1* promoter sequence had significantly lower methylation in
172 girls with high DHEAS compared to girls with normal DHEAS. None of the other 5 steroidogenesis
173 genes showed differential methylation status between girls with normal versus high DHEAS at age
174 7. Additionally, *CYP11B1* methylation correlated inversely with BMI-SDS (measured both at age 7
175 and at onset of puberty), and with fasting insulin level measured at age 7. Because *CYP11B1*
176 encodes a key enzyme in 11-oxyandrogen biosynthesis, these findings suggest that *CYP11B1*
177 methylation may be one of the mechanisms regulating 11-oxyandrogen synthesis, and that
178 differences in *CYP11B1* methylation may be a cause, or a consequence, of differences in BMI-SDS
179 and/or insulin.

180 The regulation of *CYP11B1* expression by methylation in cortisol-producing adenomas also
181 supports a functional role for *CYP11B1* promoter methylation, since the *CYP11B1* gene promoter
182 was significantly less methylated in adenoma than in adjacent unaffected adrenal tissue (31).
183 Moreover, reduced *CYP11B1* promoter activity by DNA methylation was confirmed directly in a
184 reporter assay. Notably, however, the five CpG sites analyzed by Kometani et al. were located 150
185 to 400 bp upstream of the CpG sites analyzed in our study. Similarly, an epigenetic analysis of
186 aldosterone-producing adenomas, presenting with or without hypercortisolemia, also showed that
187 the *CYP11B1* promoter was significantly less methylated in aldosterone-producing adenoma with
188 hypercortisolemia than in those without hypercortisolemia (45). Taken together, these

189 observations and the current report support the physiological significance of *CYP11B1* methylation
190 in regulating CYP11B1 expression.

191 The inverse relationship between BMI-SDS and *CYP11B1* methylation observed in our study is
192 consistent with observations in obese, diabetic *db/db* mice, in which obesity, hyperglycemia, and
193 hyperlipidemia are associated with increased adrenal Cyp11b1 and corticosterone compared to
194 control *db/+* mice (46). Also in rodents, corticosterone-induced abdominal obesity produces
195 insulin resistance (47, 48), which is consistent with our observation in girls of a significant
196 relationship between decreased *CYP11B1* methylation and increased insulin levels. However, the
197 direction of causality remains uncertain in these associations, as does the relative contribution of
198 11-oxyandrogens, glucocorticoids, and mineralocorticoids as potential mediators.

199 We observed a direct correlation between *HSD17B5* and *HSD17B2* CpG methylation status,
200 although the variability in *HSD17B2* methylation was considerably greater than that of *HSD17B5*.
201 The products of these two genes have opposing effects, with the *HSD17B2* gene product (17β-
202 hydroxysteroid dehydrogenase type 2) converting estrogens and androgens into weaker or
203 inactive steroids, while the *HSD17B5* gene product (17β-hydroxysteroid dehydrogenase type 5,
204 also known as aldo-keto reductase 1C3) does the reverse (49, 50 [Supplemental Figure 1]). Little is
205 known about *HSD17B5* epigenetic regulation, but several studies suggest that downregulation of
206 the *HSD17B2* gene by methylation may play a role in the pathogenesis of endometriosis and of
207 breast cancer (34, 51, 52). These observations are consistent with epigenetic control of "strong-
208 weak (or inactive)" sex steroid interconversion, and with the possibility that such control may
209 operate during adrenarchal development.

210 *In silico* analysis to detect transcription factor binding sites in the *CYP11B1* study sequence
211 predicted the presence of 2 potential sites for the Specificity protein 1 (SP1) (Figure 4). This

212 ubiquitous transcription factor functions by recruiting the basal transcription machinery and
213 promoting transcription (53). Several studies indicates that promoter hypermethylation affect
214 SP1's binding and transcriptional activation (54, 55). These observations would be consistent with
215 the hypothesis that diminished *CYP11B1* methylation could increase SP1 binding and consequently
216 CYP11B1 expression.

217 This study has several limitations. First, it is observational, and thus causality cannot be inferred.
218 Second, we used peripheral blood rather than adrenal tissue for DNA methylation analysis, and at
219 only a single time point that was not contemporaneous with some measurements, such as the
220 initial DHEAS levels at age 7—used to divide subjects with normal and high DHEAS—and the
221 fasting insulin levels, which were also measured at age 7. However, we have shown in earlier
222 studies that DHEAS elevation and BMI-SDS were relatively stable between ND and HD subjects
223 from age 7 to menarche (10). Lastly, serum 11-oxyandrogens have not yet been measured in
224 these subjects, although others have shown that they are elevated in premature adrenarche (28).
225 A major strength of this study was its large, unique cohort of pediatric patients—with careful
226 prospective follow-up over a long period—and the resulting ability to assess associations between
227 developmental, anthropometric, metabolic, hormonal, and molecular measures.

228 We conclude that girls with high versus normal DHEAS at age 6-7 years are hypomethylated at the
229 *CYP11B1* promoter during early puberty, and that the *CYP11B1* methylation status correlates
230 inversely with insulin levels at age 6-7 and with BMI-SDS both at age 6-7 and during early puberty.
231 These observations raise novel hypotheses regarding developmental and disease-related
232 regulation of adrenal androgen secretion.

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References

1. Witchel SF, Pinto B, Burghard AC & Oberfield SE. Update on adrenarche. Curr Opin Pediatr. 2020 32 574-581.

2. Silverman SH, Migeon C, Rosemberg E & Wilkins L. Precocious growth of sexual hair without other secondary sexual development; premature pubarche, a constitutional variation of adolescence. Pediatrics 1952 10 426-432.

3. Voutilainen R & Jaaskelainen J. Premature adrenarche: etiology, clinical findings, and consequences. J. Steroid Biochem. Mol. Biol. 2015 145 226-236.

4. Idkowiak J, Lavery GG, Dhir V, Barrett TG, Stewart PM, Krone N & Arlt W. Premature adrenarche: novel lessons from early onset androgen excess. Eur. J. Endocrinol. 2011 165 189-207.

5. Pereira A, Iñiguez G, Corvalan C & Mericq V. High DHEAS is associated with earlier pubertal events in girls but not in boys. J Endocr Soc. 2017 1 800-808.

6. Mäntyselkä A, Jääskeläinen J, Lindi V, Viitasalo A, Tompuri T, Voutilainen R & Lakka TA. The presentation of adrenarche is sexually dimorphic and modified by body adiposity. J Clin Endocrinol Metab. 2014 99 3889-3894.

7. Utriainen P, Jääskeläinen J, Saarinen A, Vanninen E, Mäkitie O & Voutilainen R. Body composition and bone mineral density in children with premature adrenarche and the association of LRP5 gene polymorphisms with bone mineral density. J Clin Endocrinol Metab. 2009 94 4144-4151.

8. Paterson WF, Ahmed SF, Bath L, Donaldson MD, Fleming R, Greene SA, Hunter I, Kelnar CJ, Mayo A, Schulga JS, et al. Exaggerated adrenarche in a cohort of Scottish children: clinical features and biochemistry. Clin Endocrinol (Oxf). 2010 72 496-501.

9. Corvalán C, Uauy R & Mericq V. Obesity is positively associated with dehydroepiandrosterone sulfate concentrations at 7 y in Chilean children of normal birth weight. Am J Clin Nutr. 2013 97 318-325.

10. Mericq V, Pereira A, Uauy R & Corvalán C. Early BMI gain and later height growth predicts higher DHEAS concentrations in 7-year-old Chilean children. Horm Res Paediatr. 2017 87 15-22.

11. Ibáñez L, Potau N, Chacon P, Pascual C & Carrascosa A. Hyperinsulinaemia, dyslipaemia and cardiovascular risk in girls with a history of premature pubarche. Diabetologia 1998 41 1057-1063.

12. Ibañez L, Aulesa C, Potau N, Ong K, Dunger DB & de Zegher F. Plasminogen activator inhibitor-1 in girls with precocious pubarche: a premenarcheal marker for polycystic ovary syndrome? Pediatr Res. 2002 51 244-248.

13. Ibañez L, Fucci A, Valls C, Ong K, Dunger DB & de Zegher F. Neutrophil count in small-for-gestational age children: contrasting effects of metformin and growth hormone therapy. J Clin Endocrinol Metab. 2005 90 3435-3439.

14. Güven A, Cinaz P & Bideci A. Is premature adrenarche a risk factor for atherogenesis? Pediatr Int. 2005 47 20-25.

15. Utriainen P, Jääskeläinen J, Romppanen J & Voutilainen R. Childhood metabolic syndrome and its components in premature adrenarche. J Clin Endocrinol Metab. 2007 92 4282-4285.

16. Sopher AV, Jean AM, Zwany SK, Winston DM, Pomeranz CB, Bell JJ, McMahon DJ, Hassoun A, Fennoy I & Oberfield SE. Bone age advancement in pre-pubertal children with obesity and premature adrenarche: possible potentiating factors. Obesity (Silver Spring) 2011 19 1259-1264.

17. Celik N, Alp H, Çamtosun E, Alp E, Çelik S & Berk E. The association between premature adrenarche and cardiovascular risk may be greater than expected. Horm. Res. Paediatr. 2017 87 7–14.

18. Liimatta J, Utriainen P, Laitinen T, Voutilainen R & Jääskeläinen J. Cardiometabolic Risk Profile Among Young Adult Females With a History of Premature Adrenarche. J Endocr Soc. 2019 3 1771-1783.

19. Potau N, Williams R, Ong K, Sanchez-Ufarte C, de Zegher F, Ibañez L & Dunger D. Fasting insulin sensitivity and post-oral glucose hyperinsulinaemia related to cardiovascular risk factors in adolescents with precocious pubarche. Clin Endocrinol (Oxf). 2003 59 756-762.

20. Lappalainen S, Utriainen P, Kuulasmaa T, Voutilainen R & Jääskeläinen J. Androgen receptor gene CAG repeat polymorphism and X-chromosome inactivation in children with premature adrenarche. J Clin Endocrinol Metab. 2008 93 1304-1309.

21. Lappalainen S, Utriainen P, Kuulasmaa T, Voutilainen R & Jääskeläinen J. ACTH receptor promoter polymorphism associates with severity of premature adrenarche and modulates hypothalamo-pituitary-adrenal axis in children. Pediatr Res. 2008 63 410-414.

22. Noordam C, Dhir V, McNelis JC, Schlereth F, Hanley NA, Krone N, Smeitink JA, Smeets R, Sweep FC & Claahsen-van der Grinten HL, et al. Inactivating PAPSS2 mutations in a patient with premature pubarche. N Engl J Med. 2009 360 2310-2318.

23. Utriainen P, Laakso S, Jääskeläinen J & Voutilainen R. Polymorphism of *POR*, *SULT2A1* and *HSD11B1* in children with premature adrenarche. Metabolism. 2012 61 1215-1219.

24. Palmert MR, Hayden DL, Mansfield MJ, Crigler JF Jr, Crowley WF Jr, Chandler DW & Boepple PA. The longitudinal study of adrenal maturation during gonadal suppression: evidence that adrenarche is a gradual process. J Clin Endocrinol Metab. 2001 86 4536-4542.

25. Remer T, Boye KR, Hartmann MF & Wudy SA. Urinary markers of adrenarche: reference values in healthy subjects, aged 3-18 years. J Clin Endocrinol Metab. 2005 90 2015 2021.

26. Rege J, Nakamura Y, Satoh F, Morimoto R, Kennedy MR, Layman LC, Honma S, Sasano H & Rainey WE. Liquid chromatography–tandem mass spectrometry analysis of human adrenal vein 19-carbon steroids before and after ACTH stimulation. J. Clin. Endocrinol. Metab. 2013 98 1182–1188.

27. Turcu AF, Nanba AT, Chomic R, Upadhyay SK, Giordano TJ, Shields JJ, Merke DP, Rainey WE & Auchus RJ. Adrenal-derived 11-oxygenated 19-carbon steroids are the dominant androgens in classic 21-hydroxylase deficiency. Eur. J. Endocrinol. 2016 174 601-609.

28. Rege J, Turcu AF, Kasa-Vubu JZ, Lerario AM, Auchus GC, Auchus RJ, Smith JM, White PC & Rainey WE. 11-Ketotestosterone is the dominant circulating bioactive androgen during normal and premature adrenarche. J. Clin. Endocrinol. Metab. 2018 103 4589–4598.

29. Janner M, Sommer G, Groessl M & Flück CE. Premature adrenarche in girls characterized by enhanced 17,20-Lyase and 17β-Hydroxysteroid Dehydrogenase activities. J Clin Endocrinol Metab. 2020 105 dgaa598.

30. Jaenisch R & Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet. 2003 33 Suppl 245-254.

31. Kometani M, Yoneda T, Demura M, Koide H, Nishimoto K, Mukai K, Gomez-Sanchez CE, Akagi T, Yokota T & Horike S-I, et al. Cortisol overproduction results from DNA methylation of CYP11B1 in hypercortisolemia. Sci Rep. 2017 7 11205

32. Lazo de la Vega-Monroy M-L, Solís-Martínez M-O, Romero-Gutiérrez G, Aguirre-Arzola VE, Wrobel K, Wrobel K, Zaina S & Barbosa-Sabanero G. 11 beta-hydroxysteroid dehydrogenase 2 promoter methylation is associated with placental protein expression in small for gestational age newborns. Steroids 2017 124 60-66.

33. Appleton AA, Armstrong DA, Lesseur C, Lee J, Padbury JF, Lester BM & Marsit CJ. Patterning in placental 11-B hydroxysteroid dehydrogenase methylation according to prenatal socioeconomic adversity. PLoS One 2013 8 e74691.

34. Yamagata Y, Nishino K, Takaki E, Sato S, Maekawa R, Nakai A & Sugino N. Genome-wide DNA methylation profiling in cultured eutopic and ectopic endometrial stromal cells. PLoS One 2014 9 e83612.

35. Nahar MS, Kim JH, Sartor MA & Dolinoy DC. Bisphenol A-associated alterations in the expression and epigenetic regulation of genes encoding xenobiotic metabolizing enzymes in human fetal liver. Environ Mol Mutagen 2014 55 184-195.

36. Marshall WA & Tanner JM. Variations in pattern of pubertal changes in girls. Arch Dis Child 1969 44 291-303.

37. Kain J, Corvalán C, Lera L, Galván M & Uauy R. Accelerated growth in early life and obesity in preschool Chilean children. Obesity (Silver Spring). 2009 17 1603-1608.

38. de Onis M, Garza C, Victora CG, Onyango AW, Frongillo EA & Martines J. The WHO Multicentre Growth Reference Study: planning, study design, and methodology. Food Nutr Bull. 2004 25(Suppl) S15–S26.

39. Gungor N, Saad R, Janosky J & Arslanian S. Validation of surrogate estimates of insulin sensitivity and insulin secretion in children and adolescents. J Pediatr. 2004 144 47-55.

40. Wojdacz TK, Dobrovic A & Hansen LL. Methylation-sensitive high-resolution melting. Nat Protoc. 2008 3 1903-1908.

41. Smith E, Jones ME & Drew PA. Quantitation of DNA methylation by melt curve analysis. BMC Cancer 2009 9 123.

42. Krygier M, Podolak-Popinigis J, Limon J, Sachadyn P & Stanisławska-Sachadyn A. A simple modification to improve the accuracy of methylation-sensitive restriction enzyme quantitative polymerase chain reaction. Anal Biochem. 2016 500 88-90.

43. Beikircher G, Pulverer W, Hofner M, Noehammer C & Weinhaeusel A. Multiplexed and Sensitive DNA Methylation Testing Using Methylation-Sensitive Restriction Enzymes "MSRE-qPCR". Methods Mol Biol. 2018 1708 407-424.

44. Grabe N. AliBaba2: context specific identification of transcription factor binding sites. In Silico Biol. 2002 2 S1-15.

45. Kometani M, Yoneda T, Demura M, Aono D, Gondoh Y, Karashima S, Nishimoto K, Yasuda M, Horike SI & Takeda Y. Genetic and epigenetic analyses of aldosterone-producing adenoma with hypercortisolemia. Steroids 2019 151 108470.

46. Hofmann A, Peitzsch M, Brunssen C, Mittag J, Jannasch A, Frenzel A, Brown N, Weldon SM, Eisenhofer G & Bornstein SR, et al. Elevated steroid hormone production in the db/db mouse model of obesity and type 2 diabetes. Horm Metab Res. 2017 49 43-49.

47. Rafacho A, Ortsater H, Nadal A & Quesada I. Glucocorticoid treatment and endocrine pancreas function: implications for glucose homeostasis, insulin resistance and diabetes. J Endocrinol 2014 223 R49–R62.

48. London E, Lodish M, Keil M, Lyssikatos C, de la Luz Sierra M, Nesterova M & Stratakis CA. Not all glucocorticoid-induced obesity is the same: differences in adiposity among various diagnostic groups of Cushing syndrome. Horm Metab Res. 2014 46 897–903.

49. Wang CT, Li CF, Wu WJ, Huang CN, Li CC, Li WM, Chan TC, Liang PI, Hsing CH, Liao KM. High Expression of 17β-hydroxysteroid dehydrogenase type 2 is associated with a better prognosis in urothelial carcinoma of the urinary tract. J Cancer. 2016 Oct 26;7(15):2221-2230. doi: 10.7150/jca.16777

50. Penning TM. AKR1C3 (type 5 17β-hydroxysteroid dehydrogenase/prostaglandin F synthase): Roles in malignancy and endocrine disorders. Mol Cell Endocrinol. 2019; 489:82-91. doi: 10.1016/j.mce.2018.07.002

51. Bhavani V, Srinivasulu M, Ahuja YR, Hasan Q. Role of *BRCA1*, *HSD17B1* and *HSD17B2* methylation in breast cancer tissue. Cancer Biomark. 2009; 5: 207-13. doi: 10.3233/CBM-2009-0105

52. Haynes BP, Straume AH, Geisler J, A'Hern R, Helle H, Smith IE, Lønning PE, Dowsett M. Intratumoral estrogen disposition in breast cancer. Clin Cancer Res. 2010; 16:1790-801. doi: 10.1158/1078-0432.CCR-09-2481

53. Emili A, Greenblatt J & Ingles CJ. Species-specific interaction of the glutamine-rich activation domains of Sp1 with the TATA box-binding protein. Mol Cell Biol 1994 14 1582-1593.

54. Sun Z, Yu S, Chen S, Liu H & Chen Z. SP1 regulates KLF4 via SP1 binding motif governed by DNA methylation during odontoblastic differentiation of human dental pulp cells J Cell Biochem. 2019 120 14688-14699.

55. Su CW, Chang YC, Chien MH, Hsieh YH, Chen MK, Lin CW & Yang SF. Loss of TIMP3 by promoter methylation of Sp1 binding site promotes oral cancer metastasis. Cell Death Dis. 2019 10 793.

Table 1: CpG site information and methylation methodology data

Assay	*M*ethylation-*S*ensitive *H*igh *R*esolution *M*elting *A*nalysis (MS-HRMA)			
Gene	CpG island coordinates (GRCh 38.p13)	CpG count	Primers Sequence (5´ > 3´)	Amplicon (bp)
CYP11B1 (NC_000008.11)	Chr 8: 142,879,650- 142,879,819	5	Fwd: ATCTACAACAACCTCAACCACCTAT Rev: ATTGGAATGGTATTTAGGGTAAAGG	170
HSD11B2 (NC_000016.10)	Chr 16: 67,430,457- 67,430,745	31	Fwd: TTTTTGTTTTAGGTAGGTTTTGTGG* Rev: CTCAAATAAACACATACCACTCAC*	289

Assay	*M*ethylation-*S*ensitive *R*estriction *E*nzymes – *q*uantitative PCR assay (MSRE-qPCR)				
Gene	CpG site coordinates (GRCh 38.p13)	MSRE		Primers Sequence (5´ > 3´)	Amplicon (bp)
		Name (Recognition seq.)	Units in reaction		
SULT2A1 (NC_000019.10)	Chr19: 47,881,771 - 47,881,772 (negative strand)	*Hpa*II (CCGG)	15	Fwd: TTCTCCTCTAGTCTCCTAATGCT Rev: AAATGGCGGAAGGTCAGG	87
HSD11B1 (NC_000001.11)	Chr1: 209,685,894 - 209,685,895 (positive strand)	*Xho*I (CTCGAG)	30	Fwd: GATAAGTCTCCAGGGCAACC Rev: TGGGCATCAGGCATCAAT	105
HSD17B2 (NC_000016.1)	Chr16: 82,034,679 - 82,034,680 (positive strand)	*Ssi*I (*Aci*I) (CCGC)	30	Fwd: AATGGAGGCTGGCATAGAATAG Rev: GAAACATGCCAGGGAGAGATAA	142
HSD17B5 (NC_000010.11)	Chr10: 5,093,261 - 5,093,262 (positive strand)	*Mlu*I (ACGCGT)	20	Fwd: CCTTGGCATCATACTACATTTCTAC Rev: GATTTCAGCAATATGGTGGATCA	132

* primer obtained from Lazo de la Vega-Monroy et al., 2017

Table 2: Anthropometric and hormonal description of study population at Tanner 1 and Tanner 2

	Tanner 1			Tanner 2		
	ND	HD	p	ND	HD	p
n	49	48		49	48	
Age years	*6.63 (±0.05)*	*6.91 (±0.04)*	*<0.05*	9.6 (9.5-10.6)	9.9 (9.5-10.1)	0.61
Height cm	*120.04 (±0.007)*	*120.12 (±0.007)*	*0.93*	137.7 (136-141)	137.6 (135-138)	0.55
Height SDS	*0.17 (±0.13)*	*-0.15 (±0.12)*	*0.08*	*0.22 (±0.12)*	*-0.10 (±0.12)*	*0.23*
Weigh kg	23.4 (23.2-26)	24.3 (23.8-26.4)	0.42	34.7 (34.4-40.8)	34.4 (33.4-38.0)	0.61
Weigh SDS	0.48 (0.21-0.8)	0.45(0.24-0.74)	0.85	0.31 (0.19-0.84)	0.19 (0.06-0.62)	0.89
BMI	16.7(16.3-17.6)	16.7 (16.7-17.9)	0.37	17.6 (18.1-20.2)	18.1 (18.03-19.9)	0.52
BMI SDS	0.76 (0.31-0.89)	0.71 (0.54-1)	0.51	0.44 (0.27-0.91)	0.50 (0.32-0.87)	0.66
DHEAS (µg/dL)	26.4 (22.2-27.8)	59.2 (56.7-66.1)	<0.05	62.2 (50.4-64.4)	96.7 (96.8-123.5)	<0.05

T student statistic. Values expressed as mean (±standard error).
U Mann Whitney statistic. Values expressed as median (interquartile range)
p-value ≤ 0.05 is statistically significant

Table 3: Methylation status of 11-oxyandrogens biosynthetic pathway genes at T2 between girls with normal and high DHEAS levels at T1

	ND	HD	p
CYP11B1	1.00 (0.92-1.13)	0.93 (0.91-1.00)	0.02
HSD11B2	0.58 (0.33-0.79)	0.64 (0.46-0.73)	0.90
SULT2A1	1.07 (1.01-1.14)	1.07 (1.03-1.18)	0.91
HSD11B1	2.04 (1.56-2.59)	1.81 (1.41-2.19)	0.15
HSD17B2	1.52 (1.19-1.91)	1.43 (0.71-1.78)	0.66
HSD17B5	0.92 (0.82-1.04)	0.91(0.80-1.06)	0.91

U Mann Whitney statistic. Values expressed as median (interquartile range)
p-value ≤ 0.05 is statistically significant

Figure 1 *CYP11B1*

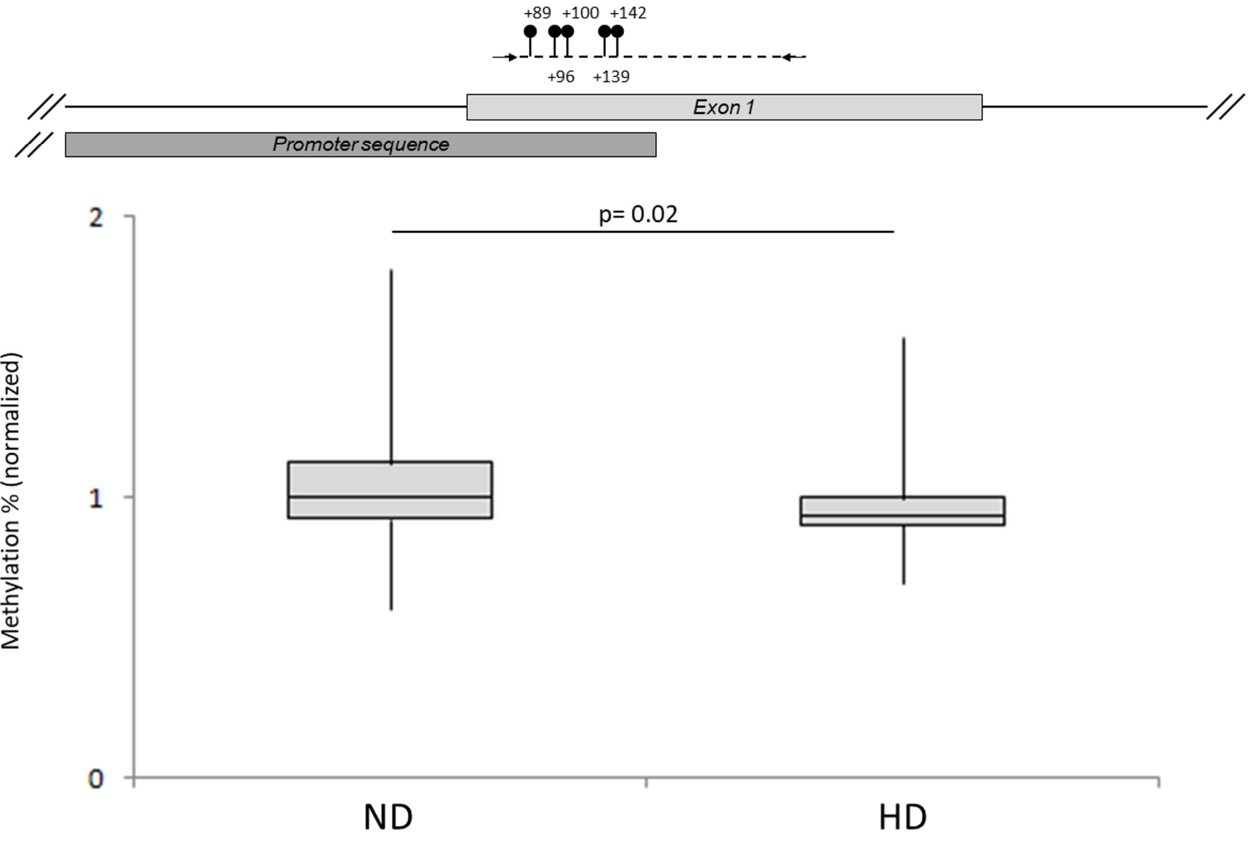


Figure 2

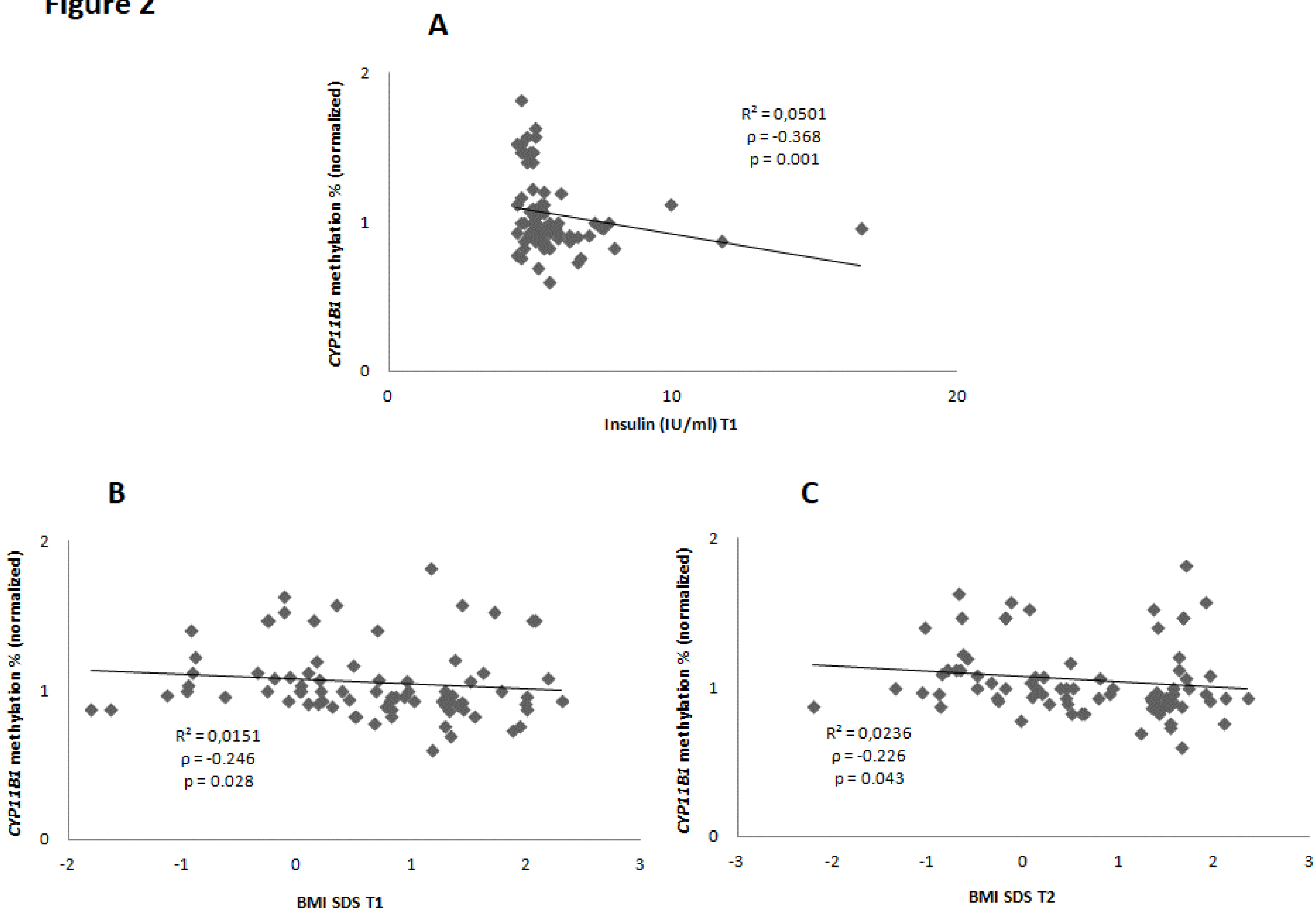


Figure 3

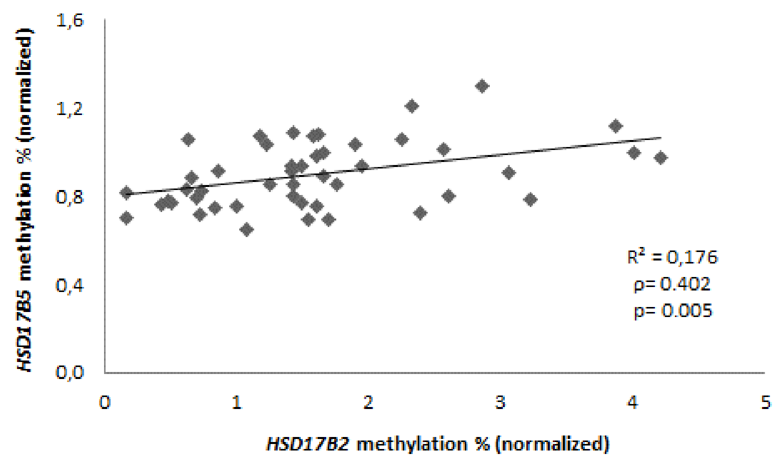
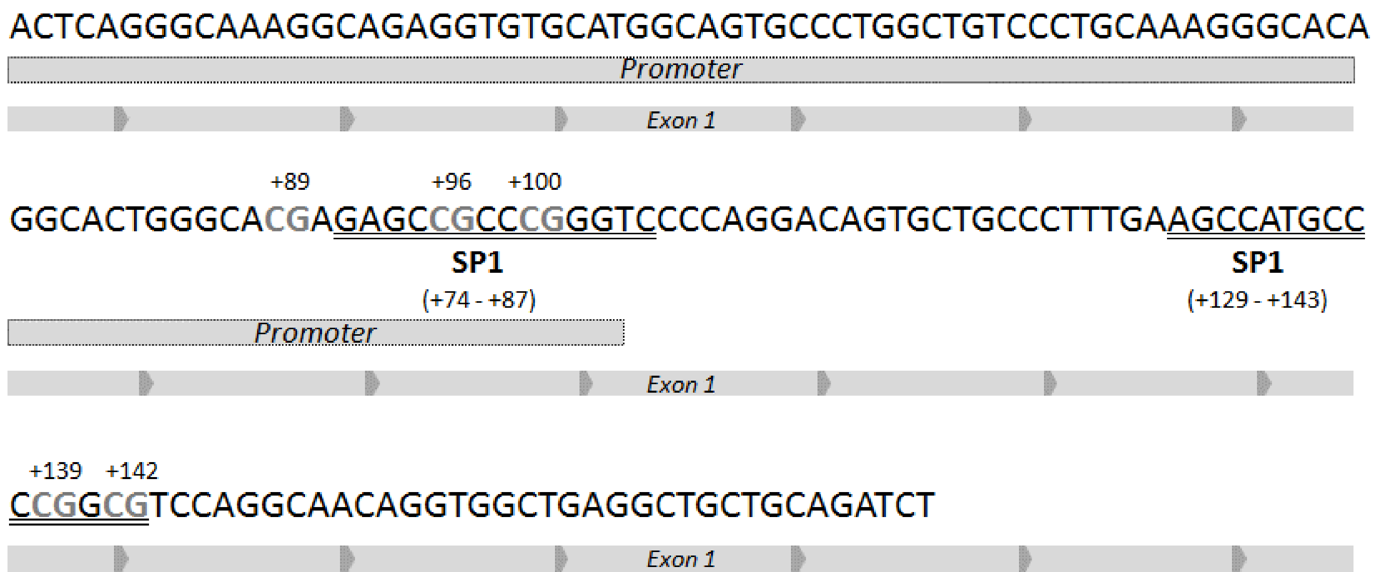


Figure 4



Figures Legends

Figure 1: *Localization of CpG sites in CYP11B1 gene (top) and methylation status comparison between normal and high DHEAS (ND vs. HD) girls (bottom)*. (*Top*) *CYP11B1* CpG sites are depicted as lollipops over a dashed line that represents amplicon analyzed by MS-HRMA (arrows represent the forward and reverse primers). Numbers indicate the localization of CpG sites according to the first nucleotide of the mRNA (+1). Note that the CpG sites of *CYP11B1* are within the final portion of the exon 1 promoter sequence. (*Bottom*) Box and whisker plot comparison of normalized methylation percentage of *CYP11B1* CpG sites in girls with normal (<42.0 mg/dL) and high (≥42.0 mg/dL) DHEAS level (U Mann Whitney)

Figure 2: *Correlations between CYP11B1 methylation and anthropometric parameters in the study cohort*. Correlation of *CYP11B1* methylation with insulin level at Tanner 1 (A), BMI SDS at Tanner 1 (B) and Tanner 2 (C). Each correlation shows the R^2, ρ and p-value. Statistic with Spearman's correlation coefficient test.

Figure 3: *Correlation between HSD17B2 and HSD17B5 methylation status in the study cohort.* The R2, ρ and p-value are shown. Statistic with Spearman's correlation coefficient test.

Figure 4: *Transcription factor recognition sites that overlap with evaluated CYP11B1 CpG sites. CYP11B1* sequence analyzed by MS-HRMA is shown, with CpG sites depicted in grey—with position indicating relation to the first mRNA nucleotide (+1). A double underline indicates sequences recognized by SP1 as predicted by AliBaba2.1 software. Coordinates of SP1 recognition sequences are shown in parentheses.

Supplementary material

Table 1: CpG site methylation correlation

	SULT2A1	*HSD11B1*	*HSD17B2*	*HSD17B5*	*CYP11B1*	*HSD11B2*
SULT2A1	1	0.197	0.016	-0.173	-0.048	-0.293*
HSD11B1	0.197	1	-0.120	-0.235*	-0.116	-0.273*
HSD17B2	-0.016	-0.120	1	0.402**	-0.152	0.326*
HSD17B5	-0.173	-0.235*	0.402**	1	-0.068	0.298*
CYP11B1	-0.048	-0.116	-0.152	-0.068	1	0.022
HSD11B2	-0.293*	-0.273*	0.326*	0.298*	0.022	1
Statistic: Spearman's correlation coefficient test *p < 0.05; ** p <0.01						

Figure 1

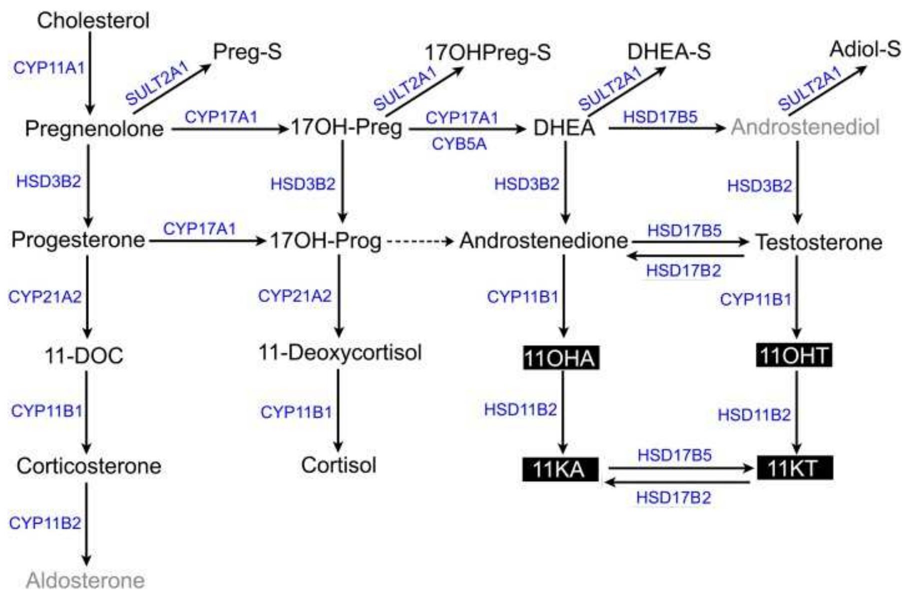


Figure 1: *Metabolites and enzymes (gene nomenclature) involved in adrenal mineralocorticoid, glucocorticoid and androgen biosynthesis (image adapted from* Rege et al. 2018)