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Prolactin and Adrenal Androgens During Adrenarche

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ABSTRACT

Introduction: Premature adrenarche (PremA), is associated with increased adiposity and in girls to earlier puberty and adverse metabolic profiles. Recently LC-MS/MS studies demonstrated adrenal production of more potent androgens: 11-oxygenated C19 steroids. Defining the mechanisms that regulate adrenal 11-oxygenated C19 steroids production has been elusive. We recently showed that genetic determinants of DHEAS during adrenarche differed from those during adulthood. One highly significant variant was at the prolactin receptor which is strongly expressed in human adrenal tissue. The aim of this study is to test whether an association exists between DHEAS/11-oxygenated C19 steroids and prolactin concentrations in pre-pubertal girls.

Methods: Two hundred and forty four prepubertal girls recruited within the 'the Food and Environment Chilean Cohort,' with normal birth weight (3.37 ± 0.02 Kg) were examined at 6.7 ± 0.6 years, including anthropometry and blood sampling. DHEAS and 11-oxygenated C19 steroids were measured by LC-MS/MS. Girls were categorized according to the DHEAS concentrations in normal DHEAS (ND, $<$ 75th percentile for the population) or high DHEAS (HD, $\geq$ 75th percentile). This definition of adrenarche subgroups allows identification of PremA independently from other factors influencing clinical manifestations (i.e ethnicity, tissue sensitivity).

Results: None of the girls presented clinical evidence of pubarche. At this age mean DHEAS concentration was 16.4 (9.6, −25) μg/dL and the 75th percentile set at 25.0 μg/dL. Girls with HD had higher weight (1.3 ± 1.2 vs. 0.7 ± 1.1, $p < 0.001$), height (0.6 ± 1.0 vs. 0 ± 0.9, $p < 0.001$) and BMI (1.3 ± 1.2 vs. 0.9 ± 1.1, $p < 0.01$) SDs compared to ND. The concentrations of all 11-oxygenated C19 steroids (ng/mL) were higher in girls with HD compared to girls with ND: 11KA by 16% [0.14 (0.12, 0.16) vs. 0.12 (0.10, 0.14)], 11OHA by 43% [0.07 (0.05, 0.08) vs. 0.04 (0.03, 0.05)], 11KT by 35% [0.19(0.15, 0.25) vs. 0.14 (0.11, 0.19)] and 11OHT by 30% [0.03 (0.02, 0.05) vs. 0.03 (0.01, 0.04)]. DHEAS levels correlated with each of the 11-oxygenated C19 steroids both in the raw and in fully adjusted model. Prolactin levels [6.5 (4.4, 10.7) vs. 5.7 (4.1, 9.3)] ng/mL and insulin [7.2 (5.7, 8.7) vs. 6.8 (5.3, −9.4)] μUI/mL did not differ in HD compared to ND girls. Prolactin levels were not associated with DHEAS concentrations but significantly associated with 11KA ($p < 0.001$) even after adjustment by covariates and was close to the limit of significance in the fully adjusted model for 11OHA ($p = 0.051$).

Conclusions: Our observations confirm that 11KT is the dominant bioactive androgen in children during adrenarche and PremA. Prolactin levels are directly correlated to the concentrations of the adrenal bioactive androgens. Conditions or medications that increase prolactin concentrations during childhood could have a role in PremA.

1 | Introduction

The adrenal cortex is the tissue of origin for three major types of steroids: mineralocorticoids, glucocorticoids and androgens. Adrenal steroid production is highly dynamic and tightly controlled by adrenocorticotropic hormone (ACTH), angiotensin II and other regulators. All adrenal steroids are synthesized de novo from cholesterol and immediately released. To ensure maximum synthetic efficiency in response to regulatory signals, the adrenal cortex is structured into three different zones, each one possessing distinct enzymatic machineries.

The *zona reticularis* (ZR) represents the innermost cortical layer, lying at the interface with the adrenal medulla. In contrast to the outer zones, this zone is not present at birth and starts to progressively grow and assume its final shape during childhood, between the age of 6 and 9 in both boys and girls a process called adrenarche. The development of a functional ZR is demonstrated by a consequent rise in dehydroepiandrosterone (DHEA) and its sulfate, DHEAS [1]. The physiological significance of adrenarche remains somewhat unclear. Some evidence suggests that DHEAS has a neuromodulatory effect, which might serve to protect certain parts of the prepubertal brain that are more active metabolically [1, 2].

Premature adrenarche (PremA) refers to increased levels of DHEA and DHEAS, above the prepubertal level before the age of 8 years in girls and 9 years in boys, and the concurrent presence of signs of androgen action including adult-type body odor, oily skin or hair or pubic hair growth and without evidence of an underlying virilizing disorder. The cut-off for DHEAS concentrations has been set at ~40 μg/dL measured by RIA (1.08 μmol/L) [3], which is within normal limits for early puberty but above average for children 6–8 years of age [1]. While PremA was thought to be a benign condition, recent studies suggest that at least a subgroup of girls with PremA may be more susceptible to the development of polycystic ovary syndrome and metabolic syndrome [4].

Recently, LC-MS/MS studies have demonstrated production of more potent androgens in the human adrenal gland, the 11-oxygenated C19 steroids, and their potential as biomarkers in hyperandrogenic disorders [5–10]. The circulating levels of these steroids were reported to be elevated in PremA [11].

In spite of great advances in the understanding of the biochemistry of adrenarche, the factors that drive this maturational process are still unknown. A limiting factor is the lack of suitable animal models, since adrenal DHEAS production is abundant only in some large mammals. Prolactin has been mentioned as one of the candidate factors modulating ZR development and function [12, 13].

In a cohort of Chilean children we have recently identified significant variants at the genome-wide level that associated with variations in serum DHEAS during adrenarche. Interestingly, these differed from those associated with DHEAS in adults [14], explaining about 30% of DHEAS concentrations at age of adrenarche (*GALR1*, *PRLR*, *PITX1*, *PTPRD*, *NR1H4*, and *BCL11B*, *SERBP1*, *CAMTA1/VAMP3*, *ZNF98*, *TRPC6*, *SULT2A1*). We also found significant reductions in age at pubarche in those children with higher PRS for DHEAS based on these newly identified variants.

Among the variants identified, one was associated with the *PRLR* gene, encoding the prolactin receptor. Since this receptor is strongly expressed in the human adrenal cortex and multiple evidence exists that prolactin is implicated in the production of adrenal androgens [13], we investigated whether there was an association between prolactin concentrations and adrenal reticularis products namely: DHEAS and 11-oxygenated in prepubertal girls.

Herein, we have used a biochemical definition of adrenarche, which allows identification of adrenarche-independent factors influencing clinical manifestations and may depend on ethnicity, steroid metabolism, sensitivity to androgens, and environmental factors [15–17].

2 | Methods

2.1 | Study Subjects

Our study sample was derived from children enrolled in the Food and Environment Chilean Cohort—FECHIC, as previously described [18]. In brief, in 2016, FECHIC was enrolled with the aim to assess if food labeling would be effective to improve diet and nutritional status of children in pre-kinder or kinder. Children from 55 public schools from the South East Area of Santiago were invited to participate. From a total of 2625 interested mothers only 962 complied with inclusion criteria. Finally, 968 children were recruited. There were no significant differences between children who agreed to participate in the study compared to the ones who did not, in terms of sex distribution, age, and anthropometric characteristics (p-value > 0.05 for all variables). We included only singletons; gestational age 37–42 weeks; birth weight ≥ 2500 g (data retrieved from medical registries, mean birth weight 3374 ± 22 g); and no physical or psychological conditions that could severely affect growth. Children with genetic, metabolic or severe chronic diseases that could affect growth were excluded from the study. This area covers six low-medium income counties of the city, which are similar to low-middle income neighborhoods from the rest of the country in terms of the level of poverty and nutritional indicators [19, 20]. For the current study we included a subset of 244 girls whose blood samples were obtained at age ~7 years. At this age, secondary sex characteristics were evaluated every year by a single female dietitian trained specifically for this purpose, with permanent supervision of a single pediatric endocrinologist (V.M.). Breast Tanner stage concordance between the dietitian and the pediatric endocrinologist was 0.9 [21]. Girls were categorized according to the DHEAS concentrations in normal DHEAS (ND, < 75th percentile) or high DHEAS (HD, ≥ 75th percentile for the population) [3].

The study protocol was approved by the Institutional Review Board of the Institute of Nutrition and Food Technology (INTA) of the University of Chile. Written informed consent was obtained from all parents or guardians of the children.

2.2 | Hormone Assays

Hormonal levels were determined by a blood sample at ~7 years of age by a fasting venous sample obtained early in the morning. Mothers were contacted the day before blood sampling to confirm the absence of fever (37.5°C) or symptoms of acute infection in the children. Samples were analyzed at the Institute of Maternal and Child Research, University of Chile.

Serum prolactin was measured by IRMA (Izotop, Budapest, Hungary); the sensitivity of this assay is 0.1 ng/mL and intra- and interassay coefficients of variation (CV) 4.3% and 5.8.%. Glycemia was measured by the enzymatic colorimetric method and expressed in mg/dL, and insulin by electrochemiluminescent immunoassay and expressed in μU/ml with a detection sensitivity limit of 1.0 μUI/mL and an intra- and interassay CV of 2.1% and 4.5% respectively. Concentrations of DHEAS were analyzed by liquid chromatography-mass spectrometry (LC-MS/MS), as we previously described [22]. In brief, the samples were resolved in a high-performance liquid chromatography (HPLC) Agilent system 1260 coupled to an AB Sciex 3200 Quantum ultra triple quadrupole mass spectrometer. The sensitivity for DHEAS weas 0.20 μmol/L. The intra- and interassay CVs were 2.9% and 5.0%. The measurement of the 11-oxygenated androgens 11-hydroxyandrostenedione (11OHA4), 11-hydroxytestosterone (11OHT), 11-ketoandrostenedione (11KT) and 11-ketotestosterone (11KT) was made by LC-MS/MS. The quantification of 11-oxygenated C19 steroids in serum samples was performed by liquid chromatography–tandem mass spectrometry including 11OHA4, 11OHT, 11KA4 and 11OHT. Unlabeled and deuterated steroids were obtained from Steraloids Inc (Newport, RI, USA) and Chromsystems GmbH (Gräfelfing, Germany), respectively. For extractions in 96 well filter plates (Chromsystem) we added 500 ul of serum sample or calibrators, 50 μL of the respectively deuterated-labeled steroids and 450 ul of extraction buffer (Chromsystem). The plates were agitated at 600 rpm for 2 min and then centrifuged at 200 g for 1 min. The plates were then washed and centrifuged as we describe below. Then the extraction plate was placed on the recollection plate and the filters were eluted with 500 μL of Elution buffer (Chromsystem) and centrifuged at 200 *g* for 1 min. The eluates were concentrated under nitrogen at 45°C and reconstituted with 100 ul. of reconstitution buffer (Chromsystem). Samples (10–40 μL) were injected via autosampler and resolved with a Intersil C4, 5 um, 4,6 I,D x 250 mm column (GL Sciences Inc., Torrance, CA, USA) on an HPLC ExionLC AE system (AB Sciex LLC, Framingham, MA USA). The column effluent was directed into the source of a Sciex 6500 triple quadrupole tandem mass spectrometer using electrospray ionization. The sensitivities for 11OHA4, 11KA4, 11OHT and 11KT were 0.010, 0.032, 0.010 and 0.032 ng/mL respectively. The corresponding intra-assay CV were 4.4%, 3.6%, 5.1% and 3.9% respectively and the inter-assays CV were 6.1%, 4.7%, 6.9% and 5.2% respectively.

2.3 | Statistical Analyses

The results are presented as mean ± SE. The distribution of anthropometric measurements across DHEAS groups was compared by the Welch´s *t*-test. Associations between prolactin levels and 11-oxo-steroids, as well as the relationships between DHEAS levels (normal vs. high), 11-oxo-steroids, and prolactin, were evaluated using multiple linear regression models. These models were sequentially adjusted for age at sample collection, BMI, and insulin levels. A *p*-value of less than 0.05 was considered statistically significant. All statistical analyses were conducted using R version 4.4.1.

3 | Results

A sample of 244 girls at a mean age of 6.6 years was evaluated. None of the girls presented clinical evidence of pubarche. On average, girls had close to +1 SDS BMI relative to the WHO reference, and 50% of the girls had excess weight (BMI SDS > 1). Girls with HD (see below) had higher weight-SDS, height-SDS and BMI-SDS compared to ND (Table 1).

At this age mean DHEAS concentration measured by LC-MS/MS was 16.4 (9.6–25.0) μg/dL (Table 1 and Figure 1A). This concentration was significantly lower than the one we have previously reported, when DHEAS concentrations were determined by RIA (29.4 ± 1.7 μg/dL) at a similar age [3]. The HD girls were then categorized by DHEAS measured by LC/MS-MS as those with a concentration greater than 25.0 μg/dL (HD, ≥ 75th percentile for the population).

The concentrations of prolactin, insulin and glucose concentrations did not differ in HD compared to ND girls (Table 1).

TABLE 1 | Clinical anthropometric characteristics and hormone concentrations of 244 girls by DHEAS levels at ~ age 7 years.

	All ($n = 244$) Mean (SE)	HD ($n = 62$) Mean (SE)	ND ($n = 182$) Mean (SE)	*p*-value
Age (y)	6.7 (0.03)	7.0 (0.6)	6.7 (0.5)	0.009
Weight (*z*-Score)	0.85 (0.08)	1.31 (0.15)	0.69 (0.09)	< 0.001
Height (*z*-Score)	0.15 (0.6)	0.63(0.11)	−0.01 (0.07)	< 0.001
BMI (*z*-Score)	1.00 (0.08)	1.30 (0.16)	0.95 (0.08)	0.058
Prolactin (ng/mL)	7.5 (4.6)	8.0 (0.6)	7.5 (0.3)	0.351
Insulin (uUI/mL)	7.8 (3.8)	8.2 (4.2)	7.7 (3.6)	0.269
Glucose (mg/dL)	81.3 (0.6)	81.4 (1.1)	81.2 (0.7)	0.569

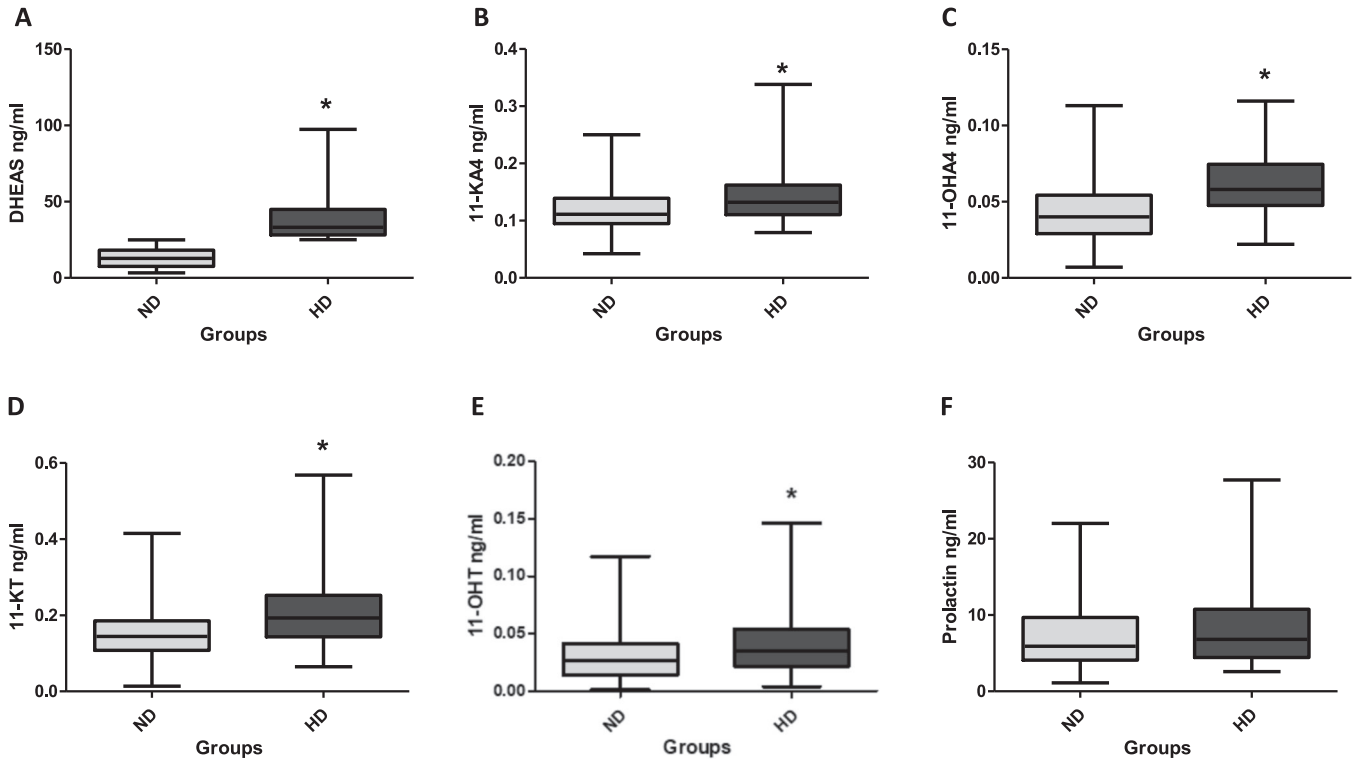

FIGURE 1 | Concentrations of DHEAS (A), 11-ketoandrostendione (B), 11-hydroxyandrostendione (C), 11-ketotestosterone (D), 11-hidroxytestosterone (E) and Prolactin (F) in prepubertal girls at age at age ~7 stratified according to biochemical adrenarche (high DHEAS (HD) vs. normal DHEAS (ND)). *$p < 0.05$.

The concentrations of prolactin did not associate to DHEAS concentrations but significantly associated with 11 KA4 (β 0.002, $p < 0.001$) even after adjustment by covariates and was close to the limit of significance in the fully adjusted model for 11 OHA4 (β: 0.001 $p = 0.051$), 11 OH T (β 0.001, $p = 0.064$) and 11 Keto T (β: 0.002, $p = 0.065$) (Table 2).

All the concentrations of 11-oxygenated C19 steroids were higher in girls with HD compared to girls with those with ND: 11KA by 16%, 11OHA by 43%, 11KT by 35% and 11OHT by 30% (Figure 1).

In girls with HD, all 11-oxygenated C19 steroids were higher but not prolactin concentrations (Table 3).

4 | Discussion

In this large cohort of girls with normal birth weight we studied DHEAS and 11-oxygenated C19 steroids levels by LC MS/MS at ~around age 7, a physiological age of adrenal reticularis maturation. We confirmed that those girls presenting higher DHEAS also present higher concentrations of 11-oxygenated C19 steroids such as 11b-hydroxyandrostenedione (11OHA4), 11-ketoandrostenedione (11KA4), 11-hydroxytestosterone (11OHT), and 11-ketotestosterone (11KT) [23] and the DHEAS concentration at this age associated significantly with concentration of each of this 4 oxo steroids, especially in those with biochemical PremA. 11KT and 11KA4 were the main 11-oxygenated C19 steroids found in these girls. In addition, our definition of high DHEAS (> 75th percentile of the distribution of our population) measured by LC MS/MS is very similar to the lower concentration found in the study of Rege et al. [11] and Rosenfield [1], confirming that this cut-off is useful to define biochemical PA. This cut-off for DHEAS concentrations is ~ 40% lower than the one we and others have been using when DHEAS is measured by RIA [3, 5].

While it is well known that ACTH signaling is necessary for ZR development [24, 25], it is also clear that DHEAS levels increase at the time of adrenarche in a manner that appears independent of the ACTH and cortisol levels, which maintain a constant pattern [26, 27]. This clear age-related separation of circulating levels of cortisol and DHEAS has led many researchers to search for a so-called adrenal androgen stimulating hormone (AASH). Amplifiers of ACTH action include insulin and IGF-1, which at physiologic concentrations stimulate expression of adrenal P450c17 and 3βHSD2 [28, 29]. Other circulating factors implicated in the onset of adrenarche are leptin, that upregulates 17,20-lyase activity in adrenocortical carcinoma cells [30] and Interleukin 6, which stimulates ACTH secretion [31]. Despite interest in the clinical significance of differences in adrenal androgen secretion, only a few studies have described the genetic determinants that regulate DHEAS serum concentrations [32, 33] either involving candidate gene approaches, such as SULT2A1 [34], or genome-wide association studies (GWAS) [35, 36]. Moreover, most studies have been performed in adult rather than pediatric cohorts. We recently described in a GWAS pediatric study an intronic variant in the prolactin receptor gene (*PRLR*, rs79760761, P = 2.31 × 10−7, β = 0.13;

TABLE 2 | Multiple linear regression models to assess the association between Prolactin and concentrations of DHEAS and of each 11-oxygenated C19 steroids.

	DHEAS (μg/dL)	
Prolactin	**β [$CI_{95\%}$]**	***p*-value**
Model 1	0.33 [−0.04–0.71]	0.082
Model 1 + BMI	0.36 [−0.01–0.73]	0.056
Model 1 + BMI + insulin	0.36 [−0.02–0.73]	0.06
	11-Keto A2 (pg/mL)	
Model 1	2.00 [0.88–3.12]	< 0.001
Model 1 + BMI	2.02 [0.90–3.14]	< 0.001
Model 1 + BMI + insulin	2.07 [0.95–3.19]	< 0.001
	11-OH-A2 (pg/mL)	
Model 1	0.51 [−0.08–1.1]	0.088
Model 1 + BMI	0.55 [−0.03–1.14]	0.063
Model 1 + BMI + insulin	0.58 [0.00–1.16]	0.052
	11-Keto-T (pg/mL)	
Model 1	2.16 [−0.03–4.36]	0.054
Model 1 + BMI	2.13 [−0.08–4.33]	0.058
Model 1 + BMI + insulin	2.08 [−0.13–4.29]	0.065
	11-OH-T (pg/mL)	
Model 1	0.60 [−0.02–1.22]	0.057
Model 1 + BMI	0.60 [−0.02–1.22]	0.059
Model 1 + BMI + insulin	0.58 [−0.04–1.21]	0.065

Note: Model 1: Adjusted by Age at control. The adjusted models included age (raw model) and next: BMI and insulin concentrations at age at age ~7.

95% CI: 0.08 to 0.18) which is significantly associated with DHEAS concentrations at the age of adrenarche. Carriers of that variant possessed 11.9 μg/dL higher DHEAS (95% CI: 7.7 to 16.2 μg/dL) levels than non-carriers [14]. *PRLR* is strongly expressed in all the layers of the adrenal cortex [13, 37, 38]. Once activated by prolactin, PRLR induces a signaling cascade that induces the production of cortisol, aldosterone, and DHEA [39–42]. In our cohort prolactin did not associate to DHEAS concentrations at age of adrenarche. Nevertheless, we found a positive correlation between serum prolactin concentrations with 11KA4, which is the main 11-oxygenated C19 steroids produced in the adrenal ZR, at higher concentrations than androstenedione. In addition, correlation of prolactin levels was at the limit of significance for the other three measured oxygenated C19 androgens when adjusted for important covariants such as BMI, SDS and insulin concentrations. Although the main 11-oxygenated C19 steroids products of the ZR are 11 OHA4 and 11 OHT, a small portion of 11 KA and 11 KT is also produced in the reticularis. Each ng/ml increase of prolactin increased 2 pg/mL of 11KA4 and 11 KT, and in 0.58 pg/mL 11 OHA4 and 11 OHT.

An effect of prolactin on the production of adrenal androgenic steroids has long been known. Several studies reported selectively increased DHEAS, but not cortisol, production in patients with prolactin-secreting (PRLA) compared to non-functioning pituitary adenomas [40–43]. DHEAS levels were significantly reduced in patients with PRLA after treatment with dopaminergic agonists, which decrease circulating prolactin levels [43]. We have also recently shown that prolactin has a specific action in the regulation of the production of the 11-oxygenated C19 steroids, DHEAS and androstenedione in the human fetal adrenal, in synergy with ACTH [44].

The effects of prolactin on adrenal androgen production suggest that it may also have a role in adrenarche, Remarkably, congenital pituitary disorders causing selective PRL/GH deficiency, without ACTH deficiency, are associated with delay in (or even absence of) adrenarche [45, 46]. Conversely, premature adrenarche has been reported in a case of paediatric PRL adenoma [47]. Furthermore, we have shown that genetic variants in the prolactin receptor gene (*PRLR*) are associated with circulating DHEAS levels in children [14]. All these data in different pathophysiological contexts converge towards a strong association between prolactin and adrenal androgen production. However, the mechanisms underlying the effects of prolactin in the regulation of adrenal steroidogenesis are still unknown. An increased sensitivity of the adrenal cortex to the effects of prolactin at the time of adrenarche could also be hypothesized.

The present study is the first one to (report a physiological cut-off for DHEAS together with the 11-oxygenated C19 steroids serum concentrations by a new standard highly accurate technique (LC-MS/MS) in a large cohort of prepubertal children at the age of physiological adrenarche. Second, we confirm the association of DHEAS, which is an androgenic precursor, with the four more potent 11-oxygenated C19 steroids and, third, we provide evidence that prolactin levels are correlated to the concentrations of these adrenal ZR products. Nevertheless, we recognize the limitations of this study. In each subject, we measured prolactin and 11-oxygenated C19 steroids in a single sample, which may be limiting, particularly for prolactin serum levels. Factors that may affect their concentration, such us sleep, stress and exercise were not considered in this study. In addition, mild manifestations of PA such as axillary odor or comedones were not registered, however none had pubarche or axillary hair. Finally, a replication cohort is lacking.

These new findings plus our previous GWAS findings suggest that prolactin plays a functional role in the adrenal gland ZR modulating the secretion of 11-oxygenated C19 steroids at age of adrenarche. Conditions with higher prolactin concentrations at this age could induce premature adrenarche, a condition that at term is associated with worsened cardiovascular profile. Nowadays there is an important indication of psychotropic drugs which are associated with increased prolactin secretion and this association should alert the physician to consider the probability of PA.

Future in vitro studies are warranted to confirm the association between prolactin and 11 oxo steroids production and the mechanisms of adrenal androgen regulation.

TABLE 3 | Multiple linear regression models to assess the association between biochemical adrenarche (high DHEAS (HD) vs. normal DHEAS (ND)) and each of the 11-oxygenated C19 steroids and prolactin.

	Model 1		Model 1 + BMI		Model 1 + BMI + Insulin	
	β [$CI_{95\%}$]	*p*-value	β [$CI_{95\%}$]	*p*-value	β [$CI_{95\%}$]	*p*-value
DHEAS (μg/dL)						
ND	ref	ref	ref	ref	ref	ref
HD	24.21 [21.56–26.85]	< 0.001	23.87 [21.21–26.54]	< 0.001	23.86 [21.19–26.53]	< 0.001
11-Keto A2 (pg/mL)						
ND	ref	ref	ref	ref	ref	ref
HD	18.83 [6.81–30.84]	0.002	18.87 [6.7–31.03]	0.003	19.07 [6.92–31.22]	0.002
11-OH-A2 (pg/mL)						
ND	ref	ref	ref	ref	ref	ref
HD	0.018 [0.012–0.024]	< 0.001	17.22 [11.29–23.16]	< 0.001	17.34 [11.42–23.27]	< 0.001
11-Keto-T (pg/mL)						
ND	ref	ref	ref	ref	ref	ref
HD	49.77 [26.97–72.56]	< 0.001	52.11 [29.12–75.1]	< 0.001	51.9 [28.88–74.93]	< 0.001
11-OH-T (pg/mL)						
ND	ref	ref	ref	ref	ref	ref
HD	8.81 [2.25–15.36]	0.009	9.19 [2.57–15.82]	0.007	9.14 [2.5–15.77]	0.007
Prolactin (ng/mL)						
ND	ref	ref	ref	ref	ref	ref
HD	0.42 [−0.94–1.77]	0.545	0.5 [−0.87–1.87]	0.469	0.49 [−0.88–1.86]	0.484

Note: The adjusted models included age (raw model) and next: BMI and insulin concentrations at age at age ~7.

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Conflicts of Interest

The authors declare no conflicts of interest.

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