Journal of Pediatric Endocrinology & Metabolism, 23, 121-132 (2010)

17α-Hydroxyprogesterone and Cortisol Serum Levels in Neonates and Young Children: Influence of Age, Gestational Age, Gender and Methodological Procedures

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ABSTRACT

To determine the influence of age, gestational age, gender and methodological protocol on serum 17OHP and cortisol concentrations. 17OHP in non-extracted (NE) and extracted (E) sera was measured by RIA in 319 full-term (FT) (1 d-5 yr) infants, 38 pre-term (PT) and in 19 neonates with classical CAH at diagnosis. 17OHP (NE- and E-) decreased with age in normal children. The extraction procedure significantly reduced 17OHP by eliminating interfering steroids in children <1 year. Sexual dimorphism was only observed in NE-17OHP. 17OHP in PT was always higher than in FT up to 2 months of age ($p < 0.001$). Neither NE- nor E-17OHP in CAH overlapped with those of FT or PT ($p<0.001$) allowing to omit the extraction procedure to confirm CAH diagnosis. Cortisol levels were within normal range in neonates with CAH, thus not adding useful information about adrenal function. Chronological and gestational age, gender, and extraction for 17OHP measurement are important factors to know when assessing adrenal function during the first year of life.

KEY WORDS

17α-hydroxyprogesterone, reference ranges, cortisol, preterm newborns, CAH

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INTRODUCTION

Serum 17α-hydroxyprogesterone (17OHP) is routinely measured in endocrinological laboratories during immediate postnatal life in order to confirm the diagnosis of congenital adrenal hyperplasia (CAH) in neonates with abnormal screening results.

CAH is a family of recessive inherited disorders of adrenal steroid hormone metabolism most commonly caused by CYP21 deficiency[1,2]. Genetic variation of mutations of the *CYP21* gene can explain the wide range of clinical and biochemical expression of patients with CAH. This spectrum goes from the classic or severe phenotype presented in the newborn period, with or without salt-loss, to the non-classical or late-onset phenotype presenting in late childhood or early adulthood with signs of hyperandrogenism. Due to the decreased activity of CYP21 in CAH, 17OHP cannot be efficiently converted to cortisol and, instead, is accumulated in large amounts and shunted into androgen biosynthesis[1,2].

Although serum 17OHP measurement is considered a useful tool for biochemical diagnosis of CYP21 deficiency[3], radioimmunoassay (RIA) methods are often affected by cross-reacting with structurally similar steroids present in neonatal plasma[4-8]. The fetal zone of the adrenal gland atrophies and disappears by three months of postnatal life[9,10]. This zone produces high levels of 17-hydroxypregnenolone sulfate that carries similar immunoreactive epitopes to the 17OHP molecule[6]. This interfering steroid causes a positive deviation of the measured value of serum 17OHP during the newborn period that should not

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be ignored[4-7]. Moreover and possibly due to the same maturation process, healthy preterm infants have higher 17OHP levels than term infants[5,11].

However, in daily practice, manufacturers do not warn about possible cross-reactions occurring in infants or differences in serum 17OHP concentrations related to age, gestational age or gender, and provide reference intervals which do not take into account these possible influencing variables.

In this context, the lack of reliable reference ranges related to the newborn period in normal term and preterm children leads to the hazard of misdiagnosis.

With respect to this problem, some RIAs are able to directly detect the 17OHP present in the serum sample and also on reconstituted material extracted from serum with different organic solvent procedures[4,6,7]. Although a solvent extraction step in the original RIA procedure has been proven to markedly lower serum 17OHP results by eliminating hydrosoluble compounds produced by the fetal zone[4,6,7], the age till when this procedure should be carried out is still controversial[4-7].

The aim of the present study was to determine the influence of factors such as chronological and gestational age, gender and methodological procedures on the determination of 17OHP and cortisol as tools needed to confirm or reject pathological adrenal conditions in the neonatal period and early childhood.

INFANTS

Three hundred and nineteen full-term (FT) healthy infants (144 girls and 175 boys) from the newborn period up to 5 years of age and a group of 38 normal premature (PT) newborns (15 girls and 23 boys) under 3 months of age were cross-sectionally studied.

FT children were categorized by gender and divided into six groups according to chronological age (Table 1). PT were divided and studied in two groups according to gestational age (GA): ≤32 weeks of GA (n = 21, 10 girls, median age and range: 37 days, 7-90 days), and >32 weeks of GA (n = 17, 5 girls, median age and range: 30 days, 3-45 days). Children consulted the Endocrinology Division for presumed endocrine abnormalities and were found to be normal. Some of them, especially PT, were studied as an indication for a higher level of 17OHP in dried blood spot samples of the current CAH screening program in our center. Those receiving steroid therapy were excluded.

Nineteen children (13 girls and 6 boys, median age and range: 19 days, 7-175 days) with the classical severe form of CYP21 deficiency (15 salt-wasting type, and 4 simple virilizing type) were studied before starting therapy.

The study was approved by the local Institutional Review Board of the Children's Hospital Ricardo Gutierrez of Buenos Aires.

METHODS

Surplus serum, remaining after completing endocrinological tests, was retrieved, fully anonymized and stored at -20°C until assayed in duplicate.

Serum extraction procedure for 17OHP measurement

The serum extraction procedure for 17OHP measurement was modified from Makela *et al.*[4]. The 17OHP present in 200 µl of serum sample was extracted with 2 ml diethyl ether by vigorously shaking for 3 minutes. The aqueous phase was frozen and the organic phase was poured into a glass tube and evaporated to dryness. Finally, the residue was redissolved with 200 µl of the 'zero' standard and the RIA was carried out as recommended by manufacturer (Diagnostic System Laboratories, Inc., Texas, USA).

The analytical recovery of 17OHP was also determined to evaluate the efficiency of the extraction procedure. An aliquot of 17OHP (SIGMA) in ethanolic solution at two different concentrations was evaporated to dryness. Then, a pool of normal sera was added to the residue with mixing. Finally, supplemented sera and its extracted portion were assayed in the same run and the percentage recovery was calculated. Only recoveries above 80% were accepted (83 ± 2% and 88 ± 5% for a concentration level of 3.3 and 14.2 nmol/L, respectively).

Hormonal assessment

17OHP was measured in serum before and after diethyl ether extraction using a coated-tube RIA (Diagnostic Systems Laboratory, Inc.). The intra-assay coefficient of variations (CVs) were below 7% at concentration levels of 3.0 and 42.0 nmol/L as calculated on pooled sera. The interassay CVs were 14.9% and 8.7% for the same dosage ranges. The functional sensitivity (FS) of the RIA method for 17OHP was 1.06 nmol/L (0.35 ng/ml)[12]. The cross-reactivity of the 17OHP antiserum declared by the manufacturer was: 17-OHP: 100%, 17-hydroxypregnenolone: 4.1%, progesterone: 1.3%, prednisone: 0.23%, dihydroandrosterone: 0.09%, corticosterone: 0.05%, others: <0.01%.

Serum cortisol levels were measured in 227 children of the same FT group, both groups of PT neonates and children with CAH, using a commercial chemoluminiscent assay (IMMULITE-1; Siemens Medical Solution Diagnostics, Los Angeles, CA, USA). The intra-assay CVs were below 5% at concentration levels of 115.9, 350.4 and 761.5 nmol/L as calculated on pooled sera. The interassay CVs were 9.0%, 8.4% and 7.7% for the same dosage ranges.

Statistical analysis

Log-transformed data of non-extracted (NE)-17OHP, extracted (E)-17OHP and cortisol concentration from girls and boys in each age band (Table 1) were compared using unpaired t-test and paired t-test, as appropriate. Within each sex, changes with age were studied by one-way ANOVA, followed by Tukey or linear trend as post tests. On the basis of ages at which statistically significant changes occurred, results in the adjacent age bands were combined to derive appropriate age- and sex-related reference ranges. Simple regression analysis was also used. Statistical significance was accepted for $p < 0.05$. Data were analyzed using GraphPad Prism Version 4.00 for Windows (GraphPad Software San Diego, CA; www.graphpad.com).

RESULTS

Serum 17OHP concentration before and after extraction varied with chronological age in normal FT girls and boys (ANOVA: $p < 0.0001$), showing their highest levels by the first two weeks of postnatal life (Fig. 1). Thereafter, a trend to decrease was observed and a rise in NE-17OHP by day 40 to 50 was evident in both sexes (Fig. 2). A post test revealed a significant linear trend to lower NE- and E-17OHP values across age in girls ($r = 0.81$ and $r = 0.64$, respectively, $p < 0.0001$) and boys ($r = 0.80$ and $r = 0.59$, respectively, $p < 0.0001$). In addition, a significant positive correlation was obtained for NE- and E-17OHP concentrations ($r = 0.79$, $p < 0.0001$).

In normal FT children, gender differences for serum direct NE-17OHP were obtained. During the first year of life, boys presented higher levels of NE-17OHP concentrations than girls, this difference being statistically significant in the first two months of life (Table 2, Fig. 1). The addition of an extraction step resulted in much lower levels of 17OHP for males and females younger than 1 year of age, and no sexual dimorphism was observed in E-17OHP concentrations at all ages (Table 2, Fig. 1). Normal percentile lines of NE- and E-17OHP in children up to 5 years of age are shown in Figures 2 and 3, respectively.

Serum 17OHP ranges for premature infants are presented in Table 3. Very premature infants (≤32 weeks of GA) tend to have higher NE- and E-17OHP values compared to PT >32 weeks of GA (NE-17OHP: $p = 0.07$ and E-17OHP: $p = 0.09$), although no correlation was obtained between NE- and E-17OHP levels and GA or GA adjusted for chronological age (GAad) in PT infants. On the other hand, NE-17OHP and E-17OHP values of both groups of premature children (≤32 and >32 weeks of GA) were significantly higher than FT newborns up to 2 months although some overlap was observed ($p < 0.001$) (Figs. 2 and 3).

Samples assayed on direct serum from PT infants were always above the normal 50th percentile of FT neonates, and 5/36 (19.4%) of these samples were even above the 97.5th percentile. On extracted sera, 10% of samples of PT were above the 97.5th percentile, all of them corresponding to infants ≤32 weeks of GA. Although the extraction

TABLE 1

Median chronological ages of normal full-term children

Age range	Girls		Boys	
	n	Median age	n	Median age
1-7 days	16	3.0 d	21	2.0 d
8-15 days	17	11.4 d	24	11.0 d
16-30 days	29	24.5 d	31	21.0 d
31-59 days	13	44.0 d	24	39.5 d
2-5.9 months	26	3.0 m	37	3.5 m
6-11.9 months	21	9.5 m	17	8.0 m
1-5 years	22	2.0 yr	21	3.0 yr

d = days; m = months; yr = years.

TABLE 2

Age- and sex-specific 17OHP reference ranges [nmol/L (ng/mL)] for non-extracted (NE-17OHP) and extracted (E-17OHP) sera in normal full-term infants and children

Age range	n	Median	2.5th percentile	97.5th percentile
			NE-17OHP	
Girls				
1-30 d	62	23.3 (7.7)	7.9 (2.6)	43.8 (14.5)
31-59 d	13	18.8 (6.2)	3.6 (1.2)	44.2 (14.6)
2-5.9 m	26	5.5 (1.8)	1.0 (0.33)	25.9 (8.5)
6-11.9 m	21	1.9 (0.62)	1.1 (0.36)	4.7 (1.6)
1-5 yr	22	1.5 (0.49)	1.0 (0.33)	3.1 (1.0)
Boys				
1-30 d *	76	29.4 (9.7)	8.9 (2.9)	64.4 (21.3)
31-59 d	24	22.7 (7.5)	8.0 (2.6)	63.8 (21.1)
2-5.9 m	37	7.7 (2.5)	1.5 (0.49)	27.2 (8.9)
6-11.9 m **	17	3.6 (1.2)	1.5 (0.49)	8.4 (2.8)
1-5 yr	21	1.2 (0.39)	1.1 (0.36)	4.4 (1.4)
			E-17OHP	
Girls and boys				
1-59 d		5.4 (1.8)	1.2 (0.39)	16.0 (5.3)
2-5.9 m		2.4 (0.79)	1.2 (0.39)	11.4 (3.8)
6 m-5 yr		1.3 (0.43)	1.1 (0.36)	3.3 (1.1)

* $p < 0.05$, ** $p < 0.01$ versus girls at the same age.
d = days; m = months; yr = years.

TABLE 3

17OHP [nmol/L (ng/mL)] ranges for non-extracted (NE-17OHP) and extracted (E-17OHP) sera in normal premature neonates

Age range	n	Median	2.5th percentile	97.5th percentile
			NE-17OHP	
Preterm ≤32 wGA				
median age: 36.5 d	21	78.8 (26.0)	38.3 (12.6)	280.0 (92.5)
Preterm >32 wGA				
median age: 30 d	17	66.7 (22.0)	24.9 (8.2)	162.7 (53.8)
			E-17OHP	
Preterm ≤32 wGA				
median age: 36.5 d	21	20.9 (6.9)	5.7 (1.9)	69.9 (23.1)
Preterm >32 wGA				
median age: 30 d	17	11.5 (3.8)	3.7 (1.2)	30.4 (10.0)

d = days; wGA = weeks of gestational age.

TABLE 4

Age ranges for cortisol [nmol/L (µg/dL)] in normal full-term and premature children

Age range	n	Median	2.5th percentile	97.5th percentile
Girls and boys				
Full-term				
1-59 d	126	115 (4.1)	30 (1.1)	556 (20.1)
2-5.9 m	45	311 (11.2)	57 (2.1)	555 (20.1)
6 m-5 yr	56	275 (9.9)	180 (6.5)	612 (22.2)
Preterm ≤32 wGA				
median age: 36.5 d	21	133 (4.8)	30 (1.1)	294 (10.6)
Preterm >32 wGA				
median age: 30 d	17	117 (4.2)	34 (1.2)	353 (12.8)

d = days; m = months; yr = years; wGA = weeks of gestational age.

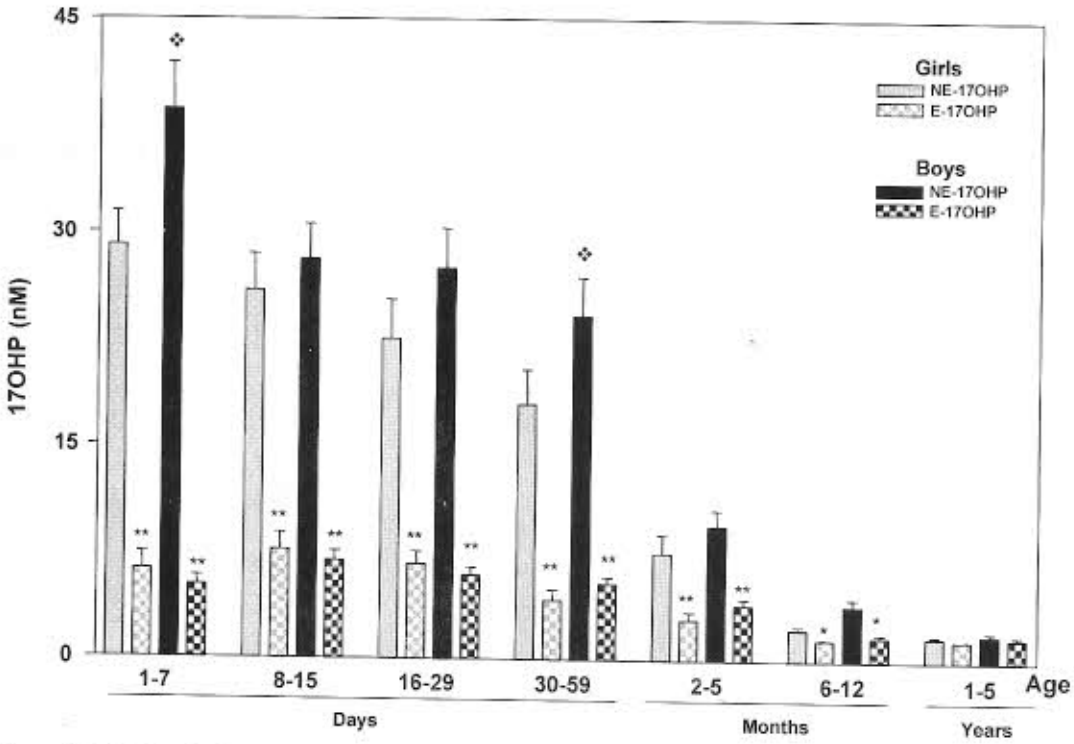

Fig. 1: Serum 17OHP levels (means ± SEM) throughout chronological age up to 5 years for full-term girls (grey bars) and boys (black bars). Significant differences (paired t-test) in direct and extracted 17OHP within each sex (** $p < 0.0001$ and * $p < 0.001$) and between sexes (❖ $p < 0.05$) are denoted in the graph.

procedure also significantly reduced 17OHP levels of children with CAH (NE-17OHP vs E-17OHP, $p < 0.0001$), infants with CAH always presented significantly higher NE-17OHP and E-17OHP levels than normal PT and FT neonates without overlap (Figs. 2 and 3).

Age-related reference intervals for cortisol levels are shown in Table 4. Cortisol levels varied with chronological age, and a linear trend towards higher cortisol concentrations was obtained across age ($p < 0.01$). Cortisol measurement by CLIA did not show gender differences. Almost all PT and all CAH neonates had cortisol concentrations within the normal range. However, some normal premature infants (2/21 PT >32 weeks of GA and 4/17 PT ≤32 weeks of GA) had cortisol concentrations below the 2.5th percentile for FT. The CAH group always presented similar cortisol levels to normal children during the neonatal period in spite of their CYP21 deficiency (median, range for FT: 115, 27.6-591 nmol/L; PT: 133, 27.6-387 nmol/L; PT ≤32 weeks GA: 27.6-251 nmol/L, CAH: 186, 56-479 nmol/L). Cortisol levels in normal children older than 6 months were always higher than 180 nmol/L (6.5 µg/dl). No correlation was found between cortisol and 17OHP levels.

DISCUSSION

CAH has been included in the neonatal screening program of several countries[13-15] and a high incidence of the classical form of CAH has recently been reported in Argentina[16]. Diagnosis of classical presentations has to be confirmed by increased serum 17OHP concentration, the most important indicator of CYP21 deficiency in human plasma[1-3,17]. Therefore, the upper limit of this steroid in serum samples according to age has to be well defined, especially for newborns. On the other hand, serum 17OHP measurement sometimes has methodological problems associ-

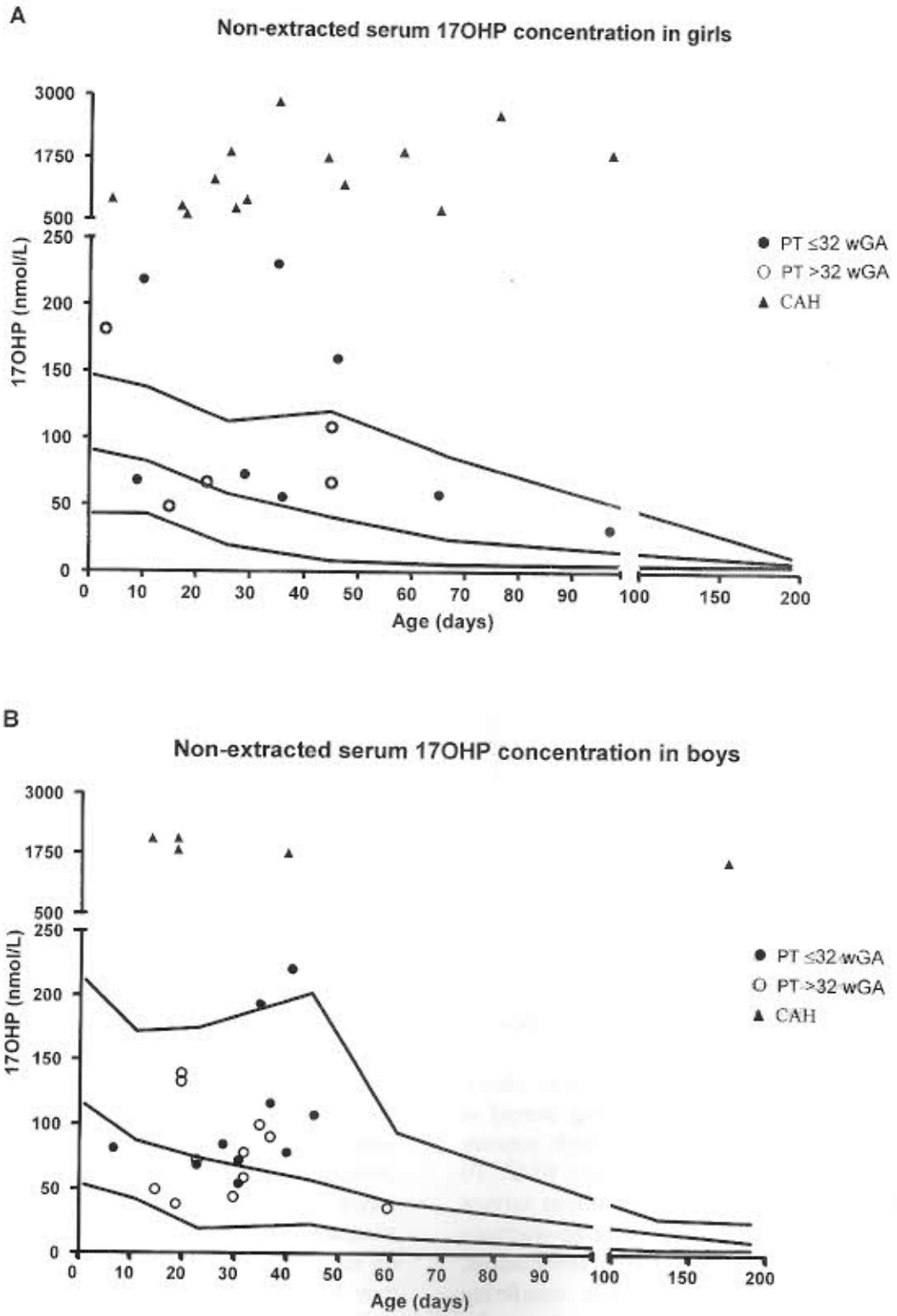

Fig. 2: Non-extracted 17OHP concentrations in individual preterm neonates ≤32 (closed circles) and >32 (open circles) weeks of gestational age (wGA) and children with CAH (triangles) compared to the normal range for the same age and sex. The lines represent the median and 2.5th and 97.5th percentiles for full-term (**A:** girls, **B:** boys).

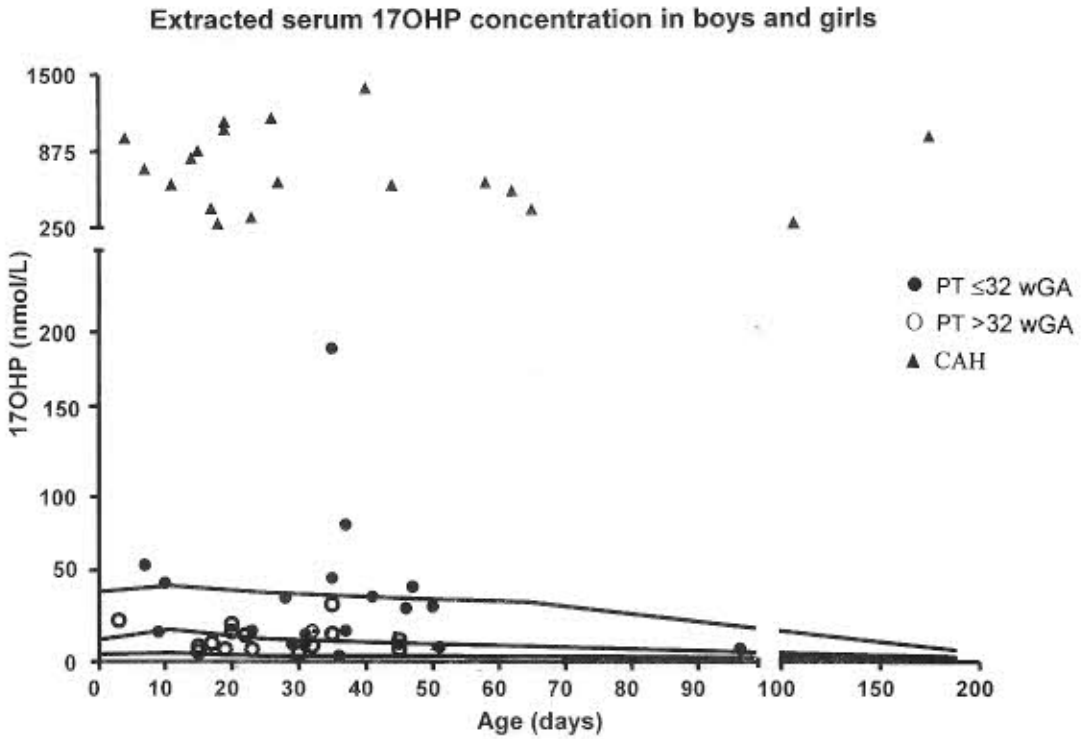

Fig. 3: Extracted 17OHP concentrations in individual preterm neonates ≤32 (closed circles) and >32 (open circles) weeks of gestational age (wGA) and children with CAH (triangles) compared to the normal range for the same age. The lines represent the median and 2.5th and 97.5th percentiles for full-term neonates.

ated with the steroid matrix in neonates, leading to false positive results. Regarding this, it is important to have reliable reference levels for serum 17OHP, especially when adrenal abnormality is suspected during the first weeks of life. Highly sensitive and accurate assays were developed for the quantification of 17OHP but this costly technology is not always available in routine laboratories[18,19].

17-Hydroxypregnenolone sulfate was identified as the most important interfering steroid in neonatal plasma causing apparent high concentrations of 17OHP measured by direct RIA[6]. To avoid this problem an adequate selection solvent extraction procedure to minimize cross-reactions has been suggested at this age[6]. Nevertheless, manufacturers do not warn about interfering steroids or suggest extraction procedures for neonatal samples. As previously reported[4-7], we found that an organic extraction procedure could efficiently eliminate hydrosoluble compounds, enhancing specificity for 17OHP measurement. Nevertheless, the concentration of these interfering substances in neonatal plasma, so much higher than that of 17OHP, could probably even affect assays in which highly specific antibodies are used[4,20]. We found that interfering substances are present at high levels especially during the neonatal period, and we provide reference data for interpreting 17OHP results at this age for both sexes. A gradual decline in both non-extractive and extractive 17OHP levels is observed throughout advancing age to reach no difference for one year-old girls and boys onwards. There is no consensus about the age of infants in whom serum samples should be extracted prior to measurement[4,5,7]. We consider that discrepancies on the age of permanence of interfering steroids may be related to differences in methodological and/or extraction protocol designs. Our data demonstrate that interfering steroids exist at highly significant levels during the neonatal period lasting until one year of age in both sexes. This finding may be explained by the fact that

most of the fetal adrenal growth and remodeling continues until 1 year of age[9,10]. Consequently, thereafter, we found that 17OHP measurement before and after extraction in normal children remained unchanged up to age 5 years.

Gender differences in 17OHP on filter paper have previously been studied in infants[11,21]. To our knowledge, this is the first time that sexual dimorphism has been reported in serum direct 17OHP measurement. Boys presented significantly higher NE-17OHP levels than girls during the newborn period. This may indicate a higher production rate of interfering compounds by the fetal zone of the adrenal cortex, another endogenous source of precursors, or a lower metabolic clearance rate of these steroids in males. The high steroidogenic activity in males during the second month of life could explain the gender differences observed in NE-17OHP at this age[22]. Conversely, Garagorri *et al.* did not find gender differences in serum 17OHP without extraction in children below 6 months of age[23]. This discrepancy may be due to less cross-reactivity with structurally similar steroids of the method used for the determination of 17OHP by these authors. On the other hand, in our screening program for CAH performed with a direct fluoroimmunoassay method in dried blood samples, 68% of the recalled children were normal boys (unpublished observations). Probably, as we suggest in this study on direct serum 17OHP measurement, gender differences in serum 17OHP levels on filter paper need also to be taken into account in order to reduce the unnecessary recall of healthy male newborns.

The differences in steroid biosynthesis by the fetal and definitive zones are based on the temporal expression of different enzymes[10]. The persistent adrenal fetal zone with relatively low expression of CYP21 in normal newborns[10] may explain the higher 17OHP concentration measured in our full-term and premature neonates. The fetal zone undergoes a rapid involution and disappears by the third month of postnatal life while enzymatic maturation takes longer[10].

Little information is available on serum 17OHP level reference ranges in preterm neonates and it is still difficult to interpret their 17OHP results[24]. As reported in neonates using 17OHP measurement in paper filter dried blood spots[11,25,26], gestational age-related changes were observed in both serum NE- and E-17OHP measurements with a wide range of 17OHP levels in preterm infants. These findings highlight the importance of taking into account gestational age to avoid misinterpretation of the 17OHP value at this age. We found that serum direct 17OHP levels of both groups of preterm infants differed significantly from those of full-term infants. The highest non-extracted 17OHP serum concentrations were found in very preterm babies (≤32 weeks of GA). These findings may indicate that interfering compounds are products of fetal metabolism. Moreover, differences in preterm >32 weeks of GA are still present as compared to 2 month-old full-term infants. For that reason, and especially when the normal range for preterm infants according to gestational age, gender, and immunoassay used is not available, we strongly recommend the extraction procedure in serum samples of premature infants, as it allows comparison with FT neonates.

Thus, further studies with larger samples are needed in order to establish whether different normal reference ranges should be advised for preterm infants. Nevertheless, obtaining reference values for premature infants is difficult for practical and ethical reasons.

Although the effect of illness was not considered separately in our premature infants, some authors reported similar 17OHP and cortisol levels in premature infants despite their stressful condition[25,27].

When children with CAH in our study were compared with normal full-term and premature neonates, NE- and E-17OHP measurements were always significantly higher without overlap. Moreover, the extraction step seemed not to be necessary to diagnose classic CAH. Nevertheless, in CAH due to genetic variation of mutations of the *CYP21* gene, a wide range of different clinical and biochemical expression may be found[1] and diagnosis may not be so clear-cut. Moreover, in this spectrum, 17OHP of patients with non-classical CAH may have some overlap with normal reference levels[28].

Some authors have already investigated the effect of prematurity[4,5,11,27,29], gender[23], and

extractive protocols of serum 17OHP concentration prior to its measurement[5,7,24]. To our knowledge, this is the first study performed simultaneously in serum samples of normal full-term, preterm and non-treated CAH neonates. According to our findings FT infants should have reference data adjusted for age of E-17OHP (avoiding gender influence). Although with a smaller number of infants, the extraction procedure allows us to handle PT infants >32 weeks of GA in this same context. Nevertheless, a higher grade of prematurity (PT ≤32 weeks of GA) would require an extra effort to set reference adjusted levels for gestational age. So we highly recommend ether extraction especially for the biochemical assessment of premature infants and biochemical/clinical discrepant patients.

Prior to birth the fetal definitive zone is the major source of cortisol biosynthesis[30]. As previously reported[23], we did not find gender differences in serum cortisol levels and an unique reference interval of cortisol for both sexes is provided. Indeed, we found that cortisol levels did not significantly vary during the first two months of life, probably due to immaturity of branch point enzymes of the fetal adrenal gland and the lack of onset of the cortisol circadian rhythm[31]. This may not only be related to the definitive zone but also to the fetal zone as higher 17OHP concentrations were also found in the same period. Perhaps, higher levels of cortisol precursor (17OHP) may be necessary to further assure adequate cortisol concentration during the neonatal period. Circadian rhythm is not present in newborn infants, and they show little pulsatility in plasma cortisol over time. Therefore, a single random plasma cortisol level is representative of the plasma cortisol levels over a prolonged period of time[27]. Cortisol levels significantly increased with advancing age in FT children. According to our findings using a commercial chemiluminescence immunoassay, a cortisol level below 180 nmol/l (6.5 μg/dl) should warn of probable adrenal insufficiency in children older than 6 months, and functional studies should be considered for further evaluation. On the other hand, cortisol levels were similar regardless of gestational age or CYP21 deficiency. Other authors also found no differences in cortisol values among preterm infants, normal full-term or children with CAH[29]. Dotsch *et al.* found that cortisol levels and cortisol/cortisone ratio were almost the same in a group of control, salt-wasting and simple-virilizing CAH children[32].

Studies on cortisol response in preterm infants suggest that both basal and peak serum cortisol concentrations are highly variable in the first 2 weeks of life, rendering it difficult to establish a normal range[33]. As some premature infants present undetectable cortisol values, perhaps transient cortisol replacement may be considered in these cases. Cortisol levels are not representative of CYP21 deficiency in CAH children due to the adrenal response to higher ACTH concentration. Accordingly, we consider that a cortisol measurement should not be used to exclude the diagnosis of CAH due to CYP21 deficiency as similar serum concentrations were obtained for both FT and CAH children.

In summary, considering that 17OHP determination is the confirmatory step for CAH neonatal screening, the strong influence found on its concentration due to chronological and gestational age, gender, and the measurement method used points out the importance of these factors when assessing samples in the first year of life.

As neonates with classical CAH have 17OHP levels significantly dissimilar from normal FT and PT neonates, the extraction step may be omitted for the diagnosis of CAH. This approach would allow shortening the time taken for the 17OHP result and it reduces the measurement's cost.

Thus, the extraction procedure would be recommended in cases when direct 17OHP results are not consistent with the patient's clinical or biochemical conditions, e.g. premature newborns and full-term infants not suspected of CAH, with a direct 17OHP concentration higher than the stipulated range for age and sex.

ACKNOWLEDGEMENTS

We acknowledge the technical expertise of Ms. Carina Morelli, Mrs. Ana Montese and Mrs. Perla Rossano. We thank Dr Laura Prieto from Fundación de Endocrinilogía Infantil (FEI) for the collection of some serum samples.

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