

CLINICAL STUDY

Estimation of the false-negative rate in newborn screening for congenital adrenal hyperplasia

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Abstract

Objective: Newborn screening based on measurement of 17 α -hydroxyprogesterone (17-OHP) in a dried blood spot on filter paper is an effective tool for early diagnosis of congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency. Its most important rationale is prevention of a life-threatening salt-wasting (SW) crisis; in moderate forms of CAH, early diagnosis and treatment may prevent permanent negative effects of androgen overproduction. Our target was to analyse if all CAH patients who had been identified clinically before puberty would have been detected by the newborn screening. **Methods:** Newborn screening cards of 110 CAH patients born between 1988 and 2000 in five Middle-European countries and diagnosed prior to puberty (77 SW and 33 moderate) and cards from 920 random, healthy newborn controls were analysed. CAH screening had not yet been introduced during this time. The diagnosis was based on clinical and laboratory signs and, in most cases, on CYP21 gene mutation analysis. All 17-OHP measurements in dried blood spots were carried out using a time-resolved fluoroimmunoassay kit.

Results: In the newborn screening blood spots, the median of 17-OHP levels was 561 nmol/l (range 91–1404 nmol/l) in subjects with the SW form and 40 nmol/l (4–247 nmol/l) in the moderate form. All 77 SW patients would have been detected by newborn screening using the recommended cut-off limits (30 nmol/l). However, 10 of 33 patients with moderate CAH would have been missed. 17-OHP levels of all controls were below the cut-off.

Conclusion: Newborn screening is efficient for diagnosing the SW form of CAH, but is inappropriate for identifying all patients with a moderate form of CAH. It appears that the false-negative rate is at least one-third in children with the moderate form of CAH.

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Introduction

Congenital adrenal hyperplasia (CAH) is a recessively inherited disorder in the biosynthesis of adrenal steroids with an incidence of 1 in 5000–18 000 newborns of a mixed population (1). In 90–95% of cases it is caused by a mutation of the CYP21 gene, which encodes the enzyme 21-hydroxylase (P450c21). A deficiency in the activity of this enzyme results in reduced cortisol production and an excess of adrenal androgens. From a clinical point of view, categorisation into three subtypes of P450c21 deficiency has evolved: (i) the salt-wasting (SW) form is characterised by a life-threatening metabolic crisis with salt-loss, hyponatraemia, hyperkalaemia, dehydration, shock and signs of ambiguous

genitalia in females but not in males; (ii) the simple virilising (SV) form is usually recognised by a variable degree of clitoris hypertrophy and posterior labial fusion in females, and pseudo-precocious puberty in males; and (iii) the non-classic (NC) form is usually suspected in females because of hirsutism and cycle irregularities and is rarely diagnosed before the onset of puberty (2, 3). Recent concepts consider CAH as a continuum of phenotypes from severe to moderate to mild, instead of three distinct subtypes (4, 5). The severity of the disease depends to a great extent on the type of gene mutation, the subsequent degree of reduction in P450c21 activity and the sex of affected subjects (6, 7).

Late diagnosis may result in death during an SW crisis or in prolonged incorrect gender assignment in

patients with the SW form. In moderate forms irreversible harm may develop during prepuberty, i.e. progressive virilisation, pseudo-precocious puberty and accelerated bone maturation with reduced final height (1, 8–10). One strategy for avoiding these disadvantages has been the introduction of newborn screening for CAH, based on the measurement of the 17 α -hydroxyprogesterone (17-OHP) level in blood samples collected on filter paper by means of a heel-stick (8, 11, 12). Although the method is encumbered with a high false-positive detection rate, its effectiveness in the recognition of SW patients is undisputed, while there is some evidence for its inability to detect all the other patients who become symptomatic before puberty. However, data on the false-negative detection rate of the current 17-OHP screening test for CAH are scant (1, 8, 13).

Our target was to estimate this number by measuring the 17-OHP concentration in screening cards from our CAH patients. The cards were collected and stored from a time period when CAH screening was not yet performed in these countries and CAH diagnosis was based on clinical methods.

Subjects and methods

Patients and controls

Paediatric endocrinologists from five Middle European countries (Austria, the Czech Republic, Hungary, Slovakia and Slovenia, with a total population of about 35 million) pooled data from the CAH patients they had treated over the previous 35 years in order to create a common databank (for detail cf. (14)).

In these countries nation-wide neonatal mass screening programmes were introduced at least two decades ago and have been in effect since then. Several drops of blood from each newborn baby are collected on a filter-paper card, dried, and mailed to a centralised laboratory where different tests for congenital metabolic and endocrine disorders are performed. The left-over cards with spare dried blood spots are stored at room temperature either in their original form or after autoclaving (15). In none of the five countries was mass screening for CAH introduced before 2001, the screening cards were hence from a period before the introduction of CAH screening. Depending on the country, screening cards are available back to 1988.

In order to create a control group, left-over cards (520 original, 400 autoclaved) from 920 newborns were located and 17-OHP concentrations estimated in a dried blood spot. The birth weights ranged from 535 to 4600 g. As previously reported, storage times up to 12 years produced a negligible influence in this group, while autoclaving reduced the 17-OHP concentration by 32% (for details cf. (15)). Thus, in the present study all autoclaved values have been

corrected for this deviation by using the equation $y = -1.117 + 0.68x$ (15).

Similarly, left-over screening cards (60 native, 50 autoclaved) from 110 CAH patients were located and 17-OHP concentrations measured. The birth weights of the patients ranged from 2600 to 4400 g.

All screening cards originated between 1988 and 2000.

Diagnosis and classification of P450c21-deficient CAH patients

The diagnosis was based on typical clinical symptoms, on elevation of serum 17-OHP levels and in most cases on CYP21 gene mutation analysis. The SW form was considered if an SW crisis occurred during the first 4 weeks of life, i.e. dehydration and serum sodium less than 125 mmol/l and serum potassium more than 6 mmol/l, requiring parenteral fluid replacement and glucocorticoid and mineralocorticoid treatment (16). A moderate form of CAH was diagnosed if clinical symptoms of CAH without SW crisis were present before the age of puberty onset, i.e. 8 years (3).

Methods

All 17-OHP measurements in dried blood spots on newborn screening filter paper cards were carried out in the same laboratory using a time-resolved fluoroimmunoassay kit (DELFLIA neonatal 17-OHP kit; Wallac Oy, Turku, Finland) and following the manufacturer's instructions. Assay performance criteria, the influence of autoclaving and storage and the correction for these influences have been previously reported (15). 17-OHP concentrations are given as nmol/l blood.

In patients with the moderate form of CAH, bone age was determined according to Greulich & Pyle (17), pubertal stages were rated according to Tanner (18), genital masculinisation was staged according to Prader (19) and 17-OHP serum measurements were performed with commercially available RIA kits. Patients' DNA samples were analysed for mutations of the CYP21 gene, as previously published (20, 21).

Statistical analysis

The statistical analysis was performed using SigmaStat, Version 2.03 (SPSS Inc., Chicago, IL, USA). Prior to any further analysis, data were tested for normality. Since normal distribution was not observed in all of the data sets, data were expressed as the median (1st–99th or 25th–75th percentile) and analysed by means of the Mann–Whitney rank sum test or the Kruskal–Wallis one-way ANOVA on ranks with a subsequent pair-wise multiple comparison procedure (Dunn's method), as appropriate.

In two CAH patients, birth weight is stated as 'normal' (Table 2). For statistical purposes, values

were set to 3300 g. In three CAH patients, pre-therapeutic 17-OHP levels were measured in dried blood spots on screening cards and not in serum. These data were not used in the statistical calculations.

Results

After correcting for autoclaving, but not for time of storage, and grouping according to birth weight, the controls showed 17-OHP levels (Table 1) similar to those measured with the same fluoroimmunoassay in screening cards which had not been stored (22, 23). Also in accord with these reports, there was a significant difference in 17-OHP levels between the various weight groups ($H = 236.9, P < 0.001$; ANOVA on ranks). Premature, low birth weight babies showed significantly higher 17-OHP concentrations than mature newborns ($P < 0.05$, Dunn's method).

In the 77 SW patients, the median 17-OHP concentration was 561 nmol/l (range 91–1404 nmol/l), measured in dried blood spots on the newborn screening cards (Fig. 1). Using 90 nmol/l, which is diagnostic and requires immediate medical action (24), as a cut-off value for term babies all SW patients would have been detected by the newborn screening.

In the 33 patients with the moderate form of CAH, the median 17-OHP level was 40 nmol/l (range 4–247 nmol/l; Fig. 1). Considering 30 nmol/l 17-OHP in blood or less as normal for mature newborns (24), 10 of 33 moderate CAH patients would not have been identified by screening (first ten patients in Table 2).

By scrutinising the clinical data of the moderate CAH patients (Table 2), it is apparent that the ten patients who would have been missed in the screening were diagnosed later than the others. Their pre-therapeutic 17-OHP serum concentrations were lower, but the acceleration in bone age and excessive growth were similar to those who would have been identified by means of the screening (Table 3). In addition, all ten patients displayed signs of virilisation or premature pubarche (Table 2).

All moderate CAH patients analysed had two mutant alleles. Most of the children were compound heterozygotes with a severe (del, conv, cluster E6, L307insT, Q318X, Q315X, i2) and a moderate (I172N) or a mild

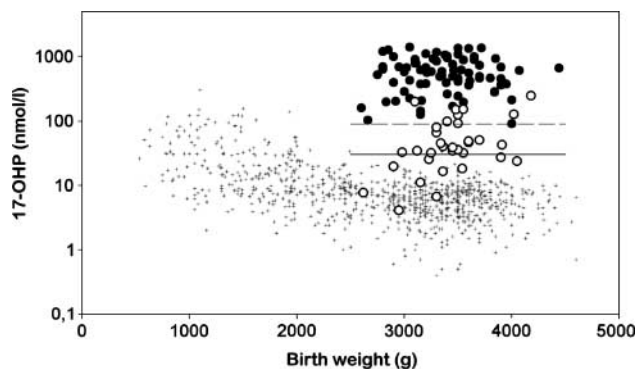


Figure 1 17-OHP concentration in dried blood on screening cards of normal newborns (+) and newborns with the SW form (●) or a moderate form (○) of CAH. The solid horizontal line is the cut-off value between normal newborns and patients with CAH (30 nmol/l); the dashed horizontal line is the cut-off value indicative for the severe form of CAH (90 nmol/l; cf. (26)).

(V281L, P30L) allele (25). The ten missed patients tended to have a more moderate/mild genotype than the detected patients (for details cf. Table 2).

Discussion

All our 77 patients with the SW form of CAH had 17-OHP concentrations exceeding 90 nmol/l blood on screening cards. Following the recommendations of the European Society for Paediatric Endocrinology (ESPE) and using 90 nmol/l as the threshold in term babies indicative of a severe form of CAH requiring immediate medical investigation and treatment (24), all our SW patients would have been identified within the first 2 weeks of life. However, using the same recommendations, where values of 30 nmol/l and below are considered as normal, 10 of the 33 patients with the moderate form of CAH would have been missed. The ten missed children, however, became symptomatic during prepuberty, showing virilisation, premature pubarche, accelerations of bone age and abnormal growth.

From these data it is evident that the time-resolved fluoroimmunoassay employed and the strategy recommended by ESPE in the CAH-screening programme have a sensitivity of 100% for mature neonates with the SW form of CAH. The sensitivity for patients with the moderate form of the disease, which still becomes clinically significant before puberty, is only 70%. In fact, the true number of unidentified patients may be somewhat higher, as indicated by our data, since not all clinically symptomatic children with CAH may have been identified and some of them – born after 1995 – may even not have had enough time to develop clinical signs. Although the missed patients seem to suffer from a more moderate form of the disease, some still may suffer lasting harm because of the late onset of treatment. At least four of them will have

Table 1 Median (1st–99th percentile) 17-OHP levels in blood spots on filter paper cards from newborn controls grouped according to birth weight. 17-OHP levels differed significantly ($P < 0.001$) among groups, while babies with lower birth weight had higher 17-OHP concentrations.

Weight (g)	n	17-OHP (nmol/l)
<1500	115	21.8 (1.9–330.0)
1500–2000	115	12.7 (2.6–145.0)
2000–2500	115	9.4 (1.0–160.0)
>2500	575	6.6 (0.7–19.3)

Table 2 Detailed clinical, hormonal and genetic data from our patients with the moderate form of CAH ordered according to 17-OHP concentration at screening. [§]Prior to onset of treatment; [&]bold print = severe gene mutation, normal print = moderate gene mutation, cursive print = mild gene mutation (27); *suspected because of an affected sibling; **new mutation with a change of a glutamine to a stop codon; [§]17-OHP measured on screening cards.

Initials	Sex	Birth weight (g)	Day of screening sampling	Serum 17-OHP [§] (nmol/l)	Chronological age [§] (years)	Bone age [§] (years)	Bone age [§] (SDS)	Height [§] (cm)	Height [§] (SDS)	Tanner [§] stage	Prader [§] stage	Genotype		Screening 17-OHP (nmol/l)
												Allele 1 ^{&}	Allele 2 ^{&}	
M.J.	M	2950	4	280	5.9	11.5	7.4	132.0	2.95	P2	–	I2	I172N	4.1
S.O.	F	Normal	4	591 [§]	0.3	0.3	0.1	65.0	2.00	P1	2	L307 ins T + R356W	V281L	6.7
H.M.	F	2620	4	60	6.2	6.8	0.8	120.0	–0.36	P2–3	1	I2	V281L	7.7
H.A.*	F	3150	4	18	7.9	8.8	1.1	122.2	–0.74	P2	1	I2	V281L	11.1
A.A.	F	3360	4	90	4.4	5.0	0.7	112.0	1.77	P1–2	1	Q315X**	P30L	16.5
D.R.	F	3540	3	105	0.0	0.0	0.0	52	1.00	P1	2	Del/conv	I172N	18.3
M.S.	M	2900	5	285	7.8	12.0	5.5	140.7	2.40	P3	–	Refused	–	19.7
V.M.	F	4050	4	51	7.1	8.0	1.1	132.0	2.01	P2	1	I2	I172N + V281L	23.8
K.F.	M	3230	4	78	6.8	13.0	8.3	121.1	0.18	P2	–	I172N	P30L	25.4
Z.N.	M	3900	5	33	5.2	13.0	10.6	130.6	4.50	P3	–	I2 + P453S	I172N	27.3
S.P.	F	3550	5	150	1.9	6.0	5.4	85.0	1.19	P2	2	Conv	I172N	31.9
S.L.	F	2980	4	573	1.6	2.0	1.4	79.0	0.80	P1	2	Del	P30L	32.7
K.P.*	M	3120	5	114	2.9	2.7	–0.5	89.0	–1.50	P1	–	I172N	P30L	34.5
O.K.	F	3450	3	522	1.3	3.0	5.7	93.9	1.00	P1	2	I172N	I172N	34.5
S.F.*	M	3050	4	144	1.2	1.3	0.3	78.0	1.00	P1	–	Conv	P30L	35.8
H.M.	F	3150	3	480	3.6	7.5	6.2	106.0	2.00	P2	1	I172N	I172N	38.7
D.M.*	M	3360	4	42	0.0	0.0	0.0	55.0	3.50	P1	–	Del/conv	I172N	40.4
S.J.*	M	3910	4	222	0.8	1.0	1.3	80.0	3.50	P1	–	Del	P30L	42.6
B.O.	M	3340	4	552	3.1	6.0	7.1	107.0	3.00	P3	–	Refused	–	45.2
G.H.	F	3250	9	43	0.0	0.0	0.0	50.0	0.00	P1	2	I2	I172N	45.4
K.E.	M	3600	5	123	6.7	11.0	5.8	132.1	2.70	P2	–	I2	P30L	46.5
F.Z.	F	3250	4	399	2.0	4.5	6.5	90.0	1.00	P1–2	2	I172N	I172N	48.8
P.B.	F	3700	4	201	0.2	0.2	–0.2	58.0	–1.00	P1	4	Del/conv	I172N	50.5
P.J.	M	3300	5	180	2.6	7.7	13.7	103.5	2.99	P2	–	I172N	I172N	66.0
K.D.	F	Normal	4	1620 [§]	0.9	1.0	0.8	83.0	4.50	P2	1	I172N	I172N	80.0
Z.D.	M	3500	4	2115 [§]	5.1	11.3	8.3	130.0	4.50	P3	–	I2	I172N	92.0
N.K.	F	3450	4	68	0.1	0.1	0.0	55.0	2.00	P1	2	Q318X	I172N	98.4
P.J.	M	3500	4	116	3.0	5.0	2.6	110.0	3.82	P2	–	Conv	I172N	124.2
K.N.	F	4020	5	75	0.0	0.0	0.0	50.0	0.00	P1	2	Q318X	I172N	126.9
P.P.*	M	3480	4	200	3.6	9.5	13.2	109.5	2.50	P1	–	Del/conv	I172N	150.5
S.J.	F	3550	5	100	0.0	0.0	0.0	50.0	0.00	P1	4	Del/conv	I172N	152.2
S.M.	F	3100	4	52	0.0	0.0	0.0	49.0	–0.50	P1	3	Del	I2	199.6
K.D.	M	4180	4	342	5.7	13.0	9.7	132.0	3.40	P2	–	Cluster E6	I172N	247.2

Del, deletion; conv, conversion.

Table 3 Median values (25th–75th percentile) of clinical and hormonal data from patients with a moderate form of CAH at diagnosis. Patients are grouped according to whether they would have been detected or missed by CAH screening.

	Patients with a moderate form of CAH		P
	Detected	Missed	
Age (years)	1.6 (0.1–3.0)	6.0 (4.4–7.1)	0.005
Bone age (years)	2.7 (0.1–7.1)	8.4 (5.0–12.0)	0.04
SDS	+ 1.4 (0.0–6.3)	+ 1.1 (0.6–7.4)	NS
Height (cm)	87 (58–107)	121 (112–132)	0.02
SDS	+ 1.6 (0.0–3.4)	+ 1.8 (0.0–2.4)	NS
17-OHP (nmol/l)	150 (93–356)	78 (46–148)	0.05
n	23	10	—

considerably reduced final height (Table 2). This fact should be a reason for further improvement of our assays.

By using a less stringent cut-off value for normals, i.e. 37.5–40 nmol/l 17-OHP in blood (7, 26), approximately half of our moderate CAH patients would not have been detected. An increased cut-off value is used by some groups in order to reduce the number of false-positive measurements and to avoid unnecessary stress for the parents. Our data indicate that such measures may be taken without losing patients with the SW form. However, a greater number of children with a moderate, but nonetheless clinically significant form of the disease, would be missed.

The value of a particular screening programme depends on both the specificity and the sensitivity of the assay employed, i.e. the rate of false-positive and false-negative identification of the patients examined. In the case of CAH screening, various direct assay systems with different antisera have been used over the years, but – to a varying extent – they are all confounded by cross-reactivity with steroids related to 17-OHP which are present in neonatal serum (11). Thus, the determination of the rate of false-positive measurements has been an important issue since the introduction of CAH screening (11, 27). Eventually, this rate could be reduced to 0.2–0.9% by the introduction of birth weight- or gestational age-adjusted cut-off values and by application of the DELFIA time-resolved fluoroimmunoassay (23, 26). This assay currently produces the most specific, but still not accurate, estimates for 17-OHP in screening (11, 28).

The problem of false-negative measurements is more difficult to deal with and very few data are available (8, 13). First, there is presently no method to identify all CAH patients, because some of them, especially with the mild form, have very few – if any – symptoms and may come to attention later on in life or never. By concentrating only on those patients who become symptomatic before puberty and who actually may benefit from early treatment, it can take up to a decade after birth until all these patients are recognised

and those missed in the screening are identified. Secondly, a prerequisite for the calculation of the false-negative rate is the usage of the same assay system and the same cut-off values over a prolonged period. A report from the Swedish CAH-screening programme (8), where 66 patients with CAH were identified and seven cases missed between 1989 and 1994, comes closest to these requirements. However, it is difficult to calculate a false-negative rate from these data, since both the assay and the cut-off level were altered during the observation period and at the closure of the study, in 1997, not all missed children with the moderate form of CAH may have been noticed.

Therrell *et al.* (13) used a different approach to estimate the false-negative rate by screening all newborns twice, shortly after birth and again at age 1–2 weeks. In fact at the first screen all SWs were detected, but only 40% of the SVs and 13% of the NC patients were identified at the second screen. The study does not specify the used assays and the cut-off values, and it is uncertain whether all CAH patients display elevated 17-OHP at age 1–2 weeks. Although this strategy may certainly help to identify additional patients with the moderate and mild form of CAH, the high incremental cost for a mandatory second screen in all newborns has been criticised (29, 30).

In the present study we provide evidence that by applying a current assay and current cut-off values in CAH screening, all patients with the SW form of CAH may be detected within the first 2 weeks of life. Apparently two-thirds of the patients with a moderate form of the disease who become clinically manifest during childhood may also be identified by screening. The remaining patients seem to suffer from an even more moderate form of the disease, although presenting signs of virilisation, pseudo-precocious puberty and accelerated bone maturation. Their early recognition remains a paramount goal. Thus, the development of more specific assays for CAH screening is important in order to further reduce the rate of both false-positive and false-negative measurements.

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