21-Hydroxylase Deficiency Detected in Neonatal Screening: High Probability of False Negativity in Late Onset Form

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ABSTRACT

Aim Despite the high sensitivity of neonatal screening in detecting the classical form of congenital adrenal hyperplasia due to 21-hydroxylase deficiency, one of the unclear issues is identifying asymptomatic children with late onset forms. The aim of this nationwide study was to analyse the association between genotype and screened level of 17-hydroxyprogesterone in patients with the late onset form of 21-hydroxylase deficiency and to quantify false negativity.

Methods In the Czech Republic, 1,866,129 neonates were screened (2006–2022). Among this cohort, 159 patients were confirmed to suffer from 21-hydroxylase deficiency, employing the 17-hydroxyprogesterone birthweight/gestational age-adjusted cut-off limits, and followed by the genetic confirmation. The screening prevalence was 1:11,737. Another 57 patients who were false negative in neonatal screening were added to this cohort based on later diagnosis by clinical suspicion. To our knowledge, such a huge nationwide cohort of false negative patients has not been documented before.

Results Overall, 57 patients escaped from neonatal screening in the monitored period. All false negative patients had milder forms. Only one patient had simple virilising form and 56 patients had the late onset form. The probability of false negativity in the late onset form was 76.7 %. The difference in 17-hydroxyprogesterone screening values was statistically significant ($p < 0.001$) between severe forms (median 478.8 nmol/L) and milder (36.2 nmol/L) forms. Interestingly, the higher proportion of females with milder forms was statistically significant compared with the general population.

Conclusions A negative neonatal screening result does not exclude milder forms of 21-hydroxylase deficiency during the differential diagnostic procedure of children with precocious pseudopuberty.

ABBREVIATIONS

17OHP	17-hydroxyprogesterone
21OHD	21-hydroxylase deficiency
21OHD-CAH	congenital adrenal hyperplasia due to 21-hydroxylase deficiency
CZ	Czech Republic
NS	neonatal screening

Introduction

Routine neonatal screening (NS) for congenital adrenal hyperplasia due to 21-hydroxylase deficiency (21OHD-CAH; MIM 201910) was launched in the Czech Republic (CZ) in 2006, to prevent life – threatening metabolic salt–wasting crisis in the most severe forms, and to avoid precocious pseudopuberty with short final height, in milder forms [1]. NS of 21OHD-CAH is performed by immunologic assay for 17-hydroxyprogesterone (17OHP). The second-tier test for confirmation of diagnosis is based on measuring metabolites of steroid spectrum by tandem mass spectrometry. Finally, early genetic confirmation with *CYP21A2* genotyping is a clue to predict disease severity, make treatment decisions and planning follow-up [2].

Despite the high NS sensitivity to detect 21OHD-CAH classical forms, one of the unclear issues is identifying asymptomatic children with late onset forms [3]. These non-classical forms have a variable phenotype depending on the age, gender and the presence of a classical mutation [4]. The aim of this nationwide study was to analyse association between the genotype and screened level of 17OHP in patients with the late onset form of 21OHD-CAH and to quantify false negativity in NS of 21OHD-CAH. To our knowledge, such a cohort of patients has not been studied before.

Materials and methods

Over a period of sixteen years from 2006 to 2022, a total of 1,866,129 neonates were screened, which represents 100 % of all live-births in CZ. Dried blood spots were tested for 17OHP using fluorescence *immuno*-assay (Delfia and AutoDelfia produced by Perkin-Elmer) [1]. Among this neonatal cohort, 159 patients were confirmed to suffer from 21OHD-CAH, employing the 17OHP birthweight/gestational age-adjusted cut-off limits (▶ **Table 1**, [5]), and followed by the genetic confirmation of pathogenic variants in the *CYP21A2* gene. Thus, the screening prevalence of 21OHD-CAH among the neonatal cohort was 1:11,737.

Fifty-seven patients who were initially NS false negative were added to the cohort after being diagnosed later based on the clinical suspicion of 21OHD-CAH. The diagnosis was confirmed by genetic testing and 17OHP values were traced back in their neonatal screening cards. The entire cohort included 216 patients with 21OHD-CAH, 103 males (47.7 %) and 113 females (52.3 %). All genetic investigations of *CYP21A2* were performed at a single Czech laboratory, therefore the completeness of the nationwide dataset of patients is high probable.

Data was evaluated in IBM SPSS 29.0 program (IBM Corporation, Armonk, New York, United States of America). Kruskal-Wallis test and Mann-Whitney test with Bonferroni correction were used for the statistical analysis of continuous variables. Obtained data are presented as numbers and percentages; the differences between groups were tested using chi-squared and binomial tests. Statistical significance was set at 0.05.

Results

The genotype data was available from all patients and was analysed separately in four patient subcohorts according to their genotype – group A: salt–wasting form (52 patients, 24.1 %), B: salt–wasting/simple virilising form (47 patients, 21.8 %), C: simple virilising form (44 patients, 20.4 %), D: late onset form (73 patients, 33.8 %) (▶ **Fig. 1**, ▶ **Table 2**) [modified according to 1,6].

▶ **Table 3** provides the proportion of false negativity NS for 21OHD-CAH. Overall, 57 patients escaped from NS in the monitored period. The probability of false negativity in late onset form was 76.7 %. *CYP21A2* variants were varied with p.V282L representing 76.8 %. Other genetic findings included p.P31L (10.7 %), c.1488 + 13 G > A (7.1 %), p.P454S (3.6 %) and p.R357Q (1.8 %).

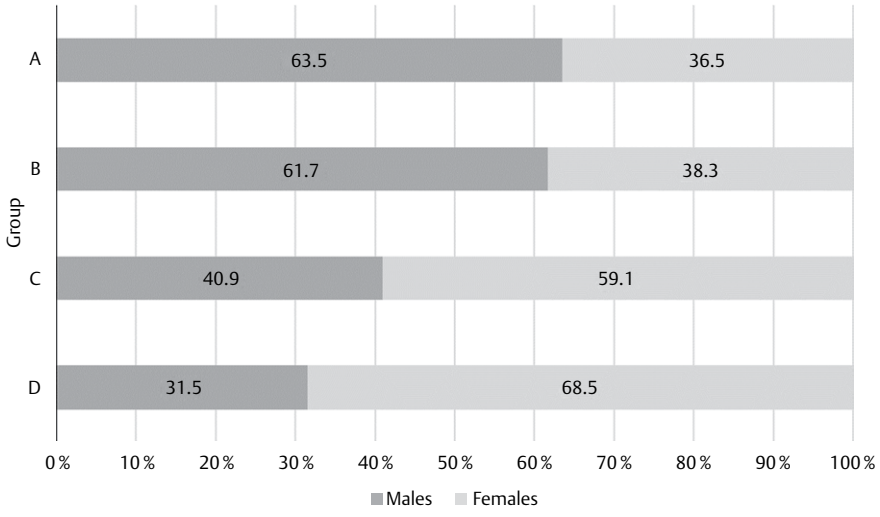
▶ **Fig. 1** summarizes the gender distribution according to genotype and shows a higher proportion of males in groups A-B (severe forms), but the difference was not statistically significant compared to the general population. On the other hand, there was a higher proportion of females with milder forms in groups C-D, and the difference was statistically significant in group D ($p = 0.002$) compared with the general population.

The association between screened 17OHP, gender and genotype are summarized in ▶ **Fig. 2**. The difference in 17OHP screening values between severe forms (group A, B, median 478.8 nmol/L) and milder forms (group C, D, median 36.2 nmol/L) was statistically significant ($p < 0.001$). On the contrary, gender was not a statistically significant factor.

▶ **Table 1** Decision limits for congenital adrenal hyperplasia due to 21-hydroxylase deficiency according to sample timing (48–72 postnatal hours), used from 2009 [5].

Gestational age (weeks)	Birthweight (g)	17-hydroxyprogesterone (nmol/L in capillary blood)
≤ 27	< 900	137
28	900–1,099	117
29	1,100–1,299	100
30	1,300–1,499	85.0
31	1,500–1,699	71.0
32	1,700–1,899	60.0
33	1,900–2,099	49.0
34	2,100–2,299	40.0
35	2,300–2,499	31.0
36	2,500–2,699	30.0
≥ 37	≥ 2,700	20.0

Decision limits in 2006–2009 were approximately 20 % higher due to the use of different kits and later sample collection.



▶ **Fig. 1** Gender distribution according to genotype of congenital adrenal hyperplasia due to 21-hydroxylase deficiency (**a**: salt–wasting form, **b**: salt–wasting/simple virilising form, **c**: simple virilising form, **d**: late onset form).

▶ **Table 2** Cohort of analysed patients with congenital adrenal hyperplasia due to 21-hydroxylase deficiency (n = 216) [modified according to 1,6].

Group	21-hydroxylase activity	Mutations *CYP21A2* gene determinating phenotype	Median and range of screening 17-hydroxyprogesterone (nmol/L)
A: salt–wasting form (52 patients)	0 %	del, chim, stop mutations, e. g. p.Q319* p.I237N + p.V238E + p.M240K c.923_924insT p.R357W	528 (140–957)
B: salt–wasting/simple virilising form (47 patients)	< 1 %	c.293-13 C > G	430 (99–1,100)
C: simple virilising form (44 patients)	1–2 %	p.I173N, p.R357Q	53 (23–397)
D: late onset form (73 patients)	20–60 %	p.V282L, p.P31L, c.1488 + 13 G > A, p.P454S	20 (2–130)

▶ **Table 3** Distribution of screening positive and false negative patients in neonatal screening for congenital adrenal hyperplasia due to 21-hydroxylase deficiency.

Group	Screening detected patients n (%)	False negative patients n (%)
A: salt–wasting form	52 (100.0)	0 (0.0)
B: salt–wasting/simple virilising form	47 (100.0)	0 (0.0)
C: simple virilising form	43 (97.7)	1 (2.3)
D: late onset form	17 (23.3)	56 (76.7)
Total	159 (73.6)	57 (26.4)

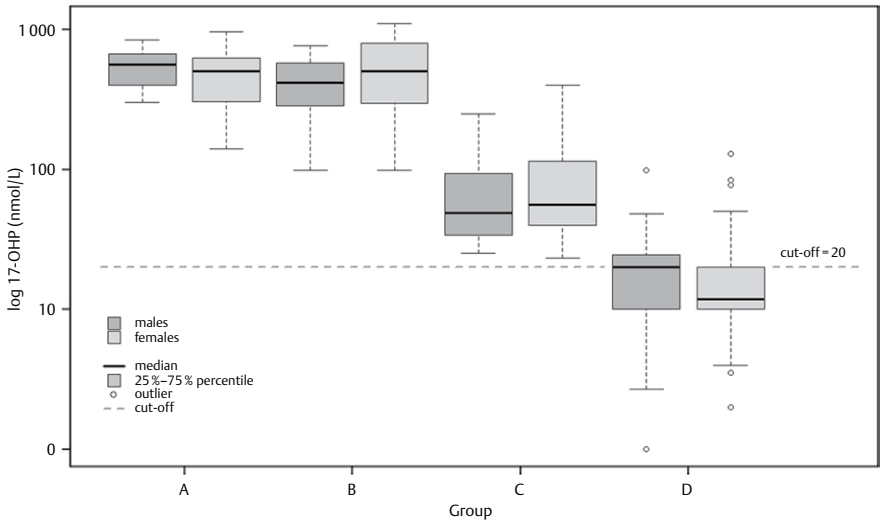
Discussion and conclusions

NS is efficient in diagnosing classical forms of 21OHD-CAH but is inadequate in identifying all patients with late onset forms [7]. It appears that the false negative rate is at least one-third in children with the moderate form of 21OHD-CAH [8]. In our study, a surprisingly high probability (76.7 %) of false negativity in the late onset form was confirmed.

In this group of false negative patients, *CYP21A2* variants were expectable with p.V282L representing almost 80 % of all detected mutations. The frequency of the underlying genetic defect in our patients was similar to that observed in other European populations [9, 10].

The above results support the statement that negative NS result does not exclude milder forms of 21OHD-CAH during differential diagnostic procedure in children with precocious pseudopuberty. In additional, in our cohort one patient with salt–wasting/simple virilising form of 21OHD-CAH escaped from NS. However, this was not a true biological false negativity, as the patient was treated with topical hydrocortisone before obtaining the dried blood spot and the rule of screening before corticosteroid administration was not followed.

On the other hand, we emphasize that the primary aim of NS is to prevent salt-wasting crises and mortality, not to identify children with milder forms. Therefore, the 17OHP cut-off limits are chosen to ensure high sensitivity for detecting severe cases. Similarly, higher 17OHP cut-of limits prevent to unnecessary hydrocortisone treatment in milder forms.



▶ **Fig. 2** Association between screening 17-hydroxyprogesterone (17-OHP), gender and genotype of congenital adrenal hyperplasia due to 21-hydroxylase deficiency (**a**: salt–wasting form, **b**: salt–wasting/simple virilising form, **c**: simple virilising form, **d**: late onset form).

Owing to differences in health care systems between nations, the experiences of NS of 21OHD-CAH in CZ are not directly transferable to all other countries. Examples include differences in sensitivity and specificity. In Australia no known false negatives were notified, and the protocol has a reported sensitivity of 100 % and specificity of 99.9 % [11]. In the Dutch national NS registry, no false-negatives were reported from 2017–2019 [12]. In Sweden, NS reports only three false negative patients from 1986–2011 [7]. On the other hand, same authors declare a high rate of false positive results in preterm infants. In such cases, we can speculate that NS diagnoses the severe forms, while milder forms escape leading to the lack of its detection by NS.

This study had several limitations that are partly linked to the retrospective design. Also, the completeness of the nationwide dataset of patients was high probable. On the other hand, there is the possibility of patients having the late onset form of 21OHD-CAH who escaped NS detection in monitored period and are not yet diagnosed. Hence, our presented data does not reflect the real false negativity of the neonatal screening program. As a nationwide study, validation on an external group was not possible. The strength of this study includes the country-wide surveillance of all possible affected patients, evaluated on the background of the national epidemiological data on 21OHD-CAH. To our knowledge, such a huge nationwide cohort of 21OHD-CAH NS false negative patients has not been documented before.

Finally, it should be noted that NS is an effective secondary prevention tool for the early detection of severe forms of 21OHD-CAH. The main purpose of NS is not to detect all forms of 21OHD-CAH. A negative NS result does not exclude milder forms of 21OHD-CAH during the differential diagnostic process of children with precocious pseudopuberty. Therefore, the diagnosis of milder forms remains in the hands of clinicians.

“Compliance with Ethical Statements”

- Informed consent: For this type of study formal consent is not required. This article does not contain any studies with human participants or animals performed by any of the authors.
- The study was approved by the Ethics Committee of the Third Faculty of Medicine, Charles University.
- Authors whose names appear on the submission have contributed sufficiently to the scientific work and therefore share collective responsibility and accountability for the results.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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