Neonatal Screening for Congenital Adrenal Hyperplasia in Turkey: A Pilot Study with 38,935 Infants
Güran T et al. Neonatal screening for CAH in Turkey

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What is already known on this topic?

Classical congenital adrenal hyperplasia (CAH) occurs in 1:13,000 to 1:15,000 live births. 21-hydroxylase enzyme deficiency (21-OHD) occurs in 90 to 95% of all cases of CAH. CAH is the most common cause of primary adrenal insufficiency in childhood.

CAH is a potentially life-threatening condition that requires accurate diagnosis and urgent treatment with glucocorticoid and mineralocorticoid replacement. Symptoms and signs may easily be overlooked particularly in male infants who do not have genital ambiguity.

In 2002 the Joint Lawson Wilkins Paediatric Endocrine Society/European Society for Pediatric Endocrinology Working Group recommended biochemical screening for CAH in the newborn period.

Neonatal screening for CAH is effective in detecting the salt-wasting form and thereby reducing mortality.

What this study adds?

The estimated incidence of classical 21-OHD CAH in the screened population was 1:7,787 in Turkey.

The incidence of CAH due to classical 21-OHD is higher in Turkey in comparison to previous reports in the literature. Thus, it may be worthwhile to add CAH to newborn screening panel in Turkey.

Abstract

Background: Congenital adrenal hyperplasia (CAH) is the most common form of primary adrenal insufficiency in children. Neonatal screening for CAH is effective in detecting the salt-wasting form and reducing mortality.

Aim: To estimate incidence of CAH in Turkey and to assess the characteristics and efficacy of the adopted newborn CAH screening strategy.

Method: A pilot newborn CAH screening study was carried out under the authority of Turkish Directorate of Public Health. Newborn babies ≥ 32 gestational weeks and ≥ 1500 gr birth weight from four cities between March 27- September 15, 2017 were included. Screening protocol included one sample two-tier testing. In the first step, 17α -hydroxyprogesterone (17-OHP) was measured by fluoroimmunoassay in dried blood spots obtained at 3-5th days of life. The cases with positive initial screening were tested by steroid profiling in dried blood spots using liquid chromatography-tandem mass spectrometry method to measure 17-OHP, 21-deoxycortisol, cortisol, 11-deoxycortisol and androstenedione as a second-tier test. The babies with steroid ratio of $(21\text{-deoxycortisol}+17\text{-OHP})/\text{cortisol} \geq 0.5$ were referred to pediatric endocrinology clinics for diagnostic assessment.

Results: 38,935 infants were tested, 2265 (5.82%) had second-tier testing, and 212 (0.54%) were referred for clinical assessment, 6 of whom were diagnosed with CAH (four males, two females). Four cases were identified as salt-wasting 21-hydroxylase deficiency (21-OHD) (2 males, 2 females), one male baby had simple virilizing 21-OHD, one male baby had 11-OHD CAH. The incidence of classical 21-OHD in the screened population was 1:7,787.

Conclusion: The incidence of CAH due to classical 21-OHD is higher in Turkey in comparison to previous reports. Thus, it is suggested to add CAH to newborn screening panel in Turkey. The use of steroid profiling as a second-tier test improves the efficacy of the screening and reduces false-positives.

Keywords: Newborn screening, congenital adrenal hyperplasia, second-tier, steroid profiling

Introduction

Congenital adrenal hyperplasias (CAH) arise from biallelic gene defects encoding the enzymes and cofactor proteins involved in the cortisol biosynthesis. The most common enzyme deficiency that accounts for more than 90% of all cases with CAH is 21-hydroxylase deficiency (21-OHD). 21-OHD is classified into 3 subtypes according to clinical severity: classical salt wasting (SW), classical simple virilizing (SV), and nonclassical CAH (NCCA; mild or late onset) (1). Data from close to 6.5 million newborn screenings worldwide indicate that classical CAH occurs in 1:13,000 to 1:15,000 live births (2). CAH is the most common cause of primary adrenal insufficiency in childhood and is a potentially life-threatening condition that requires accurate diagnosis and urgent treatment with glucocorticoid and mineralocorticoid replacement. Symptoms and signs may easily be overlooked particularly in male infants who do not have genital ambiguity. Because of delayed or missed diagnosis in affected male infants (and some very virilized female infants), in 2002 the Joint Lawson Wilkins Paediatric Endocrine Society/European Society for Pediatric Endocrinology Working Group recommended biochemical screening for CAH in the newborn period (3, 4). The majority of states in the United States and more than 50 countries are currently performing newborn screening for CAH (5). Infant screening programs have markedly decreased the time to diagnosis, theoretically decreasing morbidity (6, 7). Based on proven importance, a pilot newborn screening (NBS) programme for CAH has been initiated by the Turkish Directorate of Public Health (TDPH) on March 27, 2017 in 4 cities of Turkey. We have evaluated the database collected from this pilot study to describe the incidence of CAH in Turkey. We have also described the cases with CAH in detail identified by this pilot study. Additionally, we assessed the results in detail in regards to the characteristics and efficacy of the adopted newborn screening strategy to get some prospects for enhancing screening performance.

Methods

The pilot screening programme for CAH was carried out between March 27 and July 15, 2017 by TDPH, in 4 cities (Adana, Kayseri, Konya and Samsun) of Turkey. According to the programme, dried blood spots (DBS) were obtained using filter paper ("Guthrie" cards) between the 3rd and 5th days of life or as soon as possible after 48 hours of age by heel prick. The samples were obtained simultaneously with the ongoing nationwide newborn screening program for congenital hypothyroidism,

phenylketonuria, biotinidase deficiency and cystic fibrosis. The CAH screening algorithm was developed in consultation with a scientific committee consisting of paediatric endocrinologists from several universities in Turkey (**Figure 1**). Newborn babies ≥ 32 gestational weeks and ≥ 1500 gr birth weight from the four cities where the pilot study was conducted were included.

Initial CAH screening was based on the measurement of 17-OHP in DBS on filter paper by fluoroimmunoassay (FIA) (**Labsystems Diagnostics, Finland**). Cut-off values for 17-OHP were based primarily on gestational age and birth weight. 17-OHP values of 10 ng/mL and 15 ng/mL have been used as cut-off points for newborn babies ≥ 36 gw and/or ≥ 2500 gr birth weight, and for newborn babies between 32-36 gw and/or 1500-2500 gr birth weight; respectively (**8, 9, 10**). If the 17-OHP level was above the cut-off level in the first-tier test using immunoassay, the filter paper was directly analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) for a steroid profiling assay for simultaneous analysis of 17-hydroxyprogesterone (17-OHP), 21-deoxycortisol (21-S), cortisol (F), androstenedione (4AS) and 11-deoxycortisol (11-S). Normal values for babies 32 - 36 weeks and/or 1500-2500 gr were; 17-OHP: < 8 ng/mL, 21-S: < 1.5 ng/mL, F: > 50 ng/mL, 4AS: < 4.5 ng/mL. Normal ranges for babies ≥ 36 weeks and/or ≥ 2500 gr were; 17-OHP: < 1.5 ng/mL, 21-S: < 1.5 ng/mL, F: > 50 ng/mL, 4AS: < 4.5 ng/mL. Although all of the steroids were evaluated for each baby; (21S+17-OHP)/F ratio ≥ 0.5 was considered as the main criterion for referral to clinical evaluation for CAH signs and symptoms (**Figure 1**) (**11, 12, 13**).

Reagent, instruments, and analytical conditions for LC-MS/MS

The steroid standards for cortisol, 17-OHP, 21-S, 11-S, androstenedione, and deuterated steroid standards for d4-cortisol, d8-17-OHP, d3-testosterone were purchased from Sigma-Aldrich (MO, USA). Acetonitrile (ACN), methanol, ethanol, Isopropyl alcohol (IPA), formic acid, and LC-MS grade water were purchased from Merck (Darmstadt, Germany).

Detection and measurement were performed on a QTRAP® 5500 tandem mass spectrometer equipped with an Sciex Exion AC LC system (AB Sciex, Concord, Ontario, Canada) that was operated using electrospray ionization (ESI) source in positive and multiple reactions monitoring (MRM) mode. The column used was Phenomenex Kinetex C18, 100 mm $\times$ 2.1 mm, 2.7 μ (Phenomenex, Torrance, CA, USA) that was maintained at 50°C. The mobile phase gradient conditions consisted of water (A) (containing 0.1% v/v Formic Acid in Water) and acetonitrile (B) (containing 0.1% v/v Formic Acid in Acetonitrile). The flow rate was 0.35 mL/min, and the final injection volume of each sample was 20 μ L. All sample extracts were maintained in the autosampler at 4 °C while awaiting injection. The ionization source conditions were as follows: curtain gas (CUR): 25 psi; ion spray voltage (IS): 5500V; temperature: 500 °C; nebulizer gas (GS1): 50 psi and heater gas (GS2): 50 psi. The optimized precursor and product ion pairs, collision energy and retention times for the analytes and internal standards were listed in **Table 1**.

Sample Preparation

In order to obtain calibrators and control DBS, blood from a healthy donor was washed four times with saline to remove all plasma. The washed cells were then combined with steroid-free serum in proportions that resulted in a hematocrit of 0.50. A mix of unlabeled steroid hormones stock solutions of 1 mg/mL in ethanol was diluted in steroid-free serum to obtain 4 points for calibrations and 2 points for controls. Cortisol concentrations were 0, 4.12, 37.0, 333.33 nmol/L in calibrators and 12.3 and 111.1 nmol/L in controls. All other analyte concentrations were 0, 2.1, 18.5, 166.7 nmol/L in calibrators and 6.2 and 55.6 nmol/L in controls. Internal standard stock solutions of 1 mg/mL were prepared in ethanol for all deuterium-labeled steroid hormones and diluted to 40 mmol/L in IPA/ACN.

The blood spots, each 4x3 mm in diameter were punched were punched out of each DBS calibrator, control and sample using a manual puncher into a tube and 500 μ L of internal standard mix was added. The tubes were mixed for 60 min by an orbital shaker. Supernatant was transferred to a 96-well plate and evaporated at 50°C by vacuum centrifuge. 50 μ L of a methanol/water mixture was added to reconstitute the dry residues and 20 μ L injected by limited insert vials.

Ethics

The parents were informed about NBS. Heel-prick blood samples were collected from live-born babies after written consent from the parents were obtained. The study was carried out with the written permission of the Scientific Committee of the TDPH.

Statistical analysis

Statistical evaluation was performed using GraphPad Prism® V5.0 software (GraphPad Software Inc., San Diego, California, USA). The results for each steroid are reported as mean, SD or as median (IQR) in the text. We performed a *t-test* for the comparison of the means of two independent samples. Values were considered statistically significant when *P* value is less than 0.05.

Results

The total number of newborns that underwent CAH screening was 38,935. Of those babies, 33,967 (% 87.2) were ≥ 36 gestational weeks and ≥ 2500 gr birth weight. There were 3,022 babies (7.8%) between 1500-2500 gr birthweight, 3,684 babies (9.5%) born between 32-36 gestational weeks. 1,744 (4.5%) babies were born between 32-36 gestational weeks and had 1500-2500 gr birthweight.

Results of first-tier 17-OHP measurement in DBS of the normal newborn population (those without CAH) are summarized on **Table 2**. We have presented 99.8 and 99.5% of 17-OHP for healthy babies with an effort to define healthy cut-off values with a greater sensitivity (**14**).

2,265 (5.8%) babies had second-tier testing by LC-MS/MS steroid profiling of same DBS. During screening majority of babies born between 32-36 gestational weeks and/or 1500-2500 gr birthweight failed to pass first-tier and required second-tier testing in comparison to those with a birthweight of ≥ 2500 gr and/or gestational age ≥ 36 weeks. (**Table 3**).

Two hundred twelve babies who failed to pass second-tier testing were referred to paediatric endocrinology clinics for further evaluation, which corresponds to an overall recall rate of 0.54%.

Table 4 shows distribution of second-tier testing values of babies referred for further analysis, results are summarized with respect to gestational age and birth weight. The highest proportion of the babies referred to clinics had (21S+17-OHP)/F ratio between 0.5-1.

The babies referred to paediatric endocrinology clinics were evaluated by medical history and physical examination for CAH symptoms and signs. Serum electrolytes were measured, in most of the babies 17-OHP testing was repeated mainly by LC-MS/MS or immunoassay. Based on this evaluation, further biochemical assessments including synacthene test, ACTH, renin and detailed plasma steroid measurements by LC-MS/MS were undertaken when necessary and only for the cases suggestive of CAH. Genetic testing was performed only if the diagnosis of CAH was established by clinical and biochemical findings. Molecular analysis of the *CYP21A2* gene was performed at the diagnostic molecular genetic laboratories of university hospitals of 4 enrolled cities. The *CYP21A2* gene was screened first for the detection of the eight most common mutations [p.P30L, IVS2-13C>G (IVS-2), p.I172N, exon 6 mutation cluster (p.I236N, p.V237E, p.M239K), p.V281L, p.Q318X, p.R356W, 8-bp-deletion], and then of large deletion and conversion by MLPA or allele specific semi-quantitative PCR/enzyme restriction method and sequencing when needed.

Consequently, 6 babies were diagnosed with CAH (four males, two females). Four cases were diagnosed with classical salt-wasting 21-OHD (2 males, 2 females), one male baby had simple virilizing 21-OHD, one male baby had 11-OHD CAH. None of these babies was premature nor had low birth weight. Diagnosis of CAH was verified by molecular analysis of *CYP21A2* and *CYP11B1* genes in five of the cases (**Table 5**). The identified mutations in our patients were among the previously known and common mutations analyzed by Sanger sequencing and heterozygosity of parents was confirmed for the identified mutations. There was no report of a case with salt wasting 21-OHD missed during the period of screening in the screening area.

The estimated incidence of classical 21-OHD CAH in the screened population was 1:7,787. In 206 of 38,935 infants there was a false positive recall rate of 0.52 %.

None of the recalled babies with false-positive results had any clinical signs or symptoms related to CAH. The duration from birth to clinical evaluation of abnormal screening test results of false-positive cases was 25.8 ± 6.4 days (mean $\pm$ SD). We have compared first-tier 17-OHP and (21-S + 17-OHP)/F values of false-positive recalled babies and babies with 21-OHD. Both of these parameters were significantly higher in babies with 21-OHD compared to term babies and ≥ 2500 gr birthweight (n=101) with false-positive screening results (**Table 6**).

Discussion

The purpose of this prospective pilot study was to estimate the incidence of CAH and to assess the characteristics and efficacy of the adopted newborn screening strategy to get some prospects for enhancing screening performance in Turkey. Newborns were screened for CAH in parallel to Turkish National Newborn Screening Programme in four cities during a six-months time period. Data analysis revealed an estimate of the incidence of classical 21-OHD CAH in the screened population as 1:7,787. Data were analysed to revisit the strategy for the upcoming extended newborn screening for CAH in Turkey.

Newborn screening for CAH is universal in the United States (US) (**15**) and many other developed countries (**5, 6**). The incidence of classical CAH is approximately 1:14,000 to 1:18,000 in most populations (**5**). However, it is reported to be more prevalent particularly in populations with high rate of consanguinity. The data from newborn screening for CAH in United Arab Emirates and Saudi Arabia revealed an incidence of 1:9,030 and 1:7,908, respectively for classical CAH (**16, 17**). Our data demonstrated that incidence of classical CAH in Turkey is similar to that of Gulf Arab region. This is most probably due to high rate of consanguinity (overall rate of consanguinity is 22% in Turkey, increasing to 34% in South East Anatolia region) (**18**). Therefore, one may expect an increase in the incidence of homozygous biallelic mutations in our population.

in comparison to compound heterozygotes for two or more different mutant *CYP21A2* alleles. This is indeed the case, 3 of 5 patients identified in the current study were homozygous carriers of biallelic mutations causing classical 21-OHD. Together with the high carrier rate for classical CAH in the general population, which is ~2%, we expect to have relatively higher incidence of classical CAH in Turkey, which we found as 1:7,787. This supports the incorporation of CAH in the core programme of NBS in Turkey.

Screening markedly reduces the time to diagnosis of infants with CAH, which may prevent serious morbidity and mortality as a consequence (19 - 22). A retrospective analysis of neonatal DBS in the Czech Republic and Austria identified 3 genotype-proven cases of classical CAH among 242 samples from cases of sudden infant death that were not screened for CAH (23). Previous studies have reported a death rate of ~10% in infants with salt-wasting CAH without screening (24), but recent estimates from developed countries are lower, 0-4% (25). We had no mortality due to unrecognized classical CAH among screened cohort and only one of the cases had severe hyponatremia at the time of diagnosis. However, the fact that there was no mortality so far, is not adding any safety for the screening program in the long run since it is well known that the crisis may occur at one week of age or even earlier. Furthermore, the initiation of hydrocortisone treatment in our study ranged between 10 to 30 days of life in 4 cases with salt-wasting 21-OHD and the duration from birth to clinical evaluation of abnormal screening test results of false positive cases was 25.8 ± 6.4 days (mean $\pm$ SD). In this regards, our pilot study should be criticised for delayed recall of positive screening results. This delayed recall can partially be explained by our single sample second-tier screening approach. A potential disadvantage of single sample second-tier testing or a second specimen programmes is that the infant would have been symptomatic by the time of second-tier testing or a second specimen was collected and tested. Therefore, a significantly high 17-OHP value in the first-tier should alarm clinician for the suspicion of CAH and the neonates with such elevated 17-OHP levels should be recalled directly. Awaiting a second analysis, even on the same sample may only delay the recall passed the time when the child develops detrimental salt crisis. Furthermore, in cases with markedly elevated first 17-OHP result, a second tier does not necessarily add important information. Indeed, our first-tier 17-OHP results of 21-OHD CAH babies were very significantly higher than that found in 101 recalled term babies with false positive screening results (302.6 ± 357 ng/mL vs 13.61 ± 4.42 ng/mL, $p < 0.0001$) and in 33967 health term babies with normal first-tier results (3.92 ± 2.43).

Another reason for relatively late recall in our screening programme may be thrice-weekly postal service of samples from hospitals to screening laboratory and different location of laboratories for FIA and LC-MS/MS. However, the efforts to reduce the recall time is ongoing for the upcoming extended CAH NBS in Turkey by performing 2 steps of screening in a single central laboratory so that second-tier testing can be performed same day upon positive first screening. Moreover, the filter paper samples will be collected from hospitals every day and sent to screening laboratory by regular daily postal service. Nevertheless, since three of five cases with classical 21-OHD diagnosed through our pilot screening were male, and thus without ambiguous genitalia, it is safe to say that the diagnosis and treatment would be delayed much more without screening. What's more, the diagnosis and treatment of the two male cases with simple virilising 21-OHD and 11-OHD would likely even be later.

Another weak point of the single sample second-tier screening is high false negative rate compared to second sample testing particularly to diagnose the classical CAH cases with delayed rise in 17-OHP levels. The Minnesota program who has the longest experience in using a single sample two-tier screening algorithm with steroid profiling by LC-MS/MS as the second tier in specimens that exceeded the first-tier 17-OHP cut-off (26). If the second tier test results were negative further follow-up of the child was considered unnecessary. They have evaluated their 11 years of experience on screening and came to the conclusion that the overall false negative rate doubled with their two-tiered algorithm. This is highlighted by the finding that seven missed cases were not tested by LC-MS/MS because their first-tier 17-OHP values were within range, and four more were missed by the second tier testing after initial abnormal screening values (26). Our single sample, two-tier screening may still have a similar risk to unidentified some CAH cases with delayed rise in 17-OHP. Therefore, physicians should have a high level of suspicion in patients presenting with signs and symptoms of CAH even if they have false-negative screening results. In view of the high false negative associated with a single newborn screen two-tier approach, some programs have opted to collect and screen a second specimen as an alternative means of improving the results of CAH screening. However, this may further struggle the screening. For example, the Colorado screening program collects the sample before 3 days of life, which may be the main reason for their high false negative rate. Therefore, this program has routinely obtained a second specimen, 1-2 weeks after birth for repeat screening, which was reported to further complicate the screening due to long time for recall (27).

We have also questioned our high recall rate during NBS for CAH in comparison to previous studies (6). Recall rate was reported between 0.002-1.2 %, generally <0.5% in many developed countries with long established screening programs for CAH (6). Furthermore, such low recall rates are reported in the course of single tier DBS screening. We could achieve a recall rate of 0.54%, in the face of a higher cost adopting single DBS-two-tier screening approach. This can be explained by the lower cut-off values we

used for the first step 17-OHP FIA measurements as well as second step (21-S+17-OHP)/F ratio to increase the sensitivity of this pilot study. The lower cut-off values have the advantage of increasing screening sensitivity with a markedly increased risk of higher false positive rate, higher cost and higher likelihood of unnecessary treatment of non-classic CAH cases, and even of non-CAH healthy babies. Further analysis of our data suggests that 99.8th percentile of FIA based 17-OHP levels in our healthy population (excluding the babies with SW 21-OHD) is 50 ng/mL for 1500-2500 gr and 32-36 gw babies, and is 20 ng/mL for ≥ 2500 gr and ≥ 36 gw babies. When these 17-OHP levels were used as cut-off values in the first-tier we would have expected 253 babies failing to pass, which corresponds to only 11% of population undergoing the second-tier test. Likewise, 161 of 212 babies (75%) had (21-S+17-OHP)/F ratio < 1 in the second-tier testing while this ratio ranges between 4.6-32.4 in classical SW 21-OHD cases. Even in the single case with SV 21-OHD, this ratio was 1.31. Therefore, if we would have used 1 as the cut-off for (21-S+17-OHP)/F ratio, recall rate would decrease by 75%. This observation is similar to Janzen N, et al. who analyzed around 8000 retrospective and prospective DBS samples for (21-S+17-OHP)/F ratio in order to compare healthy newborns (including preterms) with 66 CAH cases. None of the cases with CAH had a (21-S+17-OHP)/F ratio < 1 (11). Analysis of data from the current study helped us to revisit and modify the screening strategy for the upcoming extended NBS screening for CAH in Turkey. It emerges that above-mentioned cut-off values may enable less labour intensive, and more efficient screening strategy for CAH with a better cost-benefit profile.

Immunoassays of DBS for 17-OHP are the most widely used and least costly initial screening methods for CAH. However, poor antibody specificity in addition to abundant cross-reacting hormones in the newborn circulation as well as necessity of variation in the cut-offs with respect to gestational ages (28) and/or birthweight (29) limit their use in the detection of CAH. Furthermore, stress due to prematurity or critical illness generally increases adrenal cortisol and 17-OHP secretion, which further hamper interpretation of screening results for CAH. Therefore, using 17-OHP as the sole marker may increase the recall rate as well as likelihood of false positive healthy infants who may be started on glucocorticoids unnecessarily. Employing LC-MS/MS based steroid panel appears as an effective second-tier screen that would better separate false positives, avoid false negatives and potentially save a great deal in unnecessary healthcare expenditures, which subsequently relieves much of the stress and work burden experienced by health professionals and parents with quite confusing immunoassay results (11, 13, 30-32). We adopted a modified LC-MS/MS protocol developed by Janzen N, *et al* that utilized a ratio of the sum of 17-OHP and 21-deoxycortisol levels, divided by the cortisol level as second-tier screening test (11). This protocol was reported to identify all affected children with no false positives, for a positive predictive value of 100% (11). Particularly 21-deoxycortisol which is produced by 11 β -hydroxylation of 17-OHP is not expected to be secreted in large amounts even in preterm infants, and thus elevated levels are highly specific for 21-OHD (33, 34).

It is encouraging that simultaneous measurement of 17-OHP, 21-S, and cortisol by LC-MS/MS increased the positive predictive value of our CAH screening 9-fold over that for 17-OHP FIA alone. This pilot study also measured 11-S in addition to 17-OHP, 21-S, cortisol and 4AS, which is specifically diagnostic for 11-hydroxylase deficiency. Hence, with our tandem mass spectrometry method, it was possible to detect a male newborn with (later genotype-proven) classical 11-OHD in addition to cases with 21-OHD. To our knowledge, this is the first patient with 11-OHD identified directly during NBS for CAH. Therefore, the method of steroid profiling has a potential to distinguish other rare forms of classical CAH, beyond 21-hydroxylase deficiency more efficiently. Even though the hormones measured in LC-MS/MS based panels are not specifically diagnostic for the rare forms of CAH such as 11-hydroxylase or 3 β -hydroxysteroid dehydrogenase type II deficiency, perturbations in simultaneous steroid measurements would provide preliminary information suggesting the need for further evaluation (35, 36). In fact, these apparently “rare” forms of classic CAH are far more common in the Middle East and in Turkey, due to high rate of consanguinity (37, 38).

In conclusion, this pilot study suggests that incidence of CAH in Turkey may be higher than previous reports. Hence, it may be recommended to include CAH due to 21-OHD in the screening panel for Turkish newborn screening program. Employing current LC-MS/MS based steroid panel as second-tier testing may be expected to reduce the time to diagnosis of infants with 21-OHD CAH. It may also enable detection of rare forms of classical CAH. However, further efforts are needed in our CAH screening programme for earlier clinical recall of babies with positive NBS tests which is critically important to prevent salt loss and to shorten the period of unclear sex in the classical CAH cases. Prospective analyses of screening strategy, cut-off values and results would aid to increase the sensitivity, reduce the false positive rate of screening which subsequently alleviates the medical, psychological and economic burden of CAH and its associated health problems.

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Figure 1. Flowchart for pilot neonatal CAH screening initiated by the Turkish Directorate of Public Health. Abbreviations: FIA= fluoroimmunoassay, LC-MS/MS= liquid chromatography-tandem mass spectrometry, 17-OHP= 17-hydroxyprogesterone, 21-S= 21-deoxycortisol, F= cortisol, 4AS= androstenedione and 11-S= 11-deoxycortisol. (17-Hydroxyprogesterone (17-OHP) conversion factor from ng/mL to nmol/L: Multiply by 3.02)

Table 1. Multiple reaction monitoring functions and settings for detecting steroids by LC-MS/MS

	Precursor (m/z)	Dwell time (msec)	Product (m/z)	Collision energy (eV)	Retention time (min)
17-Hydroxyprogesterone	331	100	109	43	3.8
d8-17-Hydroxyprogesterone	339	100	112	44	3.8
Cortisol	363	100	121	30	2.8
d4-Cortisol	367	100	121	30	2.8
21-Deoxycortisol	347	100	97	30	3.1

d3-Testosterone	292	100	97	30	3.6
Androstenedione	287	100	109	30	3.3
11-Deoxycortisol	347	100	97	27	3.3

Table 2. FIA based 17-OHP values of screened population according to birth weight and gestational age.

17-OHP (ng/mL) [nmol/L]	1500-2500 gr (3,022)	≥ 2500 gr (35,907)	32-36 gw (3,684)	≥ 36 gw (35,245)	32-36 gw+ 1500-2500 gr (1,744)	≥ 36 gw + ≥ 2500 gr (33,967)
(n)						
Mean (SD)	8.29 (8.68) [25.08 (26.2)]	4.07 (2.75) [12.3 (8.3)]	8.60 (8.27) [26.02 (25.0)]	3.96 (2.53) [11.9 (7.6)]	10.80 (10.11) [32.6 (30.5)]	3.92 (2.43) [11.8 (7.3)]
Min-max	0.10-137.30 [0.3-415]	0.05-56.63 [0.15-171]	0.11-137.30 [0.33-415]	0.05-57.66 [0.15-174]	0.11-137.30 [0.33-415]	0.05-56.63 [0.15-171]
Median	5.33 [16.1]	3.53 [10.6]	5.92 [17.9]	3.48 [10.5]	7.36 [22.2]	3.47 [10.5]
IQR (25- 75%)	3.40-9.96 [10.2-30.1]	2.49-4.89 [7.5-14.7]	3.84-10.52 [11.6-31.8]	2.47-4.80 [7.4-14.5]	4.58-13.72 [13.8-41.5]	2.47-4.77 [7.4-14.2]
99.5%	50.80 [17.5]	18.05 [54.6]	49.99 [151]	16.71 [50.5]	58.27 [176]	15.97 [48.1]
99.8%	63.64 [191]	23.48 [71]	59.95 [181]	21.38 [64]	77.21 [233]	20.21 [61]

Abbreviations: 17-OHP= 17-hydroxyprogesterone, FIA= fluoroimmunoassay
SI units are given in brackets.

Table 3. Rate of second-tier testing among babies based on birth weight and gestational weeks

	1500-2500 gr	≥ 2500 gr	32- 36 gw	≥ 36 gw	32-36 gw + 1500-2500 gr	≥ 36 gw + ≥ 2500 gr
Number of babies	3,022	35,907	3,684	35,245	1,744	33,967
Second-tier testing (number; %)	(722; 24)	(1,543; 4)	(973; 26)	(1,292; 4)	(607; 34)	(1,117; 3)

Table 4. Distribution of babies based on (21S+17-OHP)/F ratio adjusted for gestational age and birth weight

(21S+17-OHP)/F ratio	1500-2500 gr	≥ 2500 gr	32- 36 gw	≥ 36 gw	32-36 gw + 1500-2500 gr	≥ 36 gw + ≥ 2500 gr
0.5-1.0	54	107	68	93	45	84
1.0-2.0	19	20	22	17	18	16
2.0-5.0	8	1	9	0	8	0
> 5.0	1	2	2	1	1	1
Total (n)	82	130	101	111	72	101

Abbreviations: 21-S= 21-deoxycortisol, 17-OHP= 17-hydroxyprogesterone, F= cortisol

Table 5. Clinical characteristics and laboratory details of the patients with CAH diagnosed through NBS

Ca se No	Karyotype	Birth weight (gr)/ Gestation al week	17- OHP by FIA (ng/mL)	Second-tier testing by LC-MS/MS (ng/mL)	Day of treatme nt initiation	Blood biochemistry at diagnosis		Diagno sis	Molecul ar defect
1	46, XX	3290/38	137.3	17-OHP	10 th day	Na (mEq /L)	132	21- OHD (SW)	<i>CYP21A</i> 2 cluster E6 (c.707T> A, c.710T> A, c.716 T>A) homozyg ous
				21-S					
				F		K (mEq /L)	6.1		
				(21-S + 17- OHP)/F		17- OHP (ng/ mL)	12.5		
				4AS					
				11-S					
2	46, XX	3139/38	137.3	17-OHP	28 th day		113		ND

				21-S	36.52		Na (mEq /L)		21- OHD (SW)	
				F	9.21		K (mEq /L)	6.5		
				(21-S + 17- OHP)/F	32.47		17- OHP (ng/ mL)	>128		
				4AS	23.10					
				11-S	0.83					
3	46, XY	2900/38	96	17-OHP	908.4 5	21 st day	Na (mEq /L)	137		CYP21A 2 IVS2- 13C>G (c.293- 13C>G) homozyg ous and CYP21A 2 p.Q319X (c.955C> T) homozyg ous
				21-S	0.49		K (mEq /L)	6.6	21- OHD (SW)	
				F	41.27		17- OHP (ng/ mL)	>20		
				(21-S + 17- OHP)/F	22.02					
				4AS	346.9					
				11-S	27.84					
4	46, XY	3200/39	96	17-OHP	58.73	30 th day	Na (mEq /L)	NA		CYP21A 2 IVS2- 13C>G (c.293- 13C>G) and p.R357W (c.1069C >T) Compound heterozyg ous
				21-S	0.02		K (mEq /L)	NA	21- OHD (SW)	
				F	12.69		17- OHP (ng/ mL)	>20		
				(21-S + 17- OHP)/F	4.62					
				4AS	6.83					
				11-S	3.84					
5	46, XY	2950/39	44.29	17-OHP	19.66	19 th day	Na (mEq /L)	134	21- OHD (SV)	CYP21A 2 p.I172N
				21-S	0.05		K	5.7		
				F	14.99					

				(21-S + 17-OHP)/F	1.31		(mEq/L)			(c.518 T>A) homozygous
				4AS	1.53		17-OHP (ng/mL)	47.2		
				11-S	0.62					
6	46, XY	3250/36	16.33	17-OHP	4.82	71 th day	Na (mEq/L)	137	11-OHD	CYP11B1 p.Asn394 ArgfsX377 (c.1180_1181insGA) and p.Phe487 Cys (c.1460T>G) Compound heterozygous
				21-S	0.002		K (mEq/L)	5.3		
				F	31.31					
				(21-S + 17-OHP)/F	0.15					
				4AS	52.33		17-OHP (ng/mL)	NA		
				11-S	113.83					

Abbreviations: NBS= Newborn screening, FIA= fluoroimmunoassay, LC-MS/MS= liquid chromatography-tandem mass spectrometry, 17-OHP= 17-hydroxyprogesterone, 21-S= 21-deoxycortisol, F= cortisol, 4AS= androstenedione and 11-S= 11-deoxycortisol, 21-OHD=21-hydroxylase deficiency, 11-OHD= 11 β -hydroxylase deficiency, SW=salt wasting, SV=simple virilising, NA=Not available, ND=Not done. Conversion factors to SI units: 17-OHP, ng/mL $\times$ 3.02 $\rightarrow$ nmol/L; 4AS, ng/mL $\times$ 3.49 $\rightarrow$ nmol/L; F, ng/mL $\times$ 27.5 $\rightarrow$ nmol/L; 11-S, ng/mL $\times$ 2.88 $\rightarrow$ nmol/L.

Table 6. Comparison of first-tier 17-OHP levels and second-tier (21-S + 17-OHP)/F ratios between the 206 false-positive healthy recalled infants and 5 infants with 21-OHD.

False-positive healthy recalled babies							21-OHD CAH babies	p value*
	1500-2500 gr	$\geq$ 2500 gr	32- 36 gw	$\geq$ 36 gw	32-36 gw + 1500-2500 gr	$\geq$ 36 gw + $\geq$ 2500 gr	$\geq$ 36 gw + $\geq$ 2500 gr	
(n)	(82)	(130)	(101)	(111)	(72)	(101)	(5)	
First-tier 17-OHP (ng/mL)								
[nmol/L]								
Mean $\pm$ SD	29.52 $\pm$ 18.68 [89.3 $\pm$ 56.5]	15.11 $\pm$ 6.52 [45.7 $\pm$ 19.7]	28.00 $\pm$ 17.73 [84.7 $\pm$ 53.6]	14.02 $\pm$ 4.69 [42.4 $\pm$ 14.1]	31.09 $\pm$ 19.33 [94 $\pm$ 58.4]	13.61 $\pm$ 4.42 [41.1 $\pm$ 13.3]	302.6 $\pm$ 357 [915 $\pm$ 1080]	<0.0001

Median (IQR)	24.9 (18-35) [75 (54-105)]	12.8 (10.3-17) [39 (31-51.5)]	24.2(16-33.5) [73 (49-101)]	12.5 (10.3-16) [38 (31-48)]	27 (18.5-35) 82 (56-106)]	12.3 (10-15.3) [37 (30-46)]	262 (39-586) [793 (118-1773)]	
99.5%	117.8 [356]	39.8 [120.4]	112.8 [341]	28.9 [87.4]	120 [363]	27 [81.7]		
99.8%	129 [390]	45 [136]	127.5 [386]	29.8 [90.1]	130.3 [394]	27.5 [83.2]		
(21S+17-OHP)/F ratio								
Mean±SD	1.38±2.92	1.01±1.81	1.49±3.24	0.85±0.68	1.47±3.11	0.85± 0.70	14.1±12.94	
Median (IQR)	1.38 (0.7-1.1)	0.7 (0.6-0.9)	0.8 (0.65-1.2)	0.66 (0.6-0.85)	0.9 (0.7-1.15)	0.65 (0.6-0.86)	10.1 (3-27.2)	<0.0001
99.5%	17.9	11.6	23.3	4.2	19	4.5		
99.8%	23.17	16.64	25.35	5.89	23.61	5.99		

Abbreviations: 17-OHP= 17-hydroxyprogesterone, 21-S= 21-deoxycortisol, F= cortisol, 4AS= androstenedione and 11-S= 11-deoxycortisol, 21-OHD=21-hydroxylase deficiency

* *p* values indicate the comparison of the parameters in babies with 21-OHD with the term and ≥ 2500 gr birthweight babies (n=101) with false-positive second-tier screening results.

SI units are given in brackets.

Uncorrected proof