

# NEWBORN SCREENING FOR PHENYLKETONURIA AND CONGENITAL HYPOTHYROIDISM: RESULTS FROM CLUJ CENTER, 2011-2015

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## ABSTRACT

**Objectives.** The aim of our study was to assess the current state of newborn screening (NBS) for two innate errors of metabolism, phenylketonuria (PKU) and congenital hypothyroidism (CH) in the north-west part of Romania, in Cluj regional center (CJRC), one of the five screening centres in the country, responsible for screening from 7 counties.

**Material and methods.** The transversal descriptive study is based on data obtained from the screening of 101,739 newborns born between 2011-2015. The screening was performed for PKU and CH based on dry blood spot (DBS) collected on standardized filter paper. The concentration of phenylalanine (Phe) and thyroid stimulating hormone (TSH) in the blood was measured through a fluorometric assay. Newborns with abnormal screening results (TSH > 18 µUI/ml and Phe > 3 mg/dl) were confirmed with CH by the dosage of free thyroxine (fT4) and with PKU or hyper-phenylalaninemia (HPA) by plasma phenylalanine determination (thin-layer chromatography method coupled with densitometry video) respectively.

**Results.** We detected 10 cases with PKU (~ 1/10.000), 6 cases with (HPA), and 10 cases with CH, all being treated and none of them developed neurologic disorders. From a total of 115,779 newborns, 101,739 were screened (87.87%) while 14,040 were not tested (12.13%). The most frequent mutation in confirmed cases was R408W.

**Discussions.** Counties where screening program was deficient are: Maramures 4,811 (20.26%), Bihor 3,349 (11.33%) and Sibiu, with 6,537 (30.65%) not tested newborns. In Sibiu there are maternities where the screening program is not applied.

**Conclusions.** The high number of unscreened newborns opens the possibility of un-reversible neurologic disorders. Better organization of sampling and transportation, a correct feed-back from confirmed cases and governmental financing of the genetic analysis would increase the efficiency of the program. The number of confirmed cases claims for the future development of the program with the introduction, eventually, of other rare congenital diseases.

**Keywords:** newborn screening, phenylketonuria (PKU), congenital hypothyroidism, hyperphenilalaninemia

## INTRODUCTION

Newborn screening (NBS) programs using DBS were first developed in the 1960s, inspired by the work of Dr Robert Guthrie. Currently, NBS-PKU-CH is a well established practice in most developed countries worldwide, while it is less uniformly implemented in developing countries, which represent most of the southeastern Europe (1).

In the north-west of Romania, the implementation of NBS-PKU program started in 1 December

1978 in Cluj addressing only to newborns from Cluj county. Other counties were introduced in the NBS-PKU between 1995 and 2000: Cluj, Bihor, Salaj, Maramures and Sibiu, with a total of 25 maternities. In year 2000 screening for CH was added to the NBS-PKU program. For a short period of time (3 years), maternities from Hunedoara and Harghita counties were also sending samples to CJRC. Starting with 1 January 2015, 3 counties were transferred from Bucharest to Cluj centre

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(Harghita, Salaj and Satu-Mare). At the date, CJRC is performing the NBS-PKU-CH on samples received from 35 maternities located in 7 counties. Romanian national PKU registry was established in 2011.

Results from one survey and complementary studies (2,3) highlight that, although newborn screening is widespread, it is not yet realized on all neonates. The studies were concluding that in some countries in Europe with established NBS-PKU-CH, including Bulgaria and Romania, up to 10% of the newborns are not screened (1). In the last 4 years, the number of newborns which escape screening decreased from the rate previously published at national level. (4).

## OBJECTIVES

The aim of this study is to present the results of the program for rare diseases, NBS-PKU-CH, at the level of CJRC: number and percentage of screened vs. unscreened neonates per county and per year; the rate of positive cases at screening and the rate of confirmed cases of PKU, HPA (hyperphenylalaninemia) and CH in each county. In the same time we present the problems we faced in the organization and implementation of this program at the level of our center.

## METHODS

The study is a transversal descriptive one. Material is represented by cohorts of the newborns screened between 2011-2015 from the maternities situated in 4 counties in the north-west part of the country (Cluj, Bihor, Maramures, Sibiu), and from 3 other counties transferred to our center starting with 1 January 2015 (Satu-Mare, Harghita and Salaj). The number of tested newborns from these maternities was 101,739.

The panel of congenital disorders screened includes two diseases: PKU and CH.

The methods performed in our center are the ninhydrin method for PKU and time-resolved fluorescence (a fluoroimmunoassay) for TSH (thyroid stimulating hormone).

The assays are performed semi-automatically with a dedicated line that uses an automatic puncher, microplate incubator shaker, washer and a fluorescence reader (Anthos Labtec Instruments, Salzburg – Austria, Zenyth 3100 Multimode detector). The reader is using a time-resolved fluorescence (TRF) measurement for the quantification of TSH and top fluorescence intensity for PKU. The meas-

ured fluorescence units are transferred to the computer on-line and calculated in terms of concentrations.

The ninhydrin method for PKU is based on the enhancement of the fluorescence of a phenylalanine-ninhydrin reaction product by the dipeptide L-leucyl-L-alanine. The method measures phenylalanine quantitatively in the presence of other amino-acids, using a 390 nm wavelength for excitation and a 486 nm wavelength for emission (Neonatal Phenylalanine NP-1000/NP-4000).

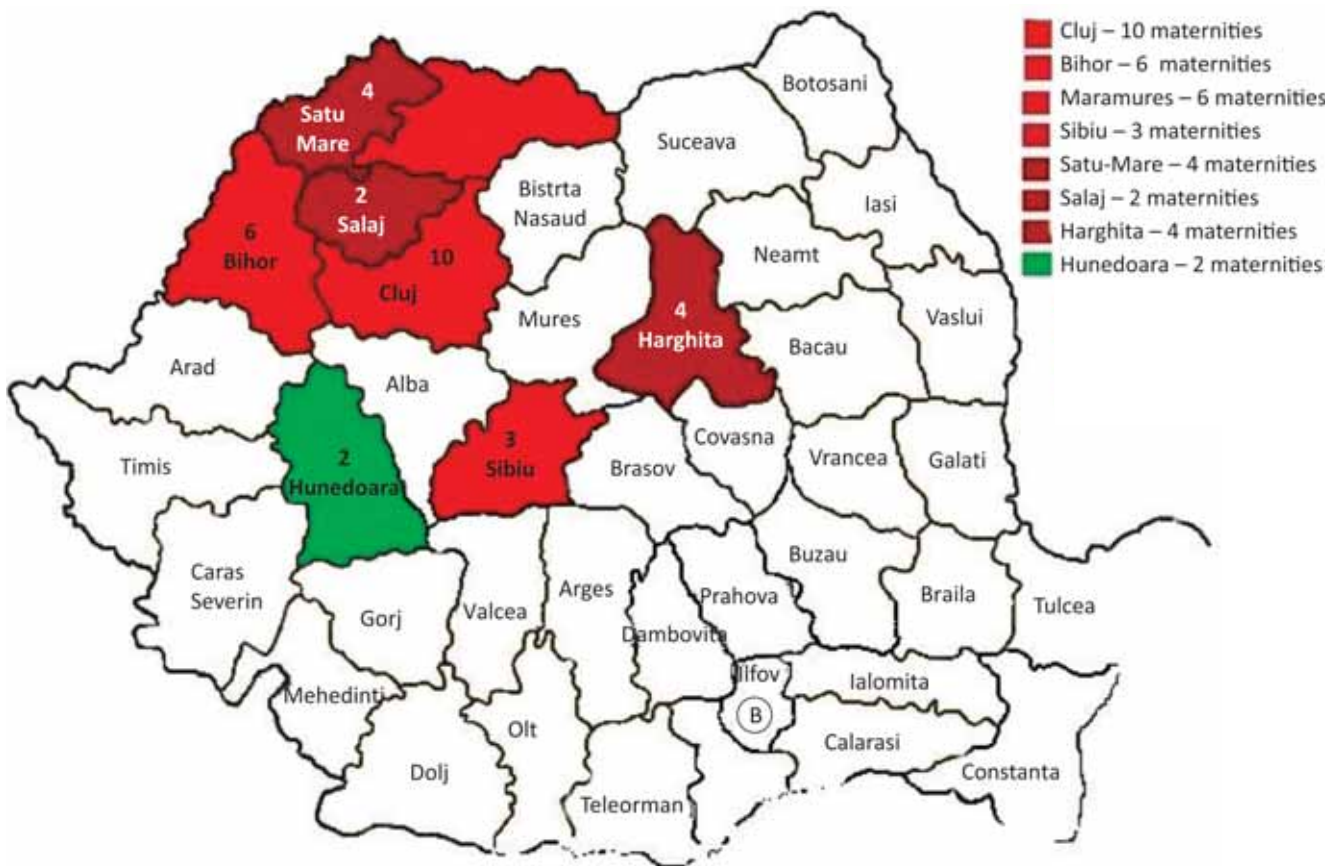
The fluoroimmunoassay for TSH is a two-site fluoroimmunoassay based on direct sandwich technique in which two monoclonal antibodies (derived from mice) are directed against two separate antigenic sites of the hormone (DELFLIA Neonatal hTSH). Standards, controls and test specimen containing hTSH are reacted simultaneously with immobilized monoclonal antibodies directed against a specific antigenic site and europium-labeled monoclonal antibodies (directed against a different antigenic site located partly on the  $\beta$  subunit). Europium ions from the labeled antibody are dissociated in a second step and they form highly fluorescent chelates. The fluorescence in each well is then measured. The fluorescence of each sample is proportional to the concentration of TSH in the sample (5,6).

In order to detect abnormal screening results cut-off values are used, which distinguishes between euthyroid and hypothyroid neonates (borderline 9-18  $\mu\text{U/mL}$ , hypothyroid  $> 18 \mu\text{U/mL}$  (7) while for PKU the borderline samples are those between 2.1-3 mg/dl and presumptive positive samples are those exceeding 3 mg/dl.

Patients with borderline and hypothyroid values should immediately be recalled for confirmatory assays. The confirmation is made by clinic examination followed by the dosage of serum free fT4 and again TSH for CH, and by the dosage of plasma phenylalanine from blood (5 ml) collected on heparin, 3 hours after the last meal of the baby, through a thin-layer chromatography method coupled with densitometry video. Patients with level of phenylalaninemia lower than 8 mg/dl at the confirmation assay are diagnosed with hyperphenylalaninemia (HPA), not classic PKU.

## RESULTS

In present, the NBS-PKU-CH is conducted in 35 maternities from 7 counties: Cluj, Bihor, Maramures, Sibiu, Satu-Mare, Salaj, and Harghita (Fig. 1).



**FIGURE 1.** The chronology of NBS introduction for PKU and CH in north-west part of Romania, 1978-2015

1978 – Cluj

1995-2000 – Cluj, Bihor, Salaj, Maramures and Sibiu

2008-2010 – Cluj, Bihor, Maramures, Sibiu, Hunedoara and Harghita

2011-2014 – Cluj, Bihor, Maramures and Sibiu

2015 – Cluj, Bihor, Maramures, Sibiu, Satu-Mare, Salaj, Harghita

From a total of 115,779 newborns, 101,739 were screened (87.87%) and 14,040 were not tested (12.13%). The sources for the total number of newborns in every county were Counties' Departments of Public Health (CDPH = DSP) (ro). The number and rate of tested and untested newborns per county and per year is presented in Table 1 and 2.

From the total number of newborns 227 were positive at screening for PKU. These newborns advance for confirmation assays. Letters were sent to parents in order to bring the child for clinical and biochemical evaluation and confirmatory assays. PKU was confirmed in 10 newborns, while 6 newborns had lower values of phenylalanine and were

**TABLE 1.** Distribution of screened vs. unscreened newborns per county

County	Total newborns	Newborns tested	Rate of tested %	Newborns untested	Rate of untested %
Sibiu	21,329	14,792	69.35	6,537	30.65
Maramures	23,745	18,934	79.74	4,811	20.26
Bihor	29,548	26,199	88.67	3,349	11.33
Cluj	32,693	34,449	*	-	*
Satu-Marea	3,490	2,522	72.26	968	27.74
Harghita	3,041	2,949	96.97	92	3.03
Salaj	1,933	1,894	97.98	39	2.02

\* from several counties mothers came to give birth in Cluj, while children are registered on the ID card of parents in the county of residence and they were reported accordingly (see discussions)

a,b,c – counties were transferred from Bucharest centre starting with 1 January 2015

**TABEL 2.** Distribution of screened vs. unscreened newborns per year

Year	Total newborns	Newborns tested	Rate of tested %	Newborns untested	Rate of untested %
2011	20,580	19,320	93.87	1,260	6.13
2012	21,119	17,988	85.17	3,131	14.83
2013	21,029	18,328	87.15	2,701	12.85
2014	21,987	19,128	86.99	2,859	13.01
2015	34,554	26,975	78.06	7,579	21.94
TOTAL	115,779	101,739	87.87	14,040	12.13

diagnosed with HPA. The confirmation rate was  $16/227 = 7.05\%$  for PKU and HPA. The incidence for PKU was approximately 1/11.600 newborns but it almost doubles if we also include the cases with HPA (6).

The cut-off values for CH were over-passed by 242 newborns and CH was confirmed in 10 of them. (Table 3).

There was no significant difference between the number of confirmed cases/county as shown in Table 4.

**TABLE 3.** The rate of confirmed cases out of positive ones tested by screening

Congenital diseases	Positive cases at screening	Confirmed cases	Rate of confirmed cases %
PKU	227	10	4.40
HPA	227	6	2.64
CH	242	10	4.13

**TABLE 4.** Confirmed cases per county

County	PKU	HPA	CH
Cluj	2	4	10
Bihor	4	0	*
Maramures	4	2	*

\* no feedback about confirmation

## DISCUSSIONS

This is the first study to assess the PKU and CH screening for a period of five years in 7 counties with a total of 35 maternities. In Romania there are five centers for NBS-PKU-CH situated in: Bucharest, Cluj-Napoca, Timisoara, Iasi and Targu-Mures. The large number of centers compared with other countries in Europe (one or two) is explained by the higher number of newborns encountered in Romania compared with all the other countries in the region (1).

The screening test is free of charge for the family, the cost per test ( $2,5 \times 2 = 5$  euro) being provided from the budget Ministry of Health within a National Health Program.

The confirmation of cases is made in other laboratory within the ECCHC.

The management and follow-up of confirmed cases is realized in the Pediatric Hospital. There are at the moment 36 pediatric patients with PKU (some diagnosed before 2011) receiving dietary treatment and being followed by the consultant specialist in Pediatrics. The majority of these patients are sending DBS weekly or once a month.

The most critical concern is the incomplete screening of newborns from several counties. The percentage of unscreened newborns in CJRC is 12.13%, which show an improvement since last reported data from south-eastern Europe in 2014 and 2015 (1,2). These newborns may face a delayed diagnosis. There are private laboratories offering a wide range of assays for the early diagnosis of congenital disorders to which parents may address independently. Some of the families communicate at the center that they have had the assay performed and the results, but others don't and we report their children as 'untested'. Even though we do not have a feedback about the number of the newborns tested by these laboratories, the newborns diagnosed will be referred to the dietary management and follow-up within the national program, except for those with HPA where the enzymatic impairment is minor and they do not need dietary treatment. For the best of our knowledge in our regional center there is no case diagnosed between 2011-2015 from other source than those within the National Program (Screening Laboratory and Genetic Laboratory explorations of the ECCHC).

Another important issue is to make a correspondence between the place of birth and testing (which might be Cluj for pregnant women residing in other counties) and the place where the birth is reported by CDPH. This inadvertence lead to the situation reported in Table 1 for Cluj county, where the number of newborns is smaller than the number of tested ones. In the same time we may speculate that the difference of newborns (1,756) tested in Cluj may be part of the newborns classified as un-



tested in their residence county (e.g. Sibiu with 6,537 (30.65%), Maramures with 4,811 (20.26%) and Bihor 3,349 (11.33% – Table 1). Nevertheless the organization of the program at national and county level should be improve because at the moment there are maternities in Sibiu which do not apply the screening program (~ 830 newborns/year).

For the improvement of the communication and reports within the NBS-PKU-CH we propose that any laboratory (state or private ones) should report to the center, screening or confirmatory assays performed for the diseases covered by the national program.

The quality of the samples received by the screening laboratory is another concern. The bad quality of 5.40% of samples is due to incorrect sample collection/transport (excessive milking, not uniformly saturated, poorly collected-smaller spots, not separated during transport) that leads to false-positive results and increases the number of confirmatory assays.

Detection of genetic abnormalities in the diagnosed cases is not covered by the national program. Parents may address to private laboratories for testing, but the cost of the assay is prohibitive for a large number of families (598-700 €). Still we have feedback regarding the genetic aspect from three

out of 16 cases. All cases had R408W mutation, the most common mutation associated with PKU in our population (8,9) and one of them had S350Y mutation, of paternal origin, for which no phenotypic association is known.

## CONCLUSIONS

A high number of unscreened newborns open the possibility of occurrence of unreversible neurologic disorders. All maternities in allocated counties to a center should collect and send samples to the screening laboratory.

The absence of feedback from cases eventually screened/confirmed in other laboratories do not allow a proper management of data regarding the efficiency of the screening program.

Proper organization of the sample collection and transport to the laboratory will allow earlier diagnosis (screening and confirmation).

Introduction of the financing for the genetic analysis in the screening and management program will allow a better management of the cases in our geographical area.

The number of confirmed cases claims for the future development of the program with introduction, of other rare congenital diseases.

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