Newborn screening for congenital hypothyroidism: the benefit of using differential TSH cutoffs in a two-screen program

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The authors have nothing to disclose

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ABASTRACT

Context. Analysis of a two-screen program for congenital hypothyroidism (CH) using differential blood TSH (bTSH) cutoffs of 10 mU/L at first screening (all infants) and 5 mU/L at second screening (selected infants).

Objectives. To characterize CH infants identified by the second screening and compare infants with bTSH 5.0-9.9 and ≥10 mU/L on second screening.

Design, Patients. Maternal and neonatal clinical features were retrospectively analyzed in 119 CH babies detected on the second screen in Lombardy region of Italy, 2007-2014.

Results. Fifty-two (43.7%) of the 119 CH neonates showed bTSH values ranging 5.0-9.9 mU/L at the second screening (Low bTSH Group) and 67 (56.3%) bTSH ≥10.0 mU/L (High bTSH Group). The frequency of thyroid dysgenesis and eutopic gland was similar in both groups, as was the frequency of permanent and transient CH. Moreover, a high frequency of extra-thyroidal malformations was found in both groups. The percentage of preterm infants (57.7% *vs* 23.9%, P<0.001) and infants admitted to NICU (50.0% *vs* 17.9%, P<0.001) was significantly higher in the Low versus the High bTSH Group. In addition, maternal treatment with glucocorticoids in pregnancy was significantly more frequent in the Low bTSH Group than in the High bTSH Group (11.5% *vs* 1.5%, P=0.042), as well as maternal hypothyroidism and/or goiter (26.9% *vs* 10.4%, P=0.036).

Conclusions. This study has demonstrated that a lower TSH cutoff at the second screening can detect additional cases of CH and that a second bTSH cutoff of 5.0 mU/L is appropriate for identifying preterm newborns and babies with associated risk factors.

Precis (200 characters included spaces)

Differential TSH cutoffs in a two-screen program for CH can detect additional cases, including infants with thyroid dysgenesis and permanent CH.

Key words

Congenital hypothyroidism, newborn screening, TSH, cutoff

INTRODUCTION

Newborn screening for congenital hypothyroidism (CH) allows the early identification and treatment of affected infants, leading to the virtual disappearance of moderate or severe intellectual disability due to neonatal hypothyroidism. The measurement of dried blood spot TSH (bTSH) is recommended as the most sensitive screening test to detect primary CH in the first days of life (1). Since the introduction of newborn screening in the mid '70s, TSH cutoff values have been progressively lowered in many screening programs (2-8). The lowering of TSH screening cutoffs together with other factors, such as the increased survival rate of preterm babies, changes in screening population demographics, and the introduction of re-screening for newborns at risk of delayed TSH rise have led to an increased incidence of CH (9-10).

To avoid missing cases among neonates at risk of delayed increase in TSH, European guidelines recommend repeat sampling at 2 weeks of life or 2 weeks after the first screening test was carried out in the following situations: preterm birth (<37 week's gestation), low birth weight (LBW) and very low-birth weight (VLBW) neonates, ill and preterm newborns admitted to neonatal intensive care units (NICU), specimen collection within the first 24 hours of life, and multiple births (1). This approach reflects concern that primary CH may be masked in babies with hypothalamo–pituitary–thyroid axis immaturity (11), medications such as glucocorticoids and dopamine (12-13), iodine-containing-drugs (14), recovery from euthyroid sick syndrome in severely ill neonates (14-15), and multiple births (16-18). Several retrospective studies have examined the impact of a repeat screening strategy using different detection methods, timing, and study populations (19-26). However, only a few of these studies have reported data on differential TSH cutoffs at the first and the second screening (3, 20, 25).

After delivery, serum TSH concentrations in term infants increase to a mean of approximately 80 mU/L at 30 minutes of life and then decrease rapidly over the next two

days (27). The serum TSH concentration is usually less than 10.0 mU/L by the end of the first week, and is usually within the normal range for children and adults by two weeks of age (28-29). Although this physiological pattern informs the timing of age-specific cutoffs for TSH concentrations within the first weeks of life, there is no consensus on the use of differential TSH cutoffs at the first and second screening. Currently in Italy there are 16 regional and inter-regional screening laboratories using bTSH as primary screening test to detect infants with CH. The bTSH cutoff used at the first screening in the various laboratories varies from 6.0 to 10.0 mU/L (3 labs with a 6.0 mU/L cutoff, 7 with a 7.0 mU/L cutoff, 1 with an 8.0 mU/L cutoff, 4 with a 9.0 mU/L cutoff, and 1 with a 10.0 mU/L). Although the strategy of repeat screening, with repeat sampling at 2 weeks, is currently adopted by all the 16 Italian screening laboratories, only 3 of these (Lombardy, Veneto, and Calabria regions) use differential cutoffs at the first and second screening. Among these, the Regional Screening Laboratory of Lombardy is the only one using differential bTSH cutoffs since 2005: 10.0 and 5.0 mU/L at the first and second screening; whereas the other two laboratories started using differential bTSH cutoffs some years later. Since 2005 about 95,000 newborns per year have been screened in the Lombardy region.

The aim of this study was to characterize those infants with CH who were identified by the second screening test in Lombardy, and to compare those showing bTSH ranging 5.0-9.9 mU/L at the second screening with CH infants showing bTSH values which were equal or higher than the TSH cutoff at the first screening (10.0 mU/L). To this end, maternal and neonatal clinical features at birth, as well as thyroid function parameters at the diagnosis were retrospectively analyzed in CH infants detected at the second screening in the Lombardy region between 2007 and 2014, and diagnosed at the pediatric endocrinology center of San Raffaele Hospital-Milan.

SUBJECTS AND METHODS

S*creening procedure*

During the study period blood samples were collected 49-120 hours after birth for the first screening, with a bTSH cutoff value of 10 mU/L, corresponding to the 99.5th percentile of a bTSH distribution previously obtained from a cohort of 73,000 at term healthy babies tested between 49 and 120 hours of life (30). A bTSH >=20.0 mU/L triggered immediate referral. Infants with a bTSH of 10-20 mU/L at first screening are recalled for a second blood spot collection (age range: 7–9 days) with a bTSH cutoff of 5 mU/L after the first week of life. Since newborns with bTSH 15-20 mU/l at first screening are considered at high risk of CH, parallel serum TSH (sTSH) and FT4 determinations are also recommended. If hyperthyrotropinaemia is confirmed on the second sample (bTSH $\geq$5 mU/L and/or sTSH $\geq$10 mU/L), the infant is considered positive. In addition, a second screening test was routinely performed in infants with a negative result on first screening test and risk factors such as gestational age < 37 weeks (preterm babies), birth weight (BW) ≤ 2000g, admission to NICU, twins, syndromes and/or malformations, use of steroid during pregnancy, and maternal thyroid disease. A second screening was also carried out in term newborns showing bTSH values at the first screening ranging 6.5-9.9 mU/L associated with further neonatal and maternal risk factors, i.e. maternal diabetes, maternal treatment with amiodarone, maternal/neonatal iodine exposure, Large Birth Size (BW>4500g), and gestational age>40 weeks (31-33). The bTSH value of 6.5 mU/L corresponded to the 97.5th percentile of the above-mentioned distribution (30).

In the study period, from January 2007 to May 2013, re-screening was carried out between 15 and 30 days of life. From June 2013 to December 2014 the timing for the sample collection on the second screen was 15±1 days of life. The bTSH cutoff value on the second

screen was 5.0 mU/L, corresponding to the 99th percentile of a bTSH distribution at 15±1 days of life obtained in a cohort of 5,000 at term healthy babies (30).

Solid phase time-resolved fluoroimmunoassay (Perkin Elmer's Auto-DELFIA technology, Waltham, Massachusetts) was used to measure TSH concentrations from dried newborn blood spots.

The ethical committee of the Istituto Auxologico Italiano approved the study protocol (Code: RF-2010-2309484) and informed consent for genetic studies was obtained from the parents.

Diagnosis confirmation

Treatment with L-T4 was started if serum TSH (sTSH) was ≥ 20.0 mU/L in a single blood collection, irrespective of the serum free thyroxine (sFT4) value, or if sTSH value was between 10.0 and 19.9 mU/L with a normal sFT4 value confirmed at least in two occasions. If sFT4 concentration was below norms for age, treatment was started regardless of sTSH values. Thyroid function at diagnosis, gender, gestational age, condition of small for gestational age (SGA) defined as BW <3rd centile for gestational age in Italy, appropriate for gestational age (AGA), large for gestational age (LGA) defined as BW >97th centile for gestational age, ethnicity, comorbidities, malformations, thyroid imaging results, and family history of thyroid diseases were recorded in all patients. According to European guidelines, the severity of CH was biologically assessed as severe, moderate, or mild on the basis of sFT4 levels of <5.0, 5.0 to <10.0, and 10.0 to 15.0 pmol/L, respectively (1). These values correspond to sFT4 levels of <0.39, 0.39 to <0.78, and 0.78 to 1.16 ng/dL, respectively. At diagnosis, thyroid ultrasound (US) was performed in all cases, whereas thyroid scintigraphy was performed if thyroid US was not immediately available or a physician with experience in pediatric thyroid US was not available.

Re-evaluation of the diagnosis

In patients with in situ (eutopic) thyroid a re-evaluation of the diagnosis was performed at 3 years of age or later after one-month withdrawal of the replacement therapy. The diagnostic re-evaluation included measurements of sTSH, sFT4, anti-thyroid antibodies, and thyroglobulin. Thyroid US was performed in all patients by a trained physician with experience in pediatric thyroid US.

Permanent CH was diagnosed if thyroid function tests showed sTSH levels above 10.0 mU/L on at least 2 occasions, or if sTSH was above 20.0 mU/L in a single blood collection. In these cases, L-T4 treatment was reintroduced. Cases in which sTSH values ranged between 5.0 and 9.9 mU/L with normal FT4 were considered indicative of persistent hyperthyrotropinemia (HT) not requiring the reintroduction of treatment. Transient CH was diagnosed if sTSH was below 5.0 mU/L with normal FT4 at least in two occasions. Patients with initial HT that subsequently showed sTSH below 5.0 mU/L on at least 3 consecutive blood collections were classified as affected by transient CH.

Genetic analysis

In the observation period (2007-2014) genetic analysis was not routinely requested in CH patients with eutopic thyroid, only infants with first-degree relatives affected by thyroid diseases, and also infants with clinical and biochemical features suggesting a variant in a specific gene associated with CH were investigated (34-36). Prior to 2014, PCR-amplified direct sequencing was the technique available but was largely superseded from January 2014 by targeted next-generation sequencing (NGS). In this study only 23 patients with eutopic thyroid were genetically characterized and the studied genes were *DUOX2*, *TSHR*, *DUOXA2, FOXE1, GLIS3, NKX2-1, PAX8, SLC26A4, TG, TPO, JAG1, SLC5A5, IYD and SLC16A2*. The clinical significance of rare detected variants, ranging from pathogenic changes to variants of unknown significance, was assigned by a bioinformatic approach as previously described (37-38).

Statistical analysis

A descriptive analysis was performed to report means ± SDs and frequencies of principal variables. The chi-squared test or t-test, where appropriate, was used to determine whether there was a significant difference between groups. Multiple linear regression analysis was performed to assess differences in birth weight between the groups taking into account gestational age at delivery, and to ascertain the relationship between sTSH (log-transformed values) or sFT4 at diagnosis and bTSH values on the second screen considering age at diagnosis and gestational age as covariates.

Statistical analyses were performed using Stata software for Windows (version 16; StataCorp, College Station, TX).

RESULTS

Subjects recruited in the study

Figure 1 shows that between 1st January 2007 and 31st December 2014, 767,157 newborns were screened for CH in Lombardy region. Among these newborns, 842 were diagnosed with CH and treated with L-T4. The incidence of CH confirmed at birth (transient and permanent CH) was 1:911 live borns. Among the 842 CH patients, 273 (32.4%) were negative at the first screening and were detected by the second screening test. Of the 273 CH patients, 119 (43.6%) were diagnosed at the pediatric endocrinology center of San Raffaele Hospital-Milan and were included in the study.

Maternal and neonatal features (see Table 1)

Among the 119 CH babies included in the study, 52 (43.7%) showed bTSH values ranging 5.0-9.9 mU/L at the second screening (Low bTSH Group) and 67 (56.3%) bTSH concentrations ≥10.0 mU/L (High bTSH Group) (Figure 1). Table 1 shows the neonatal, familial and maternal features in the two groups. Babies in the Low bTSH Group were more

frequently preterm than those in the High bTSH Group (57.7% *vs* 23.9%, P<0.001). This finding was essentially due to a higher percentage of babies with gestational age <34 weeks in the Low bTSH Group than in the High bTSH Group (34.6% *vs* 6.0%, P<0.001). Similarly, the percentage of babies admitted to NICU was significantly higher in the Low bTSH Group (50.0% *vs* 17.9%, P<0.001). Of note, BW was lower in the Low bTSH Group (2185 ± 992 g) than in the High bTSH Group (2920 ± 732 g) even after adjusting by gestational age (beta= -283.4; P=0.017); whereas no significant difference between groups was observed in the frequency of SGA.

Table 2 shows that 22 of the 119 infants had extra-thyroidal malformations or dysmorphic syndromes, with similar frequency between the Low bTSH Group (11/52; 21.2%) and High bTSH Group (11/67; 16.4%). Moreover, the frequency of babies with multiple malformations was similar in both groups (7.7% and 4.5%, respectively). The girl with septo-optic dysplasia (PL7 in Table 2) was affected by hypothalamic, rather than primary, hypothyroidism and also showed growth hormone deficiency with pituitary hypoplasia, ectopic neurohypophysis, optic nerve hypoplasia and delayed development. This patient has been included in the study because she was picked up by the second screening.

Regarding maternal features, mothers of babies in the Low bTSH Group were more frequently treated with glucocorticoids in pregnancy than mothers of babies in the High bTSH Group (11.5% *vs* 1.5%, P=0.042). The occurrence of maternal hypothyroidism and/or goiter was also significantly more frequent in the Low bTSH Group than in the High bTSH Group (26.9% *vs* 10.4%, P=0.036) and only one mother in the High bTSH Group showed hyperthyroidism. It is noteworthy that among babies born to mothers with hypothyroidism and/or goiter, 4 in the Low bTSH Group and 1 in the High bTSH Group showed permanent CH.

No significant differences between the groups were observed in the frequency of maternal diabetes, medically assisted pregnancy, and type of delivery.

Diagnostic classification, etiology and retesting

At diagnosis, the Low bTSH Group showed a median sTSH value significantly lower than that found in the High bTSH Group (16.5 *vs* 55.4 mU/L, P<0.001) and, as expected, the mean sFT4 concentration was significantly higher in the Low than in the High bTSH Group (14.7 $\pm$ 3.8 *vs* 9.1 $\pm$ 5.1 pmol/L, P<0.001). These differences were observed after adjusting for gestational age and age at diagnosis. According to the scale of biochemical severity of hypothyroidism proposed by European guidelines (1), babies with severe, moderate, and mild hypothyroidism at diagnosis were 0/48 with sFT4 (0%), 4/48 (8.3%), and 24/48 (50%) in the Low bTSH Group compared with 14/67 (20.9%), 25/67 (37.3%), and 20/67 (29.9%) in the High bTSH Group. Normal sFT4 values were found in 20/48 (41.7%) babies of Low bTSH Group and in 8/67 (11.9%) babies of High bTSH Group. Table 3 shows details on sFT4 levels in children with permanent CH, transient CH and persistent HT, and in children who were not re-evaluated. The median age at the start of the replacement therapy was 35 days (range 18-89 days) in the Low bTSH Group and 24 days (range 15-53 days) in the High bTSH Group.

Thyroid US and/or scintigraphy results showed no significant differences in the frequency of different etiologies of CH between groups. Of note, two babies in the Low bTSH Group and three in the High bTSH Group showed thyroid dysgenesis (see Table 3).

The final diagnosis was available in 101 of 119 CH babies included in the study. Of 18 patients with eutopic gland who were not retested off treatment, two had syndromes with difficult clinical management, one had hypothalamic hypothyroidism, 8 were followed up elsewhere and 7 were lost to follow up**.** The frequency of permanent (25.0% and 14.0%) and transient CH (43.2% and 59.7%) was similar in the Low and the High bTSH Groups, as well the frequency of persistent HT (31.8% and 26.3%) (Table 3). It is interesting to note that, in our study the frequency of patients with malformations was high in both groups (21.2% in the Low bTSH Group; 16.4% in the High bTSH Group) and not significantly

different between children with permanent and transient CH, both in the Low bTSH Group (27.2% and 21.0%, respectively) and the High bTSH Group (25.0% and 11.7%, respectively) (Table 3).

Genetic analysis

Genetic analysis was performed in 23 patients with eutopic thyroid, 13 in the Low bTSH Group and 10 in the High bTSH Group. In 16 of the 23 patients (69.5%) at least one rare variant in a CH-associated gene was identified. All the patients harbouring genetic variants were heterozygous and oligogenic cases were detected both in the Low and the High bTSH Group. The results of genetic analysis are summarized in Table 3.

In the Low bTSH Group, 3 patients harboured pathogenic variants in *DUOX2* (1 with permanent CH, patient PL13; 1 with transient CH, patient PL16; 1 with persistent HT, patient PL18). At diagnosis, both the patients with permanent and transient CH showed sFT4 levels in the range corresponding to moderate CH (6.1 pmol/L, 9.1 pmol/L). Two further patients in the Low bTSH Group showed pathogenic variants in *TSHR* (1 with permanent CH, patient PL14; 1 with persistent HT, patient PL17). Three patients harboured variants of unknown significance in *DUOXA2*, *TG*, *TSHR* and genetic analysis was negative in the remaining 5 patients.

In the High bTSH Group, among patients with permanent CH, one showed a pathogenic variant in *PAX8* (patient PH13), and another one harboured variants of unknown significance in *GLIS3* and *SLC26A4* genes. Among those with transient CH, 2 children showed pathogenic variants in *DUOX2* (patients PH14 and PH15) with sFT4 levels in the range corresponding to moderate CH at diagnosis; whereas genetic analysis was negative in one child. Finally, 3 patients with persistent HT showed pathogenic variants in *DUOX2* (patients PH18, PH19, PH20), one of whom showed severe CH (sFT4=1.2 pmol/L).

DISCUSSION

The goal of newborn screening is to identify all forms of CH - mild, moderate and severe - while at the same time avoiding the problem of overwhelming the health care system with false-positive screening results requiring unnecessary follow-up and investigation (1). This retrospective analysis of 8 years' experience of a two-screen program in the Lombardy region of Italy, shows that employing differential capillary cutoff values - 10.0 mU/L on the first screen and 5.0 mU/L on the second - helps to support this goal. By using a lower TSH cutoff on the second screen, additional cases of CH were detected, so that 43.7% of the 119 CH babies included in the study who were identified on second screening would have been missed if the same bTSH cutoff of 10.0 mU/L had been used on the first and second screen. Moreover, over half of these babies showed subnormal sFT4 levels at diagnosis (58.3% in Low bTSH Group and 88.0% in High bTSH Group).

These findings are in keeping with a previous study (20) which analysed 20 years of screening in the Quebec region (1990-2009) and which reported the results of a new screening algorithm adopted in 2001. Following an initial bTSH between 15.0 and 30.0 mU/L, the cutoff on the second test was decreased from 15.0 to 5.0 mU/L. In the period of observation 620 newborns were diagnosed with CH and among these 49 additional cases of CH were identified by the lower bTSH cutoff on the second screening. Importantly, 10 of the 49 additional cases had thyroid dysgenesis in the form of ectopic thyroid. Although the algorithms used in Lombardy and Quebec are different, use of differential cutoffs in both two-screen programs led to the identification of additional CH cases, including cases with thyroid dysgenesis.

Our study also shows that CH cases with bTSH ranging 5.0-9.9 mU/L at the second screening are similar to CH cases with higher bTSH concentrations, suggesting the benefit of using differential bTSH cutoffs. Specifically, the frequency of thyroid dysgenesis and eutopic gland found in the Low bTSH Group was similar to that observed in the High bTSH

Group, as well as the frequency of permanent and transient CH, and of persistent HT. In addition, CH babies harbouring rare variants in CH-candidate genes were similarly represented in both Low and High bTSH groups, with babies harbouring variants in the DUOX gene mostly showing moderate or severe CH at diagnosis. Both infants with TSHR gene variants were in the Low bTSH Group and showed mild hypothyroidism at diagnosis, whereas at diagnostic re-evaluation one now showed permanent CH and the other persistent HT. This finding suggests that a bTSH cutoff of 5.0 mU/L at the second screening can identify patients with variants in the TSHR gene affected by permanent CH who were negative at the first screening.

Another interesting result of our study was the finding of a similarly high prevalence of extrathyroidal congenital malformations in the Low (21.2%) and High (16.4%) bTSH Groups. Although the association between CH and extra-thyroidal congenital malformations is well known (39-41), ours is the first study to show that not only severe but even mild forms of CH can be associated with extra-thyroidal malformations. Our findings contrast with the work of Oakley et al (42) who reported that extra-thyroidal malformations occurred more frequently in infants with transient TSH elevation than in those with permanent CH. These different results may be partly explained by the two studies not considering the same population of infants with transient TSH elevation. Thus in our study, all children with transient CH started replacement therapy in the first weeks of life whereas in Oakley et al. the 88 infants with transient TSH elevation included 23 infants with transient CH, treated for a period with L-T4, and 65 infants with transient hyperthyreotropinemia who did not receive treatment, and the frequency of infants with malformations in these two subgroups was not specified. Therefore, a higher frequency among untreated infants cannot be excluded, as a transient TSH elevation during the first days of life may reflect a response to stress and malformation per se may be a source of stress.

Our analysis also demonstrates that preterm babies of < 34 weeks gestation are more likely to have bTSH values between 5-9.9 mU/L than ≥10 mU/L. These infants are therefore liable to be missed unless a second screen cut-off of 5 mU/L is used. A recent study conducted in the Republic of Ireland (43), where repeat bTSH samples are tested weekly until the preterm infant is term-corrected (37 weeks' gestation) even if the initial bTSH is normal, has demonstrated that with a bTSH cutoff of 8.0 mU/L on the second screen, almost half of CH babies with <33weeks' gestation included in the study had normal bTSH at 14 days of life. Another study conducted in Wisconsin State USA (44) has reported reference intervals of bTSH in the first weeks of life in very (28-31 weeks' gestation) and extremely preterm babies (22-27 weeks' gestation). This study showed that the 95th percentile value of serum equivalent TSH at 2 weeks of life was 12.7 mU/L (corresponding to 5.8 mU/L whole blood, conversion factor of 2.2 after assuming 55% hematocrit) in extremely preterm infants, and 10.6 mU/L (corresponding to 4.8 mU/L whole blood) in very preterm infants. Taken together these findings strongly support both second screening and a low second screen cutoff.

Finally, our study shows that a bTSH cutoff of 5.0 mU/L at the second screening is also able to detect CH in babies born to mothers treated with glucocorticoids during pregnancy, in whom the suppressive effect on pituitary TSH secretion is well established (12, 45). Also, while the effects of maternal hypo- and hyperthyroidism on fetal and neonatal thyroid function and preterm birth are known (46-48), few studies have been conducted to verify the effect of maternal thyroid disease on newborn screening for CH (49-50). In the Lombardy screening program, maternal thyroid disease is considered a risk factor for a delayed TSH rise, hence requiring a second screening at two weeks of life. This study shows a higher frequency of maternal hypothyroidism in the Low versus High bTSH Group, a finding which suggests that maternal treatment with L-T4, which has a half-life of about 7.5

days in hypothyroid patients (51), may have a beneficial effect on the hypothyroid fetus as indicated by the first test being negative with low bTSH on the second.

In conclusion, the routine second screening, while increasing laboratory costs, is necessary for neonates at risk for delayed TSH rise. It is particularly relevant in countries where the initial screening is obtained at 24-72 h of age (23, 25), because early timing of the first screening necessitates higher TSH cutoffs to compensate for the physiologic postnatal TSH surge. Two-screen programs with the same screening cutoff for both screens risk missing infants because of the physiologic TSH concentration changes in relation to age of the infant at specimen collection, and also infants with marginally elevated TSH levels who need to be treated (52). The analysis of 8 years' experience with a two-screen program for CH in Lombardy has shown the value and benefits of using differential TSH cutoffs, with a second bTSH cutoff of 5 .0 mU/L.

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Data Availability

Restrictions apply to the availability of data generated or analyzed during this study to preserve patient confidentiality or because they were used under license. The corresponding author will on request detail the restrictions and any conditions under which access to some data may be provided.

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LEGEND OF FIGURES

Figure 1. Flow chart reporting the number of newborns screened in the Lombardy region in the study period (2007-2014) and infants with congenital hypothyroidism identified by the second screening and included in the study.

Table 1. **Neonatal, family and maternal features in 119 infants with blood spot TSH elevation on second newborn screening, divided according to blood-spot TSH level: 5-9.9 mU/L (Low bTSH Group) and ≥10 mU/L (High bTSH Group).**

		Low bTSH Group n=52	High bTSH Group n=67	P
Neonatal Features	Males	27 (51.9%)	34 (50.7%)	0.90
	Preterm delivery <37 weeks	30 (57.7%)	16 (23.9%)	**<0.001**
	Gestational Age			
	34-36 weeks	12 (23.1%)	12 (17.9%)	0.48
	<34 weeks	18 (34.6%)	4 (6.0%)	**<0.001**
	Birth Weight (g)	2185±992	2920±732	**0.017***
	SGA	8 (15.4%)	5 (7.5%)	0.16
	AGA	43 (82.7%)	61 (91.0%)	0.17
	LGA	1 (1.9%)	1 (1.5%)	1.0
	Babies with extra-thyroidal malformations/syndromes	11 (21.2%)	11 (16.4%)	0.51
	Babies admitted to NICU	26 (50.0%)	12 (17.9%)	**<0.001**
	Twins	9 (17.3%)	5 (7.5%)	0.098
Family/Maternal Features	Parental consanguinity	0	4 (6.0%)	----
	Maternal glucocorticoid treatment	6 (11.5%)	1 (1.5%)	**0.042**
	Medically assisted pregnancy	6 (11.5%)	4 (6.0%)	0.33
	Maternal hypothyroidism and/or goiter	14 (26.9%)	7 (10.4%)	**0.036**

	Maternal diabetes	2 (3.8%)	2 (3.0%)	0.769
	Cesarean section**	34 (66.7%)	46 (70.8%)	0.635

*after adjustment for gestational age

**the type of delivery was unknown in one patient from the Low and two patients from the High bTSH Groups.

Abbreviations: SGA, Small for Gestational Age; AGA, Appropriate for Gestational Age; LGA, Large for Gestational Age.

Table 2. Details on 22 infants with extra-thyroidal malformations/syndromes among the 119 recruited infants with blood spot TSH elevation on second newborn screening, divided according to capillary TSH level: 5-9.9 mU/L (Low bTSH Group) and ≥10 mU/L (High bTSH Group).

	Cases with a single extra-thyroidal malformation					Cases with multiple extra-thyroidal malformations
GROUPS	**Cardiac malformations**	**Urogenital malformations**	**Gastrointestinal malformations**	**Hands-feets malformations**	**CNS malformations**	
Low bTSH Group	PL1. Aortic coarctation (transient CH) PL2. Down Syndrome with complete atrioventricular canal defect (permanent CH)	PL3. Hypospadias (permanent CH) PL4. Hypospadias (transient CH)		PL5. Left club foot (transient CH) PL6. Tendon agenesis of the first finger of the hand (permanent CH)	PL7. Septo-optic dysplasia (hypothalamic hypothyroidism)	PL8. Down Syndrome with anal stenosis, double urethral meatus (not re-evaluated) PL9. Jacobsen syndrome with double outlet right ventricle, ventricular septal defect, left renal agenesis (transient CH) PL10. Down Syndrome with duodenal atresia, ASD (not re-evaluated) PL11. Large ASD and dysplastic pulmonary valve (not re-evaluated)

High bTSH Group	PH1. Transposition of the great vessels (not re-evaluated)	PH2. Left megaureter (persistent HT) PH3. Bilateral cryptorchidism (transient CH)	PH4. Giant omphalocele (transient CH)	PH5. Bilateral syndactyly (permanent CH) PH6. Right hand syndactyly (transient CH) PH7. Di George syndrome with bilateral club feet (permanent CH)	PH8. Partial agenesis of the corpus callosum (persistent HT)	PH9. Angioma with metameric disposition, ASD (persistent HT) PH10. Down Syndrome with aganglionic megacolon and sensorineural hearing loss (transient CH) PH11. Down Syndrome with annular pancreas, duodenal atresia, inferior vena cava agenesis (not re-evaluated)

Abbreviations: CNS, Central Nervous System; ASD, Atrial Septal Defect, CH, Congenital hypothyroidism; HT, Hyperthyreotropinemia; PL, patients from the Low bTSH Group; PH, patients from the High bTSH Group.

Table 3. Serum TSH and FT4 at diagnosis, etiology of congenital hypothyroidism, genetic analysis, and results of re-evaluation of the diagnosis in the 119 recruited infants with blood spot elevation on second newborn screening, divided according to blood spot TSH level: 5-9.9 mU/L (Low bTSH Group) and ≥10 mU/L (high bTSH Group).

		LOW bTSH GROUP	HIGH bTSH GROUP
Permanent CH **Low bTSH Group n=11** **High bTSH Group n=8**	**Median serum TSH (mU/L) at diagnosis (range)**	19.77 (15.26 – 27.05)	58.25 (14.40 – 138.47)
	Median serum FT4 (pmol/L) at diagnosis (range)	13.64 (8.24 – 18.53)	12.48 (5.53 – 16.22)
	FT4 <5.0 pmol/L, n (%)	0	0
	FT4 5.0-9.9 pmol/L, n (%)	2 (18.2)	3 (37.5)
	FT4 10.0-15.0 pmol/L, n (%)	6 (54.5)	3 (37.5)
	FT4 >15.0 pmol/L, n (%)	3 (27.3)	2 (25.0)
	Etiology, n (%)		
	Athyreosis	0	0
	Ectopia	1 (9.0%)	0
	Hypoplasia *in situ*	0	1 (12.5%)*
	Hemiagenesis	1 (9.0%)	2 (25.0%)
	Normal/Hyperplasia *in situ*	9 (82.0%)	5 (62.5%)
	Infants with extra-thyroidal malformations, n (%)	3 (27.2)	2 (25.0)
	Genetic analysis only in 7 infants with normal *in situ* thyroid	**PL3**.** DUOXA2: c.376T>A **PL6**.** WT **PL12**. WT **PL13**. DUOX2: c.3391G>A DUOX2: c.1606C>T **PL14.** TSHR: c.122G>C	**PH12.** SLC26A4: IVS9-9pbA>C GLIS3: c.1145C>A **PH13.** PAX8: c.1090C>T
Transient CH **Low bTSH Group n=19** **High bTSH Group n=34**	**Median serum TSH (mU/L) at diagnosis (range)**	21.42 (11.47 - 67.00)	81.63 (10.31 – 756.60)
	Median serum FT4 (pmol/L) at diagnosis (range)	12.36 (8.49 – 20.85)	7.01 (1.29 – 19.18)
	FT4 <5.0 pmol/L, n (%)	0	11 (32.4)
	FT4 5.0-9.9 pmol/L, n (%)	2 (10.5)	11 (32.4)
	FT4 10.0-15.0 pmol/L,	12 (63.2)	11 (32.4)
		5 (26.3)	1 (2.9)

	n (%) **FT4 >15.0 pmol/L, n (%)**		
	Infants with extra-thyroidal malformations, n (%)	4 (21.0)	4 (11.7)
	Genetic analysis (only in 7 infants)	**PL4**.** WT **PL5**.** WT **PL15**. WT **PL16**. DUOX2: c.2895_2898delGTTC DUOX2: c.3155G>A	**PH14.** DUOX2:2895_2898delGTTC **PH15.** DUOX2:2895_2898delGTTC **PH16.** WT
		LOW bTSH GROUP	**HIGH bTSH GROUP**
Persistent hyperthyrotropinemia **Low bTSH Group n=14** **High bTSH Group n=15**	**Median serum TSH (mU/L) at diagnosis (range)**	14.54 (12.12 – 50.74)	49.47 (18.37 – 270.10)
	Median serum FT4 (pmol/L) at diagnosis (range)	16.67 (12.10 – 24.58)	8.11 (1.29 – 18.66)
	FT4 <5.0 pmol/L, n (%)	0	2 (13.3)
	FT4 5.0-9.9 pmol/L, n (%)	0	8 (53.4)
	FT4 10.0-15.0 pmol/L, n (%)	5 (41.7)	2 (13.3)
	FT4 >15.0 pmol/L, n (%)	7 (58.3)	3 (20.0)
	No data on FT4	2	0
	Infants with extra-thyroidal malformations, n (%)	0	3 (20.0)
	Genetic analysis (only in 9 infants)	**PL17.** TSHR: c.326G>A **PL18.** TPO: c.2101C>T DUOX2: c.1428C>A DUOX2: c.1516G>T TG: c.4645C>T DUOX2: c.1588A>T **PL19.** TSHR: c.1451A>G **PL20.** TG: IVS2+5pbG>T	**PH17.** DUOX2: IVS17-1G>C **PH18.** DUOX2: c.3329G>A DUOX2: c.1232G>A FOXE1: c.682C>G **PH19.** DUOX2: c.4552G>A **PH20**. DUOX2: c.3251G>A **PH21.** WT
Not re-evaluated children with normal *in situ* thyroid	**Median serum TSH (mU/L) at diagnosis (range)**	14.75 (10.61 – 16.94)	39.75 (12.33 – 184.52)

Low bTSH Group n=8 **High bTSH Group n=10**	**Median serum FT4 at diagnosis (range)**	17.52 (12.36 – 18.02)	10.94 (1.93 – 23.17)
	FT4 <5.0 pmol/L, n (%)	0	1 (10.0)
	FT4 5.0-9.9 pmol/L, n (%)	0	3 (30.0)
	FT4 10.0-15.0 pmol/L, n (%)	1 (16.7)	4 (40.0)
	FT4 >15.0 pmol/L, n (%)	5 (83.3)	2 (20.0)
	No data on FT4	2	0
	Infants with extra-thyroidal malformations, n (%)	4 (50.0)	2 (20.0)

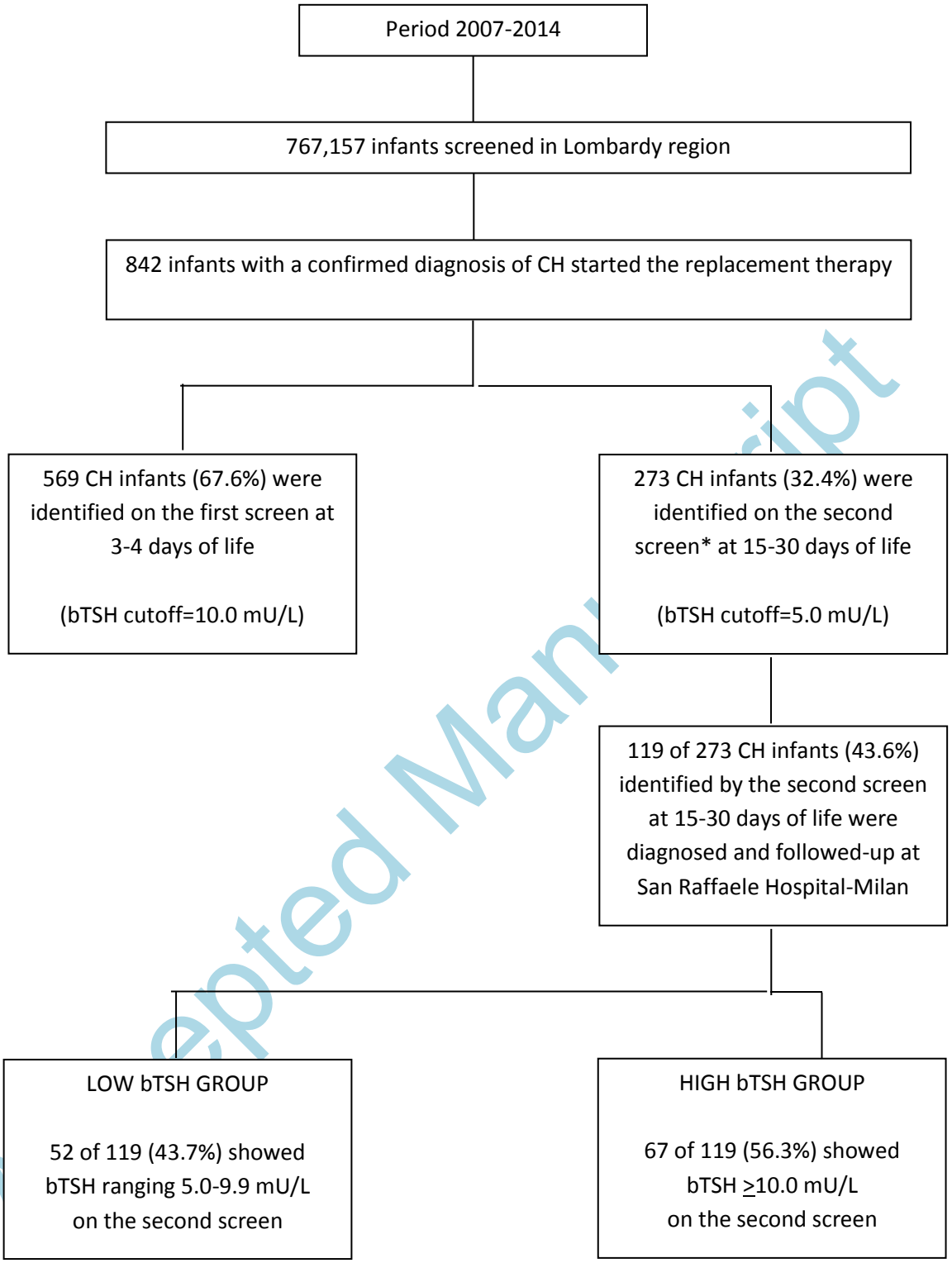
*Diagnosis of hypoplasia *in situ* obtained on thyroid ultrasound; since thyroid scintigraphy was not carried out in this patient, a possible presence of ectopic tissue cannot be excluded *(ref 53).*

**see associated extra-thyroidal malformations in Table 2.

In genetic analysis, variants with pathogenic significance are underlined.

Abbreviations: PL, patients from the Low bTSH Group; PH, patients from the High bTSH Group.

FIGURE 1



*The second screen was performed only in selected infants with risk factors