Research Article

Biological reference values for newborn screening parameters in accordance to gestational age and birth weight- a prospective study

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Article Info

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Keywords

term, preterm, normal birth weight, low birth weight, male newborns, female newborns, Central India

Abstract

Background: Disease specific biomarkers are ideal tool to detect the presence of the disorder. Timely detection of disorders can improve the health outcome. The metabolic arrangements in pre-term (PT) and low-birth weight (LBW) newborns differ from those term-born and have normal-birth weight (NBW). Hence, it is crucial to compare the values across study groups and establish a dedicated reference values for each group considering the gestational-age and birth weight.

Methods: The prospective study was conducted on the cohort of 2860 newborns who underwent newborn screening (NBS) in dried-bloodspot samples within five days of birth. The study groups included were TERMNBW, TERMLBW, PTNBW and PTLBW.

Results: The central tendency measures and the comparison of the NBS parameters across the study groups are presented. Males recorded a higher n17-OHP ($p<0.001$) median(range) compared to female newborns whereas nIRT ($p=0.008$) and nMSUD ($p<0.001$) were higher in female newborns. nTSH values was higher in TERMNBW than the PTLBW group ($p=0.03$). n17-OHP levels in TERMNBW and TERMLBW groups were lower than PTNBW and PTLBW (<0.001) newborns. nBIOT range of 378.8U and nG6PD range of 17.1U/gHb was highest in TERMNBW. The reference value observed for nTSH, n17-OHP and nIRT were respectively, 9.2mIU/L, 48.6nmol/L, 95.0µg/dL in TERMNBW and 16.9mIU/L, 70.2nmol/L, 76µg/dL in PTLBW. nG6PD reference level were respectively 2.0 and 1.6u/gHb in TERMNBW and PTLBW groups. The nBIOT levels were 52.7U and 48.0U respectively. Reference values were nearly similar for nPKU, nGAL and nMSUD.

Conclusion: The study has provided a detailed comparison and reference levels observed in various study groups and sub-groups considering the gestational-age and birth weight of the newborns.

Introduction

Nearly 75% of death in children under 5 years of age occur in newborns within a week of birth. 40% of the neonatal deaths are commonly associated with prematurity, perinatal complications, sepsis and birth defects [1]. Although mortality has declined since last decade, yet maternal and child health remains a public health problem. Disease specific biomarkers are an ideal tool to detect the presence of the disorder. These markers assist the lab physicians and clinicians to correlate with disease phenotype. Therefore, early diagnosis of the disorder should be the ultimate objective for infants so that appropriate management can be initiated. Timely detection of disorders can improve the health outcome. However, confirmatory diagnosis required high-end equipment. In India, availability of laboratories with high-end infrastructure, such as availability for liquid chromatography mass spectrometry (LCMS) and gene analysis, is very limited and unaffordable to the patients. Most rural and urban health centres have basic equipment such as enzyme linked immunosorbent assay (ELISA)/microplate reader. Therefore, it should be a mandate to have a reference value that could be extrapolated at all rural and urban health centres in Indian set-up.

Preterm (PT) newborns are at high risk due to inadequate development of body parts. Similarly, newborns with low birth weight (LBW) have some degree of immaturity [2]. The metabolic arrangements in PT and LBW newborns differ from those born full term and with normal birth weight (NBW) [3]. Hence, it is crucial to compare the levels of NBS parameters among various groups and sub-groups, and establish a dedicated cut-off values for each group considering the gestational age and birth weight. Establishment of such cut-off levels in the lab would aid in improving detection of analyte and diagnosis, implicate appropriate interventions and genetic counselling to the parents.

Materials and Methods

The prospective study was conducted on the cohort of newborns who underwent NBS in our department, in last five years (June 2019-June 2024). Nearly five thousand NBS samples were tested during this period. All samples were received in the form of dried blood spot (DBS). The DBS samples were collected after 24 hours and within five days of birth. Five days sampling time was considered based on goals suggested by Advisory Committee on Heritable Disorders in Newborn and Children (ACHDNC) that presumptive results for time-critical conditions need to be immediately reported within five days of birth [4]. The samples were checked for the quality of the sample specimen, as per the standard protocol [5].

During the entire study period, our lab has been satisfactorily performing the proficiency testing under Center of Disease Control (CDC), United States, under the Newborn Screening Quality Assurance Program (NSQAP).

The following parameters were performed for NBS testing in newborn (n), thyroid stimulating hormone (nTSH), glucose 6-phosphate dehydrodenase (nG6PD), 17-hydroxyprogesterone (n17-OHP), biotinidase (nBIOT), phenylketonuria (nPKU), immunoreactive trypsinogen (nIRT), galactose (nGAL), and maple syrup urine disease (nMSUD). nTSH, nG6PD, n17-OHP, nBIOT, nPKU, nIRT, and nGAL were analyzed by immunofluorescence method based neonatal kits by Labsystems Diagnostics Oy, Finland. The nMSUD was analysed by immunoassay method-based kit from ZenTech SA, Belgium. Newborns with positive results were called for re-testing with a fresh DBS sample and processed in duplicate. If the results were within the cut-off value the newborn was considered negative. If the values were beyond the cut-off value, they were counselled for confirmatory testing as per the guideline [4]. The newborns who did not respond for re-test and confirmatory report, were excluded. Newborns born to mothers with history of antenatal complications like gestational diabetes mellitus, pre-eclampsia, under medication for thyroid disorders, severe grade anemia and others were excluded.

Inclusion criteria included newborns within five days of birth, those who have a confirmatory report of NBS parameters as per the advice, not diagnosed with any sort of complications during the NBS testing. In Indian scenario, the prevalence of preterm (PT) and low birth weight (LBW) is high and therefore, a reference range of those with no other associated complications is also crucial. Hence, PT born and LBW newborns were also included in the study. Newborns beyond five days of birth, who did not respond for re-testing for confirmation, newborn on top-up feeding, any therapeutic interventions initiated were excluded from the study.

According the gestational age (GA) of birth, the newborns born after 37 weeks of gestation were termed as term born and those born $\leq$ 37 weeks of gestation were termed as PT born. PT newborns were classified as moderate PT (32 to 37 weeks) and very PT (28 to < 32 weeks) for further sub-analysis [2,6]. According to the birth weight (BW), newborns $\geq$ 2500 g were considered as normal birth weight (NBW) and those <2500 g were considered as low birth weight (LBW) (7,8). The LBW newborns were further classified for sub-analysis as very low birth weight (VLBW) for newborns <1500 g and extremely low birth weight (ELBW) for those <1000 g [9].

After applying the inclusion and exclusion criteria, a total of two thousand eight hundred sixty (2860) newborns were included for the study. The newborns were categorized into four study groups. Term born with NBW newborns were grouped as TERMNBW, term born with LBW were grouped as TERMLBW, PT born with NBW were termed as PTNBW and PT with LBW were termed as PTLBW.

Statistical analysis

The data was extracted in MS excel workbook. The statistical analysis was done using Microsoft excel and IBM SPSS 26. The measures of central tendency, mean, median, standard deviation (SD), the minimum and maximum values, and the range, were computed for all the NBS parameters. The values

without the outliers were extrapolated in box-plot graphs. Each of the parameter was compared across the study groups and between male and female neonates. Gender wise comparison in each study groups were performed. All the NBS parameters were checked for normality distribution. The quantitative data were found to be skewed and hence, non-parametric tests were applied. Mann-Whitney U test was performed for gender wise comparison. For comparison of values across the groups, Independent-samples Kruskal-Wallis one way analysis of variance (ANOVA) test after Bonferroni correction for multiple tests was applied. Ideally a reference interval include 2.5th to 97.5th percentile values of the desired population [10]. But, in NBS testing, articles have suggested to consider 99th percentile upper reference limit (99th URL) to identify the otherwise treatable cause at an early age, For, nG6PD and nBIOT, where lower valued suggest positive testing for the disorder, hence, 1.0th percentile lower reference limit (1.0th LRL) was considered [11–13]. A p-value less than 0.5 was considered statistically significant.

Results

The study population consisted of 2860 newborns in which 1396 (48.8%) and 1464 (51.2%) were female and male newborns, respectively. The number of term-born and PT-born newborns included in the study were respectively, 1992 (69.7%) and 868 (30.3%). In the PT-born, 840 (29.4%) were moderate PT (32 – 37 weeks) and 28 (1.0%) were very PT (<32 weeks) born. 2177 (76.1%) newborns had NBW and 683 (23.9%) had LBW. In the LBW category, 660 (23.1%) had LBW (<2500g), 15 (0.5%) had VLBW (<1500g), and 8 (0.3%) had ELBW (<1000g).

The study groups comprised of 1724 (60.3%) TERMNBW newborns, 268 (9.4%) TERMLBW, 453 (15.8%) PTNBW, and 415 (14.5%) PTLBW newborns.

The mean (SD) and median (min.-max.) of all the NBS parameters of the study population are detailed in Table 1.

Table 1: The mean (SD) and median (range) values of the NBS parameters in the study population in DBS samples.

NBS Parameters	Gender	Mean	SD	Median	Range	Min-Max	p-value
nTSH (mIU/L)		3.2	2.1	2.7	16.8	0.1-16.9	
	F	3.2	2.1	2.7	16.8	0.1-16.9	0.44*
	M	3.2	2.1	2.8	16.8	0.1-16.9	
nG6PD (IU/gHb)		8.9	2.1	9.1	17.1	0.1-17.2	
	F	9	1.9	9.2	14.2	0.2-14.4	0.87*
	M	8.9	2.2	9.1	17.1	0.1-17.2	
n17-OHP (nmol/L)		21.4	9.4	20.3	142.8	0.2-143.0	
	F	20	8.5	18.9	72.6	0.2-72.8	<0.001*
	M	22.8	9.9	21.8	142.8	0.2-143.0	
nBIOT (U)		207.7	87.5	196.9	385.7	7.2-392.9	
	F	207.8	88.6	197.2	367.8	25.1-392.9	0.24*
	M	203.2	85.4	190	378.8	7.2-386.0	
nPKU (mg/dL)		1.2	0.5	1.1	13.3	0.1-13.4	
	F	1.2	0.5	1.1	13.3	0.1-13.4	0.64*
	M	1.2	0.6	1.1	11.9	0.1-12.0	
nIRT (µg/L)		24.8	20	19.8	341.8	0.1-341.9	
	F	25.9	20.9	20.6	341.8	0.1-341.9	0.008*
	M	23.7	19	19.2	255.9	0.1-256.0	
nGAL (mg/dL)		2.2	3.6	1.1	66.1	0.1-66.2	
	F	2.2	3.2	1.1	66.1	0.1-66.2	0.61*
	M	2.4	4	1.2	66.1	0.1-66.2	
nMSUD (mg/dL)		1.8	0.9	1.8	20.9	0.1-21.0	
	F	1.9	1	1.9	20.9	0.1-21.0	<0.001*
	M	1.8	0.7	1.8	4	0.1-4.1	

*denotes significance value of Mann-Whitney U test; F denotes female newborns and M denotes male newborns

The minimum – maximum values observed in the study population for nTSH was 0.1 – 16.9 mIU/L, nG6PD was 0.1 – 17.2 IU/gHb, n17-OHP was 0.2 – 143.0 nmol/L, nBIOT was 7.2 – 392.9 U, nPKU was 0.1 – 13.4 mg/dL, nIRT was 0.3 – 341.9 µg/L, nGAL was 0.1 – 66.2 mg/dL and nMSUD was 0.1 – 21.0 mg/dL. Gender wise differences were also delineated in Table 1. Significant differences in values were recorded for n17-OHP ($p<0.001$), nIRT ($p=0.008$) and nMSUD ($p<0.001$).

Males recorded a higher n17-OHP median (range) compared to female newborns whereas the values of nIRT and nMSUD were higher in female newborns.

A descriptive detail of the central tendency measures and the comparison of the NBS parameters across the study groups has been delineated in Table 2.

Table 2: Comparison NBS parameters across the study groups.

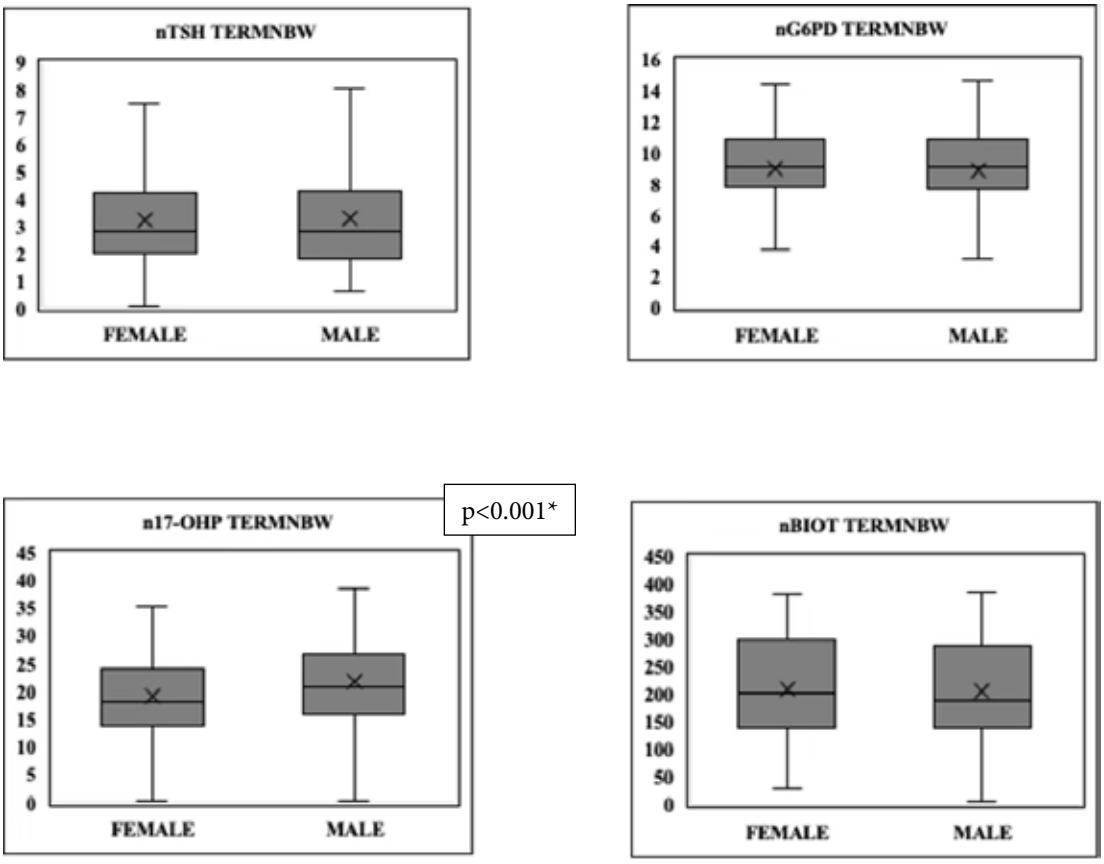
		Study Groups	Mean	SD	Median	Range	Min-Max	P value
nTSH								0.025*
	1	TERMNBW	3.2	2.1	2.8	16.8	0.1-16.9	1v4=0.03*
	2	TERMLBW	3.2	2.1	2.7	16.8	0.1-16.9	
	3	PTNBW	3.1	1.9	2.6	16.8	0.1-16.9	
	4	PTLBW	3	2.3	2.6	16.3	0.6-16.9	
nG6PD								0.57*
	1	TERMNBW	8.9	2	9.1	17.1	0.1-17.2	
	2	TERMLBW	8.9	1.9	9	14.3	0.3-14.6	
	3	PTNBW	8.9	2.2	9.4	14	0.2-14.2	
	4	PTLBW	8.9	2	9.1	14	0.2-14.2	
n17-OHP								<0.001*
	1	TERMNBW	20.4	8.5	19.5	86.8	0.2-87.0	1v3<0.001*
	2	TERMLBW	20.6	8.8	19	48.4	0.2-48.6	1v4<0.001^
	3	PTNBW	22.5	8.5	21.8	47.7	0.9-48.6	2v3=0.003*
	4	PTLBW	24.9	12.7	23.4	142.8	0.2-143.0	2v4<0.001*
nBIOT								0.017*
	1	TERMNBW	209.5	86.9	199.6	378.8	7.2-386.0	2v3=0.054*
	2	TERMLBW	195.3	84.6	179.2	334.5	48.0-382.5	
	3	PTNBW	214	88.9	207.5	344.9	48.0-392.9	
	4	PTLBW	201.1	89.3	184.9	358.5	24.0-382.5	
nPKU								0.85*
	1	TERMNBW	1.2	0.6	1.1	13.3	0.1-13.4	
	2	TERMLBW	1.2	0.4	1.1	1.6	0.7-2.3	
	3	PTNBW	1.2	0.4	1.1	5.5	0.1-5.6	
	4	PTLBW	1.2	0.6	1.1	11.3	0.7-12.0	
nIRT								0.15*
	1	TERMNBW	25.2	19.7	20	170.5	0.3-170.8	
	2	TERMLBW	26.2	26.7	20.6	341.8	0.1-341.9	
	3	PTNBW	22.9	19.3	18.6	255.9	0.1-256.0	
	4	PTLBW	24.7	16.8	20.4	106.2	0.2-108.2	
nGAL								0.73*

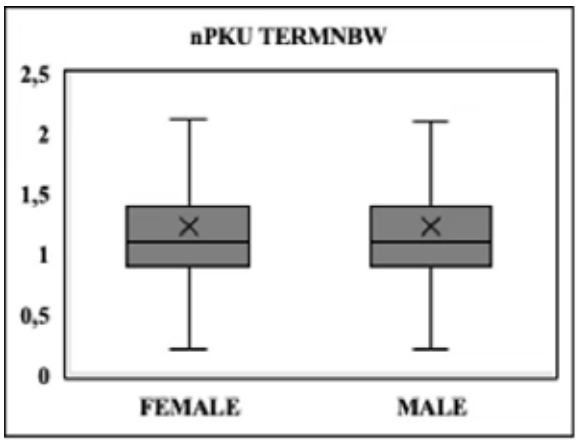
	1	TERMNBW	2.3	3.8	1.1	66.1	0.1-66.2	
	2	TERMLBW	2.3	3.3	1.2	19.8	0.1-19.9	
	3	PTNBW	1.9	2.7	1.1	22.5	0.1-22.6	
	4	PTLBW	2.1	4.1	1.1	66.1	0.1-66.2	
nMSUD								0.004*
	1	TERMNBW	1.9	0.8	1.9	14.9	0.1-15.0	1v4=0.017*
	2	TERMLBW	1.8	0.6	1.8	3.4	0.2-3.6	
	3	PTNBW	1.8	1.2	1.7	20.9	0.1-21.0	
	4	PTLBW	1.7	0.6	1.8	3.6	0.1-3.7	

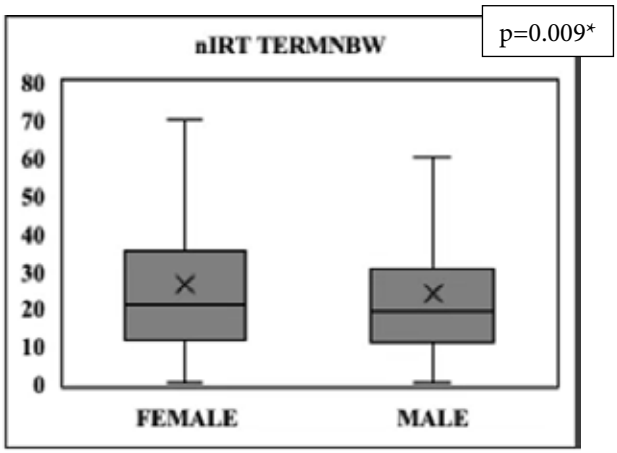
*denotes significance value of Independent-samples Kruskal-Wallis one way ANOVA test after Bonferroni correction for multiple tests

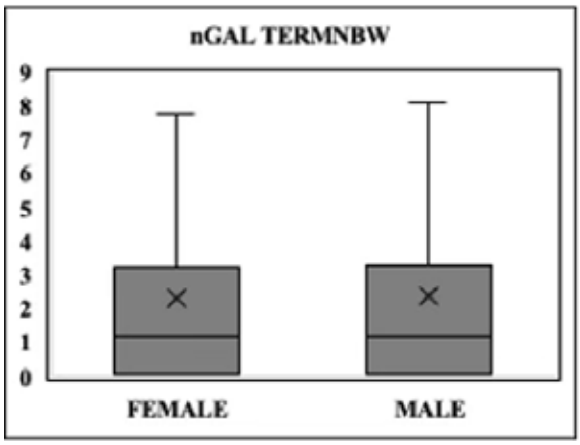
nTSH values showed a significant difference across the study groups (p=0.025) where the values were higher in TERMNBW than the PTLBW group (p=0.03). Among the study groups, PTLBW newborns recorded a lowest nTSH levels with a median (range) level of 2.6 (16.3) mIU/L. nG6PD values were nearly similar among the four study groups. The TERMNBW depicted a wider range, 0.1-17.2 IU/gHb compared to others. Gross differences in n17-OHP were observed in the study groups (p<0.001). n17-OHP levels in TERMNBW and TERMLBW groups were lower than PTNBW and PTLBW (<0.001) newborns. PTLBW newborns reported a wider range of n17-OHP, 0.2-143.0 nmol/L. The study groups showed a significant difference in their nBIOT levels (p=0.017). nBIOT range of 378.8 U was highest in TERMNBW than the other study groups. No significant dissimilarities were recorded for nPKU, nIRT and nGAL levels in the study groups. TERMNBW newborns demonstrated a highest nPKU of 13.3 mg/dL. Highest nIRT level (341.9 µg/L) was designated in TERMLBW group, whereas highest nGAL value of 66.2 mg/dl was seen in both TERMNB and PTLBW. A significant variation in nMSUD values were observed among the study groups (p=0.004). The TERMNBW group reported a higher nMSUD [median (range) of 1.9 (14.9) mg/dL] than the PTLBW group (p=0.017). Figures 1 – 4 illustrates the gender wise comparison of NBS parameters in different study groups (outliers hidden for proper reflection of values).

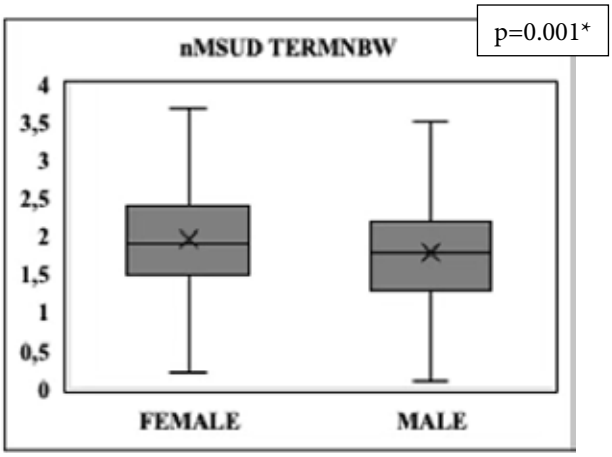
Figure 1: Gender wise comparison of DBS NBS parameters in TERMNBW study group.









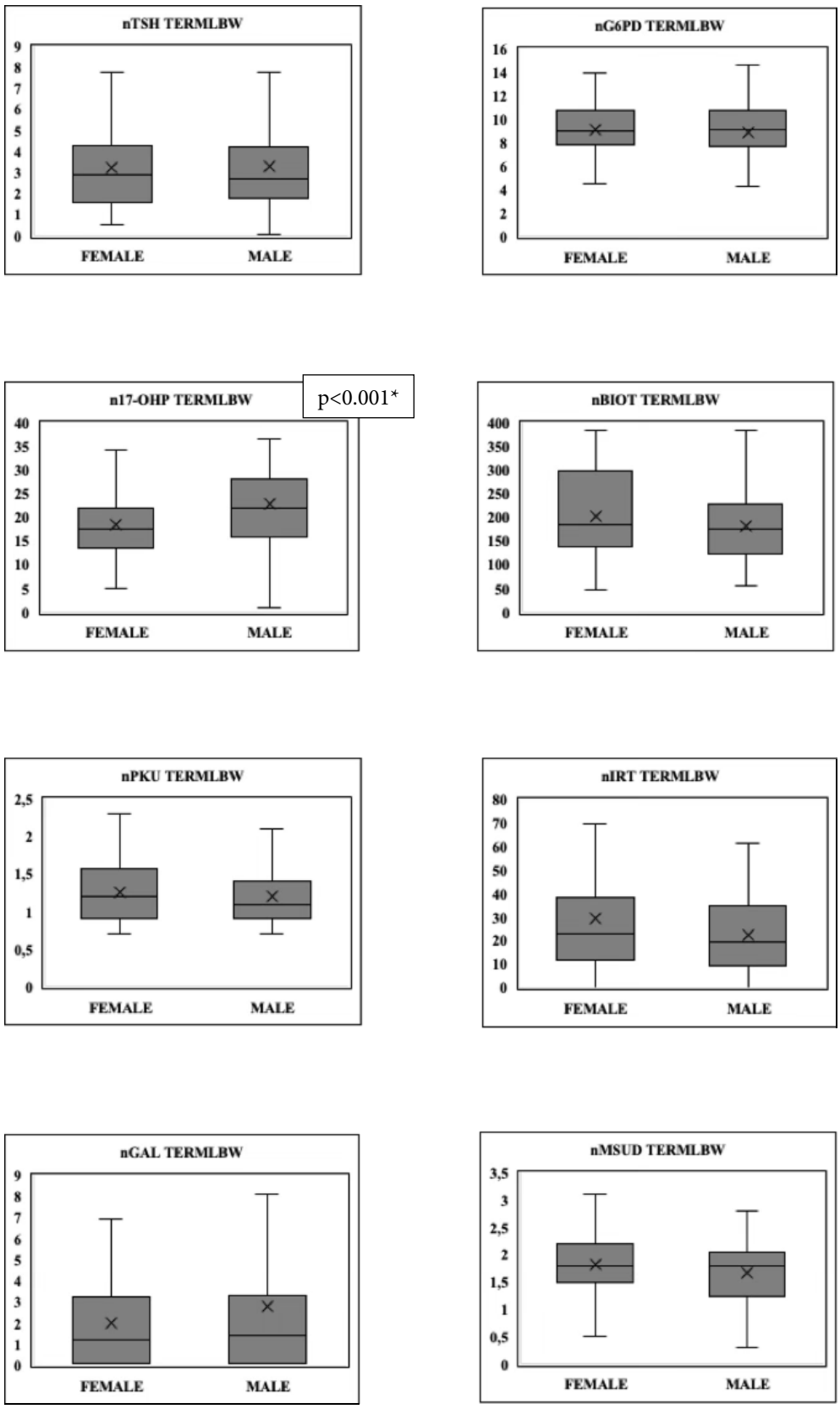


*denotes significance value of Mann-Whitney U test

Figure 1 reflects the comparison between male and female newborns of TERMNBW group. nTSH, nG6PD, nBIOT, nPKU, and nGAL did not differ significantly between male and female newborns. The male and female nTSH median (range) was 2.8 (16.8) mIU/L. The same for nG6PD were 9.1 (13.5) and 9.1 (17.1) IU/gHb, respectively. The median (range) n17-OHP of 20.9 (86.8) nmol/L in male newborns was greatly higher than 18.1 (51.4) nmol/L in female newborns ($p<0.001$). The maximum value in male and female TERMNBW for n17-OHP were respectively, 86.8 and 51.4 nmol/L. On the contrary, the median (range) of female nIRT ($p=0.009$) and nMSUD ($p=0.001$) were greater than their male counterparts. The median (range) of nIRT in female was 21.4 (168.8) µg/L and in male was 19.2 (170.5) µg/L. 1.9 (14.9) and 1.8 (4.0) mg/dL were the respective nMSUD values. The highest nMSUD values observed were 14.9 and 4.0 mg/dL respectively in female and male newborns of this group. The median (range) of nBIOT in female was 202.2 (353.0) and in male was 190.2 (378.8) U. The value of nGAL for both genders was 1.2 (66.1) mg/dL in this group.

The male newborns of TERMLBW group also reflected a higher n17-OHP level [22 (47.7) nmol/L] than the female newborns [17.7 (48.4) nmol/L] ($p<0.001$), as depicted in Figure 2.

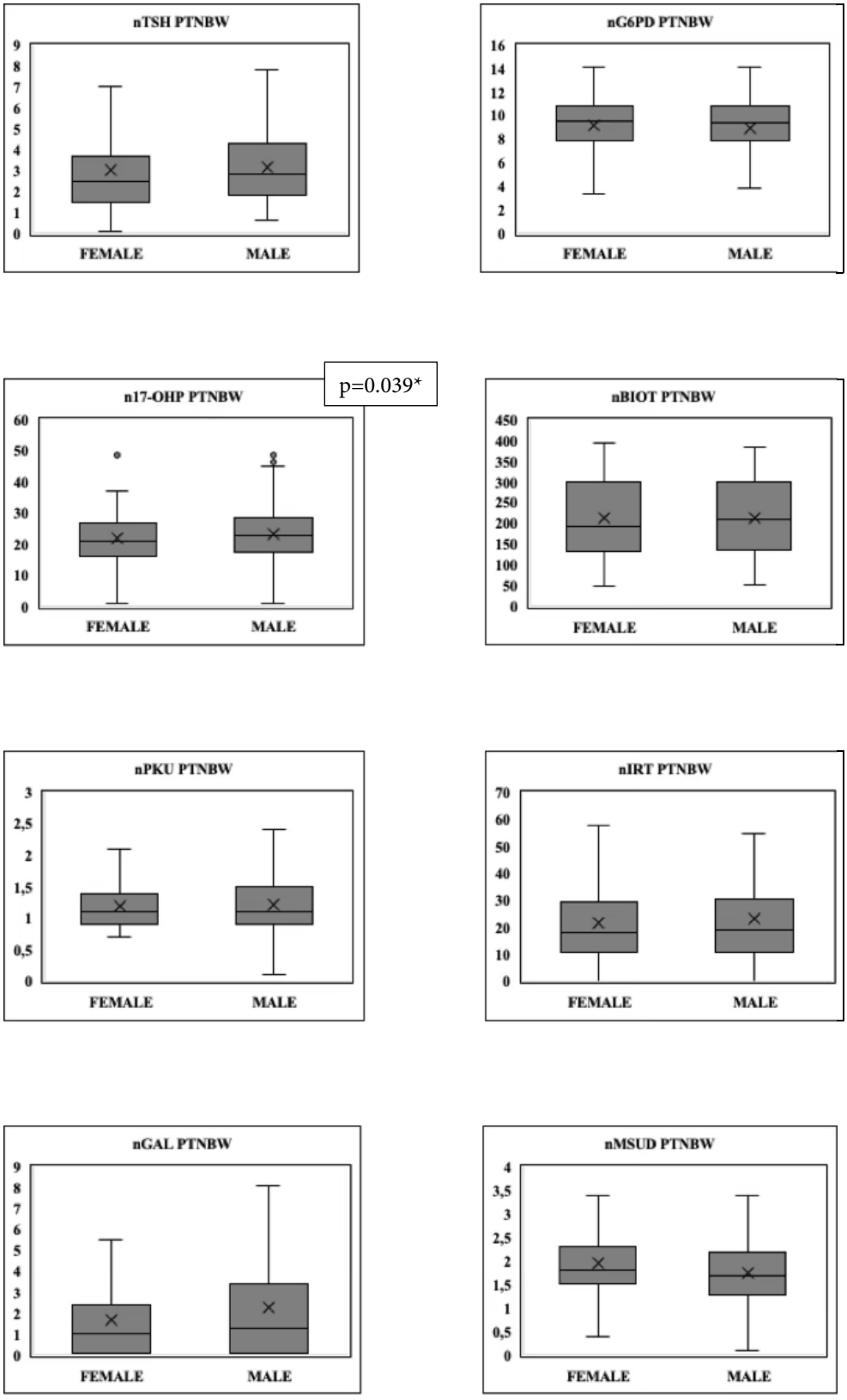
Figure 2: Gender wise comparison of DBS NBS parameters in TERMLBW study group.



*denotes significance value of Mann-Whitney U test

Similarly, PTNBW male newborns recorded a higher median (range) of 22.9 (47.7) nmol/L compared to female [20.8 (47.7) nmol/L] counterparts in this group (p=0.039) (Figure 3).

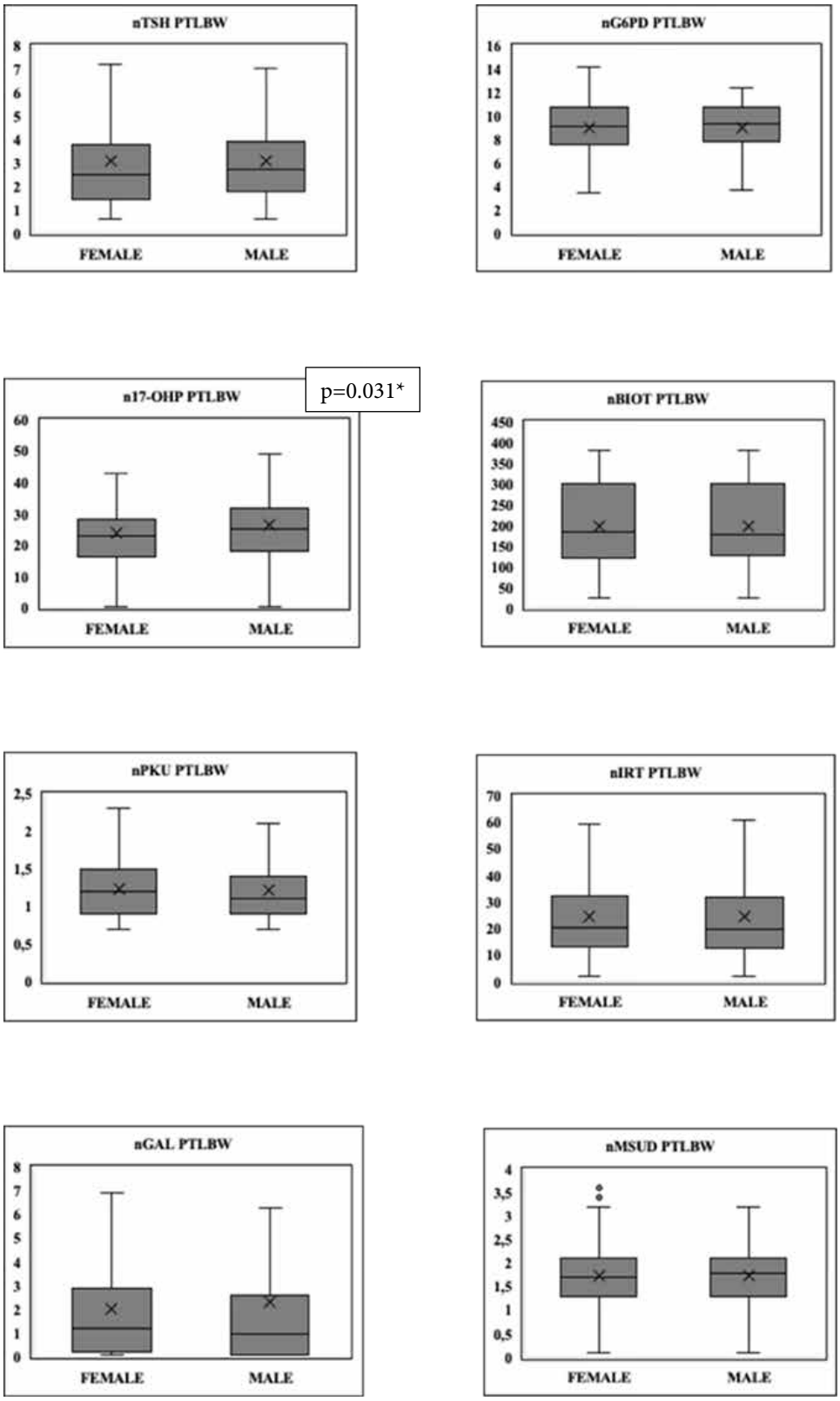
Figure 3: Gender wise comparison of DBS NBS parameters in PTNBW study group.



*denotes significance value of Mann-Whitney U test

Other NBS parameters did not show very significant variations between the genders in both the groups. Likewise, no significant differences for NBS parameters between male and female PTLBW were observed except for n17-OHP as revealed in Figure 4.

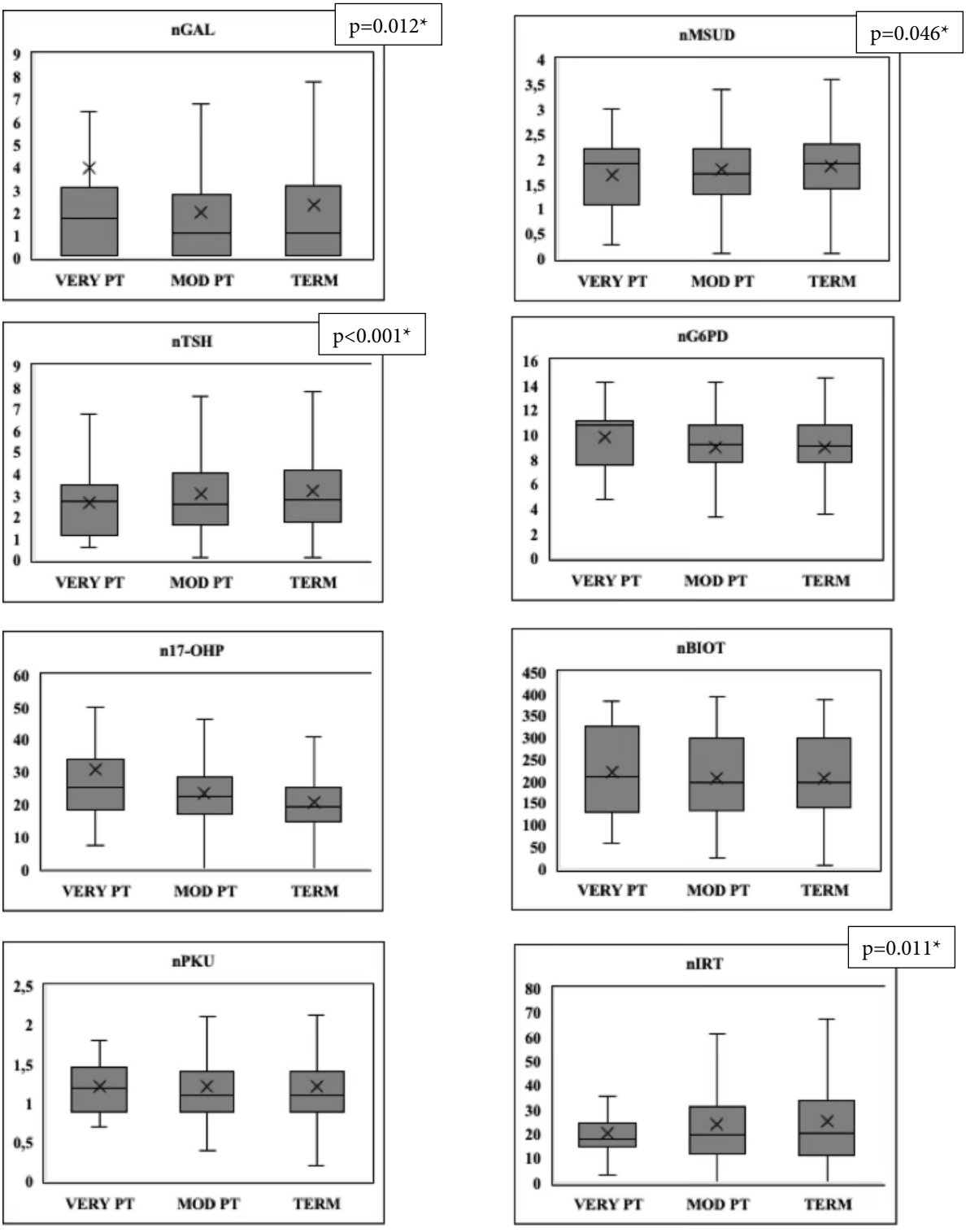
Figure 4: Gender wise comparison of DBS NBS parameters in PTLBW study group.



*denotes significance value of Mann-Whitney U test

In this group, the male newborns reported a median (range) value of 25.3 (142.8) nmol/L n17-OHP and females reported a value of 22.9 (72.3) nmol/L (p=0.031). The maximum valued detected in male and female newborns were 143.0 and 72.8 nmol/L, respectively.
The NBS parameters were also analysed for differences in values as per the gestational age and birth weight of the newborns included in the study, as illustrated in Figures 5,6. The median (range) nTSH showed significant differences among the term, moderate PT and very PT born newborns (p=0.012), as shown in Figure 5.

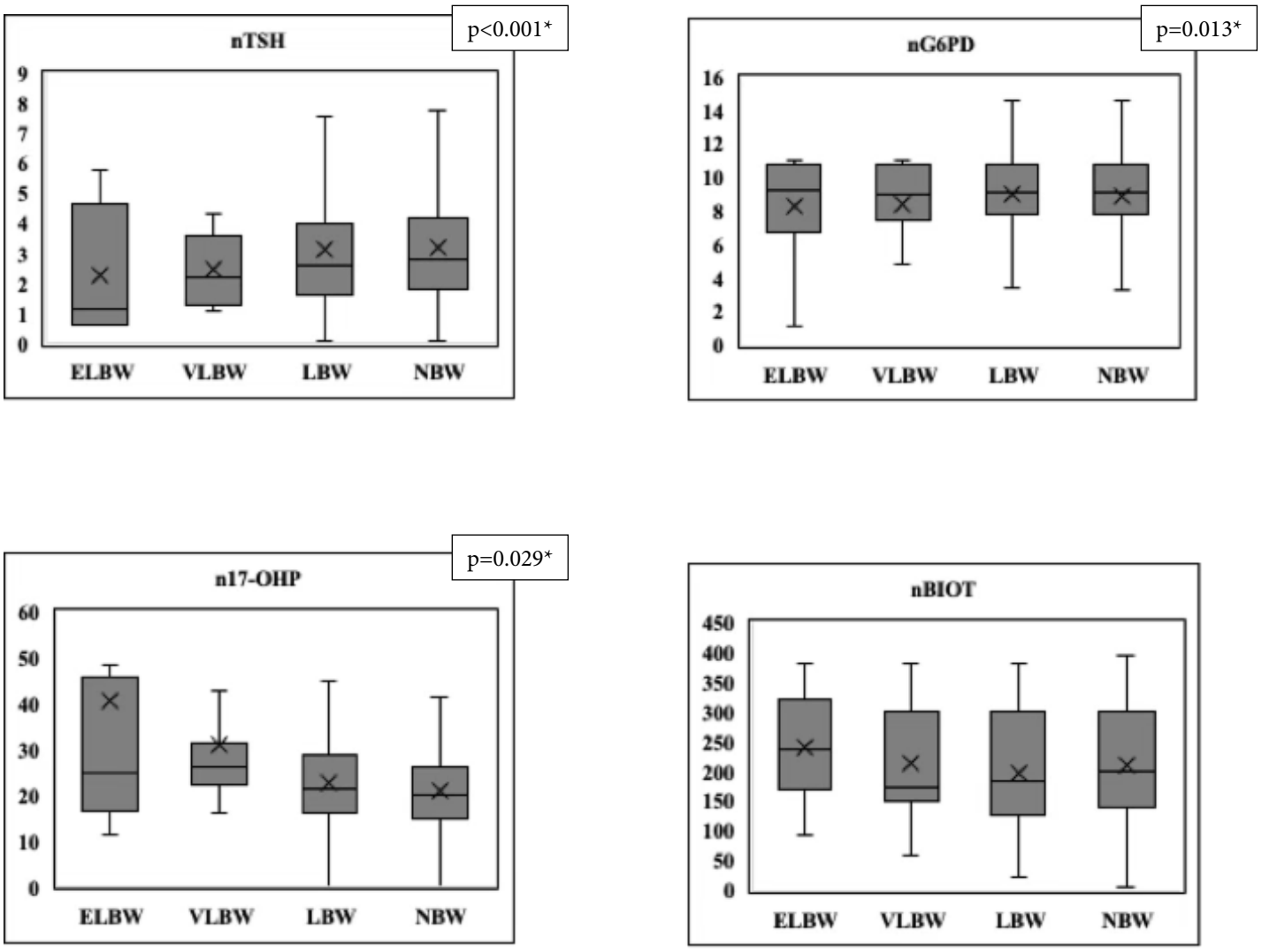
Figure 5: Distribution of NBS parameters as per the gestational age of the newborns in the study population.

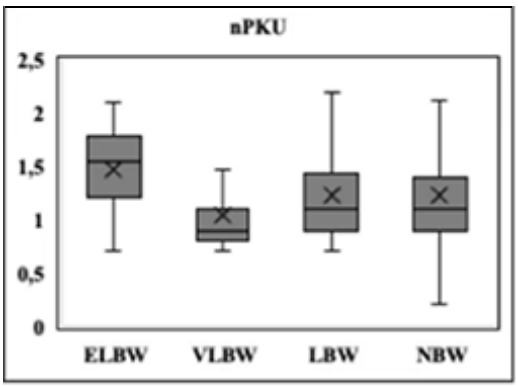


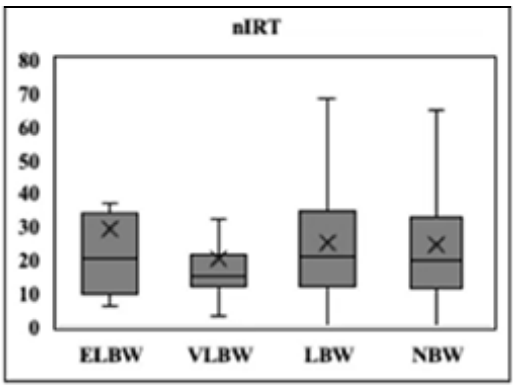
*denotes significance value of Independent-samples Kruskal-Wallis one way ANOVA test after Bonferroni correction for multiple tests

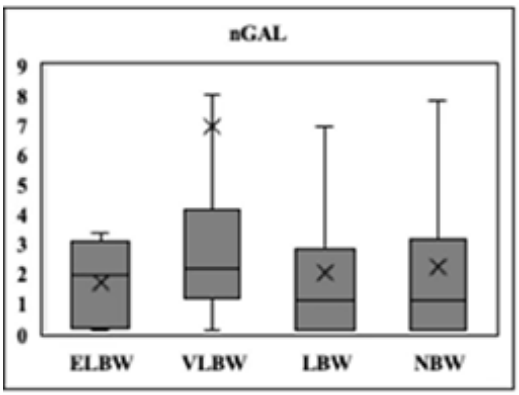
2.8 (16.8) mIU/L in term born was higher than 2.6 (16.8) mIU/L in moderate PT born (p=0.02). The median (range) in very PT was 2.7 (6.2) mIU/L. The nG6PD values varied as per the gestational age (p=0.046). The median (range) of 10.8 (9.4) IU/gHb observed in very PT was slightly higher than 9.1 (17.1) IU/gHb reported by term newborns (p=0.05) and 9.2 (14.0) IU/gHb reported by moderate PT (p=0.08). n17-OHP depicted significant variation as per the gestational age (p<0.001). Compared to the term born, the moderate PT (p<0.001) and very PT (p=0.016) recorded significantly higher levels. The median (range) values in term, moderate term, and very PT were respectively, 19.4 (86.8), 22.7 (78.6), and 25.2 (135.5) nmol/L. The respective nBIOT values were 196.9 (378.8), 196.1 (368.9), and 209.3 (322.8) U. For nPKU the levels were 1.1 (13.3), 1.1 (11.9), and 1.2 (1.6) mg/dL respectively. The nIRT values observed in the three groups were 20.2 (341.8), 19.7 (255.9), and 18.2 (46.0) µg/L. The respective values for nGAL were 1.1 (66.1), 1.1 (22.5), and 1.8 (66.1) mg/dL. No statistical differences were observed for nBIOT, nPKU, nIRT, and nGAL in the newborns as per their gestational age. nMSUD [1.9 (14.9) mg/dL] in term born was slightly higher than the moderate PT [1.7 (20.9) mg/dL] (p=0.011) born newborns. The value in very PT newborns was 1.9 (2.7) mg/dL. Distribution of NBS parameter according to their birth weight has been graphed in Figure 6.

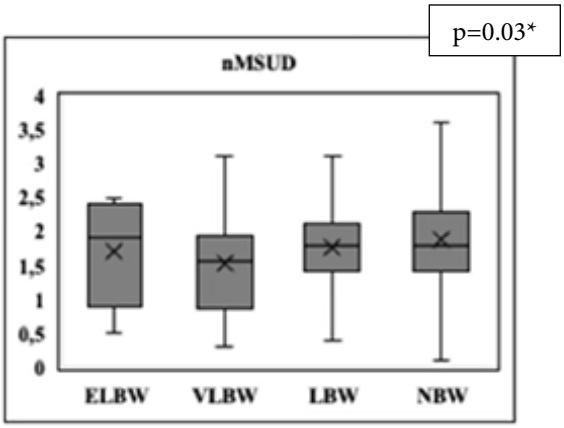
Figure 6: Distribution of NBS parameters as per the birth weight of the newborns in the study population.











*denotes significance value of Independent-samples Kruskal-Wallis one way ANOVA test after Bonferroni correction for multiple tests

nTSH and nG6PD values did not vary significantly as per the birth weight of the newborns. The NBW, LBW, VLBW, and ELBW values for nTSH were 2.8 (16.8), 2.6 (16.8), 2.2 (3.2), and 1.2 (5.2) mIU/L respectively. The median (range) of nG6PD in these newborns were 9.1 (14.2), 9.1 (14.4), 9.0 (10.3), and 9.3 (10.0) IU/gHb, respectively. The respective levels for n17-OHP were 19.9 (86.8), 21.6 (75.6),26.0 (62.8), and 24.9 (131.6) nmol/L, respectively ($p<0.001$). VLBW newborns showed a higher value compared to LBW ($p=0.019$) and NBW (0.002). Similarly, in LBW newborns n17-OHP was higher than NBW ($p<0.001$). nBIOT median (range) value in NBW [201.3 (385.7) U] was significantly greater than 181.8 (358.5) U in LBW newborns($p=0.01$). The levels in VLBW and ELBW were 171.5 (324.6) and 236 (291.1) U. A lower nPKU level was observed in VLBW newborns compared to NBW ($p=0.023$), LBW ($p=0.019$), and ELBW ($p=0.004$). The nIRT median (range) values were 19.6 (255.9), 20.7 (341.8), 15.3 (62.5), 20.4 (102.6) µg/L in NBW, LBW, VLBW, and ELBW newborns respectively. The respective nGAL were 1.1 (66.1), 1.1 (19.8), 2.2 (66.1), and 2.0 (3.3) mg/dL. nIRT and nGAL showed no significant variations among the newborns as per their birth weight, but nMSUD did show differences ($p=0.03$). nMSUD in NBW newborns depicted a median (range) of 1.8 (20.9) mg/dL compared to LBW newborns 1.8 (3.6) mg/dL ($p=0.047$).

The percentile values of the NBS parameters have been detailed in Table 3.

Table 3: Percentile distribution of NBS parameters in the study groups.

	Study Groups	1.0th	2.5th	3.0th	99th
nTSH (mIU/L)					
	TERMNBW	0.6	0.6	0.7	9.2
	TERMLBW	0.5	0.6	0.6	10.6
	PTNBW	0.6	0.6	0.7	8.6
	PTLBW	0.6	0.6	0.6	16.9
nG6PD (IU/gHb)					
	TERMNBW	2	4.4	4.8	12.9
	TERMLBW	1.6	4.4	4.6	13.6
	PTNBW	1.4	2.8	4.1	13.2
	PTLBW	1.6	4.3	4.5	12.4
n17-OHP (nmol/L)					
	TERMNBW	0.9	6.4	7.3	48.6
	TERMLBW	0.7	6.9	8.2	48.6
	PTNBW	4.9	7.8	8	48.6
	PTLBW	1.1	5.7	6.5	70.2
nBIOT (U)					
	TERMNBW	52.7	63.7	66.7	382.5
	TERMLBW	55.3	62.7	65.3	382.5
	PTNBW	54.2	64.9	67.4	382.5
	PTLBW	48	57.9	58.9	382.4
nPKU (mg/dL)					
	TERMNBW	0.7	0.7	0.7	2.4
	TERMLBW	0.7	0.7	0.7	2.1
	PTNBW	0.7	0.7	0.7	2.3
	PTLBW	0.7	0.7	0.7	2.3
nIRT (µg/L)					
	TERMNBW	2	2	2.8	95
	TERMLBW	0.23	2	2	99.6
	PTNBW	1.2	2	2	72.8
	PTLBW	2	3.7	4.3	76
nGAL (mg/dL)					
	TERMNBW	0.1	0.1	0.1	14.9
	TERMLBW	0.1	0.1	0.1	18.4
	PTNBW	0.1	0.1	0.1	12.7
	PTLBW	0.1	0.1	0.1	14.3
nMSUD (mg/dL)					
	TERMNBW	0.2	0.4	0.5	3.6
	TERMLBW	0.3	0.4	0.5	3.5
	PTNBW	0.1	0.4	0.5	3.4
	PTLBW	0.3	0.5	0.6	3.4

The 99th URLvalue was considered the highest reference value for nTSH, n17-OHP, nPKU, nIRT, nGAL, and nMSUD because higher levels are considered as screening positive. The 1.0th LRL was taken as the lowest reference value for nG6PD and nBIOT where lower values are said to be positively screened for the disorders. The 99th URL of nTSH in TERMNBW was 9.2mIU/L. The same in TERMLBW, PTNBW, and PTLBW were 10.6, 8.6, and 16.9 mIU/L respectively. The 1.0th LRL

for nG6PD in the four groups were respectively, 2.0, 1.6, 1.4, and 1.6 IU/gHb. The 99th percentile newborns depicted a value of 48.6 nmol/L for n17-OHP in all groups except PTLBW in which the value was 70.2 nmol/L. The 1.0th LRL value of nBIOT in TERMNBW was 52.7 U, in TERMLBW was 55.3 U, in PTNBW was 54.2 U and PTLBW was 48.0 U. For nPKU, 99th URL value was close to 2.3 mg/dL. The same for nIRT in the four groups were respectively, 95.0, 99.6, 72.8, and 76.0 µg/L. The 99th URL for nGAL were 14.9 mg/dL for TERMNBW, 18.4 mg/dL for TERMLBW, 12.7 mg/dL for PTNBW, and 14.3 mg/dL for PTLBW. nMSUD values for the same were close to 3.5 mg/dL in the four groups.

Discussion

NBS is not widely accepted in Indian scenario compared to the western countries. Data regarding the cut-off range is not much studies in Indian hospital setup. Lack of awareness and lack of infrastructure are the most common causes to limit NBS testing. However, in recent years, lab clinicians have become active in establishing metabolic lab to aid the physicians in providing diagnostic set up for the inborn errors of metabolic disorders (IEMD). But till date, a defined reference value for NBS parameters is yet to be determined. It is high time to generated population-based reference value for these parameters. As per inclusion criteria, we evaluated 2860 newborns who have undergone NBS in our set up and had a confirmatory report for the parameter.

The TERMNBW group reflected higher median level than the other study groups (Table 2). Term born neonates depicted higher median than PT born neonates. Similarly, the levels were highest in NBW and lowest in ELBW neonates (Supplementary Table 1). Studies have depicted normal nTSH in the first sample of preterm and LBW newborns, but in later weeks the levels are elevated and returns back to normal term neonate level after 6-8 weeks [14]. Delayed TSH elevation, usually 11 – 176 days of birth, has been reported in LBW, VLBW, and ELBW in New England NBS Program and Wisconsin NBS program [15,16]. Most NBS program do not perform a second testing if nTSH in the first sample is within normal cut-off in preterm/LBW newborns. It is now recommended that a second specimen at 2 – 4 weeks is required is these newborns and sometimes a third sample also at 6 – 8 weeks of birth [17]. LaFranchi et al explained about reduced TSH surge in preterm babies than term babies due to immature hypothalamic-pituitary-thyroid (HPT) axis and slow recovery to normalization of this physiological axis. This axis starts maturing in later half of pregnancy, but normal feedback mechanism is not active until term gestation [18]. The highest reference value for cut-off (99th percentile) in TERMNBW was 9.2 mIU/L whereas for PTLBW was 16.9 mIU/L (Table 3). The lab cut-off value as per kit brochure was 10 mIU/L. Nascimento et al study on 74123 children in Santa Catarina, suggested to decrease the cut-off from 10 to 6 mIU/L for a better sensitivity for detecting the newborns with thyroid dysfunctions [19]. On the contrary, Verma et al study on 174000 neonates in various hospitals in North India reported an optimal cut-off value of ≥ 20 mIU/L for deciding further clinical evaluation, balancing the recall rate and false negativity [20]. The recall rate for nTSH screening increased by 2% when reference value was reduced from 20 mIU/L to 10 mIU/L [21]. The availability, affordability and awareness are the mainstay for NBS program to be a success. A very high recall rate (at a very low reference value of 6 mIU/L) might influence a high-cost burden on health system and a very low sensitivity (due to very high cut-off of 20 mIU/L) might lead to mis-diagnosis of thyroid disorder. Hence, it is suggested that a reference value of 10 mIUL should be considered as an optimal level for all categories of newborn. Due to a high dynamicity observed in nTSH levels in the first few weeks of birth, it is also recommended to adopt at least 2-screen settings at 4-6 weeks, especially PT and LBW newborns.

nGDPD in TERMNBW showed a higher median and range than the other study groups (Table 2). The levels in very PT and ELBW newborns were lowest with least range (Supplementary Table 2). Yang et al study reported the mean (SD) G6PD activity level in term infants under 30 days of age to be 13.6 (3.7) U/g Hb. The suggested cut-off value to differentiate normal from G6PD deficient heterozygotes in infants below 30 days of age was 9.35 U/g Hb. The implied cut-off to differentiate G6PD deficient female heterozygote and male hemizygote was 3.85 U/g Hb [22]. Algur et al performed the quantitative neonatal G6PD screening on healthy term newborns born to Sephardic Jewish mothers. The analysis was performed in umbilical cord sample and presented a median (range) value of 0.28 (0.01-6.2) U/g Hb in G6PD deficient male newborns and 18.76 (9.0-34.66) U/g Hb in normal male newborns. Similarly, the values were respectively 4.84 (0.06-6.96) and 18.36 (9.54-35.5) U/g Hb in deficient and normal female newborns. The study reported that the enzyme activity values were significantly lower in these newborns compared to African-American male neonates with G6PD variant [2.55 (2.15-3.05) U/g Hb, $p<0.001$], implying the role of ethnicity on different G6PD variants and severity (23). Mukherjee et al study on NBS for G6PD deficiency in Eastern India published a prevalence of 0.48% in term and late preterm newborns. The cut-off values used was 6.95 U/g Hb in the cord blood sample [24]. In the present study, the G6PD values differed significantly in newborns as per the gestational age ($p=0.046$, Supplementary Table 2), but not so considering their birthweight (Supplementary Table 2) or gender (Table 1). Our study presented a cut-off (1.0th percentile) of 2.0 U/g Hb in TERMNBW and 1.6 U/g Hb for others (Table 3). However, a genotype analysis for various G6PD variants would provide a more accurate insight regarding the type and severity.

Hayashi et al study observed the highest median (range) n17-OHP levels in newborns with BW ≤ 1500 g [38.7 (1.5-155.5) nmol/L] and the lowest in NBW neonates [15.6 (0.5-169) nmol/L]. The cut-off at 99.8th percentile determined for NBW was 60 nmol/L and 117 for LBW newborns [25]. van der Kamp

et al study suggested that the cut-off for n17-OHP should be based on BW and gestational age. The observed median (range) values in DBS samples in ELBW newborns was highest, 86 (9 – 603) nmol/L compared to newborns with higher BW. The lowest values were observed in newborns with BW ≥ 4000 g [17 (1-45) nmol/L]. Similarly, highest levels, 99 (9-603) nmol/L was seen in neonates ≤ 28 weeks of GA and lowest, 16 (2-32) nmol/L in those born ≥ 41 weeks. The study revealed a significant negative correlation between n17-OHP with BW and GA. However the regression model suggested GA be a better predictor for n17-OHP than BW because adrenal gland development is related to GA of fetus [26]. Choi et al study too noted significant inverse association of n17-OHP levels with GA and the number of false positive to be higher in PT born neonates [27]. Similar finding was also reported by Seeralar et al study [28]. Prasad et al study suggested a cut-off >30 nmol/L for term newborns, ≥ 40 nmol/L for PT newborns weighing <2500 g and ≥ 30 for PT weighing > 2500 g [1]. In agreement, we observed a significant negative correlation with GA and BW ($p<0.001$, data not shown). The present study figured out a cut-off of 48.6 nmol/L for the study population.

A noteworthy finding observed in the present study was the significant differences in 17-OHP values among the various study groups and subgroups. Studies have reported that newborn 17-OHP levels to be greatly influenced by various factors. The anxiety and trauma associated with child delivery leads to elevated physiological 17-OHP levels which gradually decreases in next 12-48 hours [29]. Similarly, higher values typically recorded in male newborns, PT babies, babies with LBW, babies exposed to stressful events during antenatal period [29,30]. Besides, the 17-OHP values are also influenced by the laboratory methodology used for screening, where presence of other steroids might show some degree of antibody cross reactivity [31]. Therefore, it is suggested that a careful consideration is required for interpreting the results. The laboratories should establish an adjusted cut-off value based upon time of collection of samples, baby's gender, gestational age, birth weight, antenatal history, and type of laboratory method, to minimize the false positives. A second-tier testing method like LCMS would increase the accuracy of diagnosis.

nBIOT levels depicted a significant difference among the study groups (Table 2), more so because the median (range) was significantly higher in NBW group than the LBW neonates (Supplementary Table 4). The biotinidase enzyme cleave the dietary biocytin to release biotin to be available for various enzymes involved in catalysing important metabolic reactions [1]. Semeraro et al study recorded a high incidence, 1:8797 of partial biotinidase deficiency in Italy, and carried 1330G>C (D444H) variant in the neonates. The false positive rate was 0.3% with a cut-off of 85 U/dL. The median (IQR) levels were lower in LBW [203.65 (75.75) U/dL] than NBW newborns ($p<0.05$) and in PT [213.7 (75.8) U/dL] than the term neonates ($p<0.05$). nBIOT values correlated positively with GA, BW and age of DBS specimen. Besides, the study also observed a seasonal variation of nBIOT levels with a significantly lower values in summer seasons. Hence it is recommended that each lab must set a cut-off value based on the GA and BW and season. A repeat specimen is suggested within 40 days of first NBS for a early diagnosis of partial biotinidase deficiency [32]. The cut-off value suggested by Prasad et al study was <58.5 U for term and PT newborns weighing >2500g, and <55 U for PT weighing <2500g (1). Considering the overall 1.0th percentile in all study groups and sub-groups, the present study considers 48 U, the 1.0th value in NBW group, as the cut-off value (Table Supplementary 4).

No significant differences in nPKU level were observed between the study groups (Table 2). However, the ELBW depicted a higher median level than the other sub-groups (Supplementary Table 5). The finding could be due to the fact that ELBW newborns are usually under parenteral preparations supplemented with essential amino acids or enteral feeding with fortified human or bovine milk [33]. This might be challenging in ELBW newborns and hence, need to be closely monitored.

Newborn screening for cystic fibrosis (CF) through measurement of IRT is crucial for early diagnosis and quality of life in affected patients. Arrundi-Moreno et al study obtained a cut-off value of 76.2 ng/mL for predicting CF with a sensitivity of 95.7% and specificity of 64.5%. The median nIRT values were higher in very PT (78.35 ng/mL) and late PT (73 ng/mL) newborns compared to term (70.12 ng/mL) newborns [34]. On the contrary, the values were lower in PT group in our study. Prasad et al study established a cut-off of >60 mg/mL based on the 98.5th and 99.6th percentiles for term and PT newborns [1]. The cut-off obtained for early diagnosis for our population was 72.8 µg/L for PT and 95 µg/L term newborns. Variation of nIRT in newborns requires more studies to determine the initial cut-off for screening CF.

Early diagnosis of galactosemia and restricted dietary content of galactose and lactose is crucial for preventing long term complications. It was observed that even in presence of residual galactose-1-uridyltransferase (GALT) activity, symptoms of galactosemia persists which might be due to different variants. Dilemma in determining factors and variants suggested a lower cut-off limit for NBS for TGAL [35]. Succoio et al published a total blood galactose (TGAL) cut-off of 7 mg/dL in 44334 newborns screened in Campania that led to 50% increase galactosemia cases [36]. Present study observed a cut-off of 14.9 mg/dL for term and 12.7 mg/dL for PT newborns (Table 3).

Children diagnosed with MSUD present with different symptoms and severity and thus diagnosing at an early age is indispensable to prevent neurological damage and its sequel [37]. The levels depicted significant differences between TERMNBW and PTLBW groups. PT newborns showed a wide range compared to others. Initiation of parenteral nutritional supplement with required amino acids in PT newborns might have influenced the results of MSUD in DBS specimen. A

value of 3.4 mg/dL might be considered as the cut-off level for MSUD cases. Hence, a judicious evaluation is required for PT newborns undergoing NBS testing.

The major limitation of the study is that sensitivity and specificity could not be determined for the cut-off values as recall were made for those who were screened positive in the initial screening. Secondly, genetic analysis was not performed for confirmation of the variants. However, the major strength of the study is the large sample size and the various study groups and sub-groups included such as LBW, VLBW, ELBW, Moderate PT and very PT in male and female newborns born in a tertiary centre in Central India. A detailed quantitative determination in so many groups for all the eight NBS parameters in Indian newborns yet to be published. Hence, the study provides a foundation for researchers from various other region to report the overall cut-off for their respective newborn population.

Conclusion

The study has provided a detailed comparison and reference values observed in various study groups and sub-groups. The findings suggest more accurate reference cut-off levels to determined considering the gestational age and birth weight of the newborns. More, so each lab must set up the cut-off levels based on their methodology and population inflow as significant variation across individuals might be there. Significant differences in 17-OHP values among the study groups and sub-groups was noteworthy indicating the need to establish an adjusted cut-off value based upon gender, birth weight, and gestational age. NBS is crucial for a newborn to have a healthy quality life. Genetic diversity might influence few parameters like G6PD, biotinidase and CF and thus there is need for improvising detection and genetic guidance to the parents. Future research is required to be undertaken in large cohort from different geographical regions of a country to have more accurate adjusted cut-offs and biological references for the NBS parameters in newborns.

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The project was approved as intramural non-funded project. All tests undertaken for the research purpose were performed free of cost for the participants.

Ethical consideration

The study was reviewed and approved by the Institute Research Cell and Institute Ethics Committee and had been performed in accordance with the ethical standards described in an appropriate version of the 1975 Declaration of Helsinki, as revised in 2000. Written/verbal informed consent was taken from their legal representatives for the study.

Consent for publication

As per the IEC approved patient information sheet and informed consent, all participants have been explained that the research study shall be published in scientific meetings and journals and they were enrolled only after their consent. All authors have reviewed and agreed to the manuscript in the present form for submission.

Availability of data and material

The data is presently available with the principal investigator (Corresponding Author). It is not available in the public domain due to privacy or ethical restrictions. It be made available from the corresponding author upon reasonable request. The letter shall be reviewed and approved by all the authors and the Head of the Institution, following which it shall be made available.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Authors contribution

SP, and RN developed the concept proposal. SP and EM formulated the study design. RN and EM provided intellectual content for the study. SP and SS performed the literature search. SP, NV, SS and RN performed data acquisition, sample collection and laboratory analyses. SP, NV and RN performed the statistical data analysis. SP and RN prepared the first draft of the manuscript. EM and SS performed the manuscript editing and all authors performed the final manuscript review. All authors read and approved the final manuscript.

Abbreviations

ACHDNC – Advisory Committee on Heritable Disorders in Newborn and Children
ANOVA – one way analysis of variance
BW – birth weight
CDC – Center of Disease Control
CF – cystic fibrosis
DBS – dried blood spot
ELBW – extremely low birth weight
ELISA – enzyme linked immunosorbent assay
GA – gestational age
GALT – galactose-1-uridyltransferase
HPT – hypothalamic-pituitary-thyroid
IEMD – inborn errors of metabolic disorders
IQR – Interquartile range
LBW – low birth weight
LCMS – liquid chromatography mass spectrometry
LRL – lower reference limit
n17-OHP – newborn 17-hydroxyprogesterone
nBIOT – newborn biotinidase
NBS – newborn screening
NBW – normal birth weight
nG6PD – newborn glucose 6-phosphate dehydrodenase
nGAL – newborn galactose
nIRT – newborn immunoreactive trypsinogen
nMSUD – newborn maple syrup urine disease
nPKU – newborn phenylketonuria
NSQAP – Newborn Screening Quality Assurance Program
nTSH – newborn thyroid stimulating hormone
PT – preterm

TGAL – total blood galactose
URL – upper reference limit
VLBW – very low birth weight

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Supplementary Tables

Supplementary Table 1: Mean (SD) and median (range) and percentile values of the DBS nTSH (mIU/L) in the study groups.

	Study groups	Mean	SD	Median	Range	Min-Max	P value	1.0th
Gestational age							0.012*	
1	**Term**	3.2	2.1	2.8	16.8	0.1-16.9	1v2=0.02*	0.6
2	**Moderate PT**	3.1	2.2	2.6	16.8	0.1-16.9		0.6
3	**Very PT**	2.7	1.7	2.7	6.2	0.6-6.8		0.6
Birth weight							0.03*	
1	**NBW**	3.1	2.3	2.6	16.8	0.1-16.9		0.6
2	**LBW**	2.5	1.1	2.2	3.2	1.1-4.3		1.1
3	**VLBW**	2.5	1.1	2.2	3.2	1.1-4.3		1.1
4	**ELBW**	2.3	2.1	1.2	5.2	0.6-5.8		0.6

*denotes significance value of Independent-samples Kruskal-Wallis one way ANOVA test after Bonferroni correction for multiple tests;
PT denotes preterm; NBW denotes normal birth weight (BW); LBW denotes low BW; VLBW denotes very LBW; ELBW denotes extremely LBW; Term denotes > 37 gestational weeks (GW); Moderate PT denotes 32 to 37 GW; Very PT denotes 28 to <32 GW; NBW denotes BW ≥ 2500 g; LBW denotes BW 1500 to < 2500 g; VLBW denotes BW 1000 to <1500 g; ELBW denotes BW <1000 g.

Supplementary Table 2: Mean (SD) and median (range) and percentile values of the DBS nG6PD (IU/gHb) in the study groups.

	Study groups	Mean	SD	Median	Range	Min-Max	P value	1.0th	2.5th	3.0th	99th
Gestational age							0.046*				
1	**Term**	8.9	2	9.1	17.1	0.1-17.2	1v3=0.052*	1.9	4.4	4.8	13
2	**Moderate PT**	8.9	2.1	9.2	14	0.2-14.2		1.3	4	4.3	12.4
3	**Very PT**	9.8	2.3	10.8	9.4	4.8-14.2		4.8	4.8	4.8	4.8
Birth weight							0.77*				
1	**NBW**	9	1.9	9.1	14.4	0.2-14.6		2.7	4.4	4.8	12.4
2	**LBW**	8.4	2.9	9	10.3	0.8-11.1		0.8	0.8	0.8	
3	**VLBW**	8.4	2.9	9	10.3	0.8-11.1		0.8	0.8	0.8	
4	**ELBW**	8.3	3.4	9.3	10	1.1-11.1		1.1	1.1	1.1	

*denotes significance value of Independent-samples Kruskal-Wallis one way ANOVA test after Bonferroni correction for multiple tests, PT denotes preterm, NBW denotes normal birth weight (BW), LBW denotes low BW, VLBW denotes very LBW, ELBW denotes extremely LBW, Term denotes > 37 gestational weeks (GW), Moderate PT denotes 32 to 37 GW, Very PT denotes 28 to <32 GW, NBW denotes BW ≥ 2500 g, LBW denotes BW 1500 to < 2500 g, VLBW denotes BW 1000 to <1500 g, ELBW denotes BW <1000 g.

Supplementary Table 3: Mean (SD) and median (range) and percentile values of the DBS n17-OHP (nmol/L) in the study groups.

	Study groups	Mean	SD	Median	Range	Min-Max	P value	1.0th	2.5th	3.0th	99th
Gestational age							<0.001*				
1	**Term**	20.5	8.5	19.4	86.8	0.2-87.0	1v2<0.001*	0.9	6.6	7.3	48.6
2	**Moderate PT**	23.5	9.9	22.7	78.6	0.2-78.8	1v3=0.016*	1.6	6.3	7.9	48.6
3	**Very PT**	30.6	25.3	25.2	135.5	7.5-143.0		7.5	7.5	7.5	
Birth weight							<0.001*				
1	**NBW**	22.9	10.4	21.6	75.6	0.2-75.8	2v3=0.037*	1	6.1	7.2	48.9
2	**LBW**	30.8	15.8	26	62.8	16.0-78.8	1v3=0.002*	16	16	16	
3	**VLBW**	30.8	15.8	26	62.8	16.0-78.8	1v2<0.001*	16	16	16	
4	**ELBW**	40.6	43.1	24.9	131.6	11.4-143.0		11.4	11.4	11.4	

*denotes significance value of Independent-samples Kruskal-Wallis one way ANOVA test after Bonferroni correction for multiple tests, PT denotes preterm, NBW denotes normal birth weight (BW), LBW denotes low BW, VLBW denotes very LBW, ELBW denotes extremely LBW, Term denotes > 37 gestational weeks (GW), Moderate PT denotes 32 to 37 GW, Very PT denotes 28 to <32 GW, NBW denotes BW ≥ 2500 g, LBW denotes BW 1500 to < 2500 g, VLBW denotes BW 1000 to <1500 g, ELBW denotes BW <1000 g.

Supplementary Table 4: Mean (SD) and median (range) and percentile values of the DBS nBIOT (U) in the study groups.

	Study groups	Mean	SD	Median	Range	Min-Max	P value	1.0th	2.5th	3.0th	99th
Gestational age							0.74*				
1	**Term**	207.6	86.7	196.9	378.8	7.2-386.0		53.2	63.5	65.9	382.5
2	**Moderate PT**	207.4	88.8	196.1	368.9	24.0-392.9		51.7	59.6	63.3	382.5
3	**Very PT**	220.9	103.8	209.3	322.8	59.7-382.5		59.7	59.7	59.7	
Birth weight							0.006*				
1	**NBW**	198	86.9	181.8	358.5	24.0-382.5	1v2=0.005*	48	60.7	62.8	381.4
2	**LBW**	211.8	107.1	171.5	324.6	57.9-382.5		57.9	57.9	57.9	
3	**VLBW**	211.8	107.1	171.5	324.6	57.9-382.5		57.9	57.9	57.9	
4	ELBW	239.4	97.7	236.3	291.1	91.4-382.5		91.4	91.4	91.4	

*denotes significance value of Independent-samples Kruskal-Wallis one way ANOVA test after Bonferroni correction for multiple tests, PT denotes preterm; NBW denotes normal birth weight (BW), LBW denotes low BW, VLBW denotes very LBW, ELBW denotes extremely LBW, Term denotes > 37 gestational weeks (GW), Moderate PT denotes 32 to 37 GW, Very PT denotes 28 to <32 GW, NBW denotes BW ≥ 2500 g, LBW denotes BW 1500 to < 2500 g, VLBW denotes BW 1000 to <1500 g, ELBW denotes BW <1000 g.

Supplementary Table 5: Mean (SD) and median (range) and percentile values of the DBS nPKU (mg/dL) in the study groups.

	Study groups	Mean	SD	Median	Range	Min-Max	P value	1.0th	2.5th	3.0th	99th
"Gestational age"							0.83*				
1	**Term**	1.2	0.5	1.1	13.3	0.1-13.4		0.7	0.7	0.7	2.3
2	**Moderate PT**	1.2	0.6	1.1	11.9	0.1-12.0		0.7	0.7	0.7	2.3
3	**Very PT**	1.2	0.4	1.2	1.6	0.7-2.3		0.7	0.7	0.7	
"Birth											
weight"							0.68*				
1	**NBW**	1.2	0.6	1.1	11.3	0.7-12.0		0.7	0.7	0.7	2.2
2	**LBW**	1	0.4	0.9	1.6	0.7-2.3		0.7	0.7	0.7	
3	**VLBW**	1	0.4	0.9	1.6	0.7-2.3		0.7	0.7	0.7	
4	**ELBW**	1.5	0.4	1.5	1.4	0.7-2.1		0.7	0.7	0.7	

*denotes significance value of Independent-samples Kruskal-Wallis one way ANOVA test after Bonferroni correction for multiple tests, PT denotes preterm, NBW denotes normal birth weight (BW), LBW denotes low BW, VLBW denotes very LBW, ELBW denotes extremely LBW, Term denotes > 37 gestational weeks (GW), Moderate PT denotes 32 to 37 GW, Very PT denotes 28 to <32 GW, NBW denotes BW ≥ 2500 g, LBW denotes BW 1500 to < 2500 g, LBW denotes BW 1000 to <1500 g, ELBW denotes BW <1000 g.

Supplementary Table 6: Mean (SD) and median (range) and percentile values of the DBS nIRT (µg/L) in the study groups.

	Study groups	Mean	SD	Median	Range	Min-Max	P value	1.0th	2.5th	3.0th	99th
Gestational age							0.55*				
1	**Term**	25.3	20.7	20.2	341.8	0.1-341.9		2	2	2.5	95.3
2	**Moderate PT**	23.8	18.4	19.7	255.9	0.1-256.0		2	2	2	75.6
3	**Very PT**	20.3	10.6	18.2	46	3.4-49.4		3.4	3.4	3.4	
Birth weight							0.35*				
1	**NBW**	25.3	21.2	20.7	341.8	0.1-341.9		2	2	2	82.2
2	**LBW**	20.2	15.2	15.3	62.5	3.4-65.9		3.4	3.4	3.4	
3	**VLBW**	20.2	15.2	15.3	62.5	3.4-65.9		3.4	3.4	3.4	
4	**ELBW**	29.5	33.4	20.4	102.6	6.2-108.2		6.2	6.2	6.2	

*denotes significance value of Independent-samples Kruskal-Wallis one way ANOVA test after Bonferroni correction for multiple tests, PT denotes preterm, NBW denotes normal birth weight (BW), LBW denotes low BW, VLBW denotes very LBW, ELBW denotes extremely LBW, Term denotes > 37 gestational weeks (GW), Moderate PT denotes 32 to 37 GW, Very PT denotes 28 to <32 GW, NBW denotes BW ≥ 2500 g, LBW denotes BW 1500 to < 2500 g, LBW denotes BW 1000 to <1500 g, ELBW denotes BW <1000 g.

Supplementary Table 7: Mean (SD) a and median (range) and percentile values of the DBS nGAL (mg/dL) in the study groups.

	Study groups	Mean	SD	Median	Range	Min-Max	P value	1.0th	2.5th	3.0th	99th
Gestational age							0.51^				
1	**Term**	2.3	3.7	1.1	66.1	0.1-66.2		0.1	0.1	0.1	14.9
2	**Moderate PT**	1.9	2.7	1.1	22.5	0.1-22.6		0.1	0.1	0.1	12.8
3	**Very PT**	4	12.3	1.8	66.1	0.1-66.2		0.1	0.1	0.1	
Birth weight							0.13^				
1	**NBW**	2.1	2.9	1.1	19.8	0.1-19.9		0.1	0.1	0.1	13.9
2	**LBW**	6.9	16.5	2.2	66.1	0.1-66.2		0.1	0.1	0.1	
3	**VLBW**	6.9	16.5	2.2	66.1	0.1-66.2		0.1	0.1	0.1	
4	**ELBW**	1.7	1.4	2	3.3	0.1-3.4		0.1	0.1	0.1	

*denotes significance value of Independent-samples Kruskal-Wallis one way ANOVA test after Bonferroni correction for multiple tests, PT denotes preterm, NBW denotes normal birth weight (BW), LBW denotes low BW, VLBW denotes very LBW, ELBW denotes extremely LBW, Term denotes > 37 gestational weeks (GW), Moderate PT denotes 32 to 37 GW, Very PT denotes 28 to <32 GW, NBW denotes BW ≥ 2500 g, LBW denotes BW 1500 to < 2500 g, VLBW denotes BW 1000 to <1500 g, ELBW denotes BW <1000 g.

Supplementary Table 8: Mean (SD) and median (range) and percentile values of the DBS nMSUD (mg/dL) in the study groups.

	Study groups	Mean	SD	Median	Range	Min-Max	P value	1.0th	2.5th	3.0th	99th
"Gestational age"							0.011*				
1	**Term**	1.9	0.8	1.9	14.9	0.1-15.0	1v2=0.01*	0.2	0.4	0.5	3.6
2	**Moderate PT**	1.8	1	1.7	20.9	0.1-21.0		0.1	0.5	0.6	3.4
3	**Very PT**	1.7	0.7	1.9	2.7	0.3-3.0		0.3	0.3	0.3	
"Birth weight"							0.017*				
1	**NBW**	1.8	0.6	1.8	3.6	0.1-3.7	1v2=0.023*	0.3	0.5	0.5	4.5
2	**LBW**	1.5	0.8	1.6	2.8	0.3-3.1		0.3	0.3	0.3	
3	**VLBW**	1.5	0.8	1.6	2.8	0.3-3.1		0.3	0.3	0.3	
4	**ELBW**	1.7	0.8	1.9	2	0.5-2.5		0.5	0.5	0.5	

*denotes significance value of Independent-samples Kruskal-Wallis one way ANOVA test after Bonferroni correction for multiple tests, PT denotes preterm; NBW denotes normal birth weight (BW), LBW denotes low BW, VLBW denotes very LBW, ELBW denotes extremely LBW, Term denotes > 37 gestational weeks (GW), Moderate PT denotes 32 to 37 GW, Very PT denotes 28 to <32 GW, NBW denotes BW ≥ 2500 g, LBW denotes BW 1500 to < 2500 g, VLBW denotes BW 1000 to <1500 g, ELBW denotes BW <1000 g.