Neonatal Endocrine Labomas - Pitfalls and Challenges in Reporting Neonatal Hormonal Reports

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This review highlights pitfalls and challenges in interpreting neonatal hormone reports. Pre-analytical errors contribute to nearly 50% of all errors. Modern chemiluminescence assay are more accurate, have lower risk of Hook's effect, but continue to have problems of assay interference. Liquid chromatography mass spectroscopy is gold standard for most hormone assays. Neonatal hypoglycemia diagnostic cut-offs are lower than adults. Random growth hormone testing is of value in diagnosing growth hormone deficiency in neonates. 17-hydroxy-progesterone testing in first three days of life for congenital adrenal hyperplasia (CAH) remains a challenge due to cross-reactivity with maternal circulating steroids, prematurity and lack of adrenal maturation. Both T4 and TSH testing is encouraged after 48 hours of delivery for diagnosing neonatal hypothyroidism; repeat testing should be done immediately for confirmation of diagnosis. There is an urgent need to develop age- sex- and ethnicity-based normative data for different hormone parameters in neonates. Laboratory should develop their own neonatal references and avoid using ranges from manufacturers. In neonatal endocrinopathies, the clinical scenario should primarily dictate the treatment formulation with hormonal assay to supplement treatment.

Key words: *Birth defect, Diagnosis, Neonate, Thyroid function tests*.

No other clinical speciality is as integrally associated with laboratory sciences as Endocrinology. Laboratory endocrinology is a critical part of endocrinology care services; however, laboratory assays have their own pitfalls and fallacies. Further, interpretation of hormonal studies in neonates (first 4 weeks after birth) can often be complicated by prematurity, birth weight, infection, lesser developed immune system, immaturity of hypothalamic-pituitary-end gland axis, and issues relating to growth and development, which are not encountered in adults [1,2]. Lack of age-matched ethnicity-based normal data of many pediatric hormone parameters also makes the interpretation of a particular assay difficult. Generating normative data of hours, days and weeks after birth for different hormonal parameters is a difficult task to accomplish.

Hence it has been aptly stated that "Neonates are not little adults" [1]. Parameters which behave differently in neonates as compared to older children and adults have been elaborated in ***Box* I**. Lack of their recognition can lead to diagnosis of laboratory error-related non-existent diseases (labomas), and interventions directed against such reports can lead to easily avoidable clinical complications. The aim of this review is to highlight the modern-day pitfalls and challenges in interpreting neonatal hormone reports, and to highlight that all biochemical investigations should be interpreted with a clinical perspective; at the end of the day, we should treat the neonate and not the reports

PRE-ANALYTICAL CHALLENGES

More than 50% of all errors are believed to be due to pre-analytical errors, of which 20% are due to sampling errors [3]. The hormonal values estimated may be confounded by the timing, method used, type of sample (capillary, arterial, venous), medium of collection (plain, clot activator used, heparinized or EDTA), transport (room temperature or cold chain), age of the neonate, associated illness, stress, and even posture during sampling (supine, sitting or standing) [4]. Collection of samples like urine (especially 24 hour urine) and saliva are a challenge in neonates. Sample volume is a unique challenge, especially in premature neonates. Squeezing while collecting capillary blood sample can lead to hemolysis and spuriously high potassium levels. Pooling of samples is essential for hormones with fluctuating levels *e.g.*, luteinizing hormone (LH) and follicle stimulating hormone (FSH). For LH and FSH, it is recommended that

BOX 1 BIOCHEMICAL PARAMETERS WHICH DIFFER IN NEONATES AS COMPARED TO OLDER CHILDREN AND ADULTS

Cortisol	No diurnal variation in neonates
Tri-iodothyronine (T3)	Levels are more in neonates
Thyroid stimulating hormone	High in initial 3 days after birth
Glucose	Low in neonates with lower cut offs for hypoglycemia
Insulin like growth factor-1	Varies with age, nutrition and maturity
17-hydroxy-progesterone	Varies with gestational age and prematurity
Calcium	Very low values in neonatal period
Alkaline phosphatase	Levels are high in neonates
Albumin	Levels are low in neonates
Magnesium	Levels are low in neonates

three serum samples should be collected at 30 minutes apart, and the samples should be pooled before analysis to avoid missing of LH, FSH spikes, which can lead to false low values. Labile peptide hormones (parathyroid hormone, adrenocorticotrophic hormone) should be collected in pre-chilled syringes and vials, transported to laboratory in cold chain with immediate estimation to avoid false low values [3]. Growth hormone (GH), being a large peptide, is less susceptible to spontaneous breakdown at room temperature. However, if the sample needs to be transported to another laboratory, or if a delay is expected in sample processing and assessment, it is always a good practice to maintain cold chain to avoid false low values. Parathyroid hormone (PTH) and adreno-corticotrophic hormone (ACTH) should preferably be collected as plasma samples, to reduce processing time for immediate analysis and reporting. All other hormones can be tested from serum samples. Serum samples have increased processing time as the sample has to be left alone for some time for it to clot and serum to separate. This time may be especially increased in winters and cold temperatures. Timing of blood sampling is critical to correct interpretation of hormone reports. Basal cortisol, ACTH, PTH, basal sex steroids (including 17-hydroxy-progesterone) should ideally be measured in the early morning (6-9 am) due to diurnal variations, leading to highest levels in the early morning hours. Hormones assessing the renin-angiotensin-aldosterone axis (serum aldosterone and plasma renin activity) are not typically measured in neonates as this axis itself is not mature in the first year of life. However, whenever we are testing for serum aldosterone and plasma renin activity in older children and adults, the sensitivity of the test is increased if the sampling is done is sitting/standing position as compared to supine position. Long-term storage of blood samples should be done in the form of serum with -80^{0}C being the preferred temperature for storage. Repeated freezing and thawing of samples should be avoided to prevent hormone breakdown. Steroid hormones are stable molecules and can be measured safely in properly stored samples even years later. However, long-term storage is difficult for peptide hormones due to spontaneous breakdown. Hence, it is important for the treating doctor to be aware of the correct time, method of sample collection and processing when requesting for a particular hormone assay.

ANALYTICAL FACTORS

Sample Volume

Laboratories are usually not equipped to handle small volume samples from the neonates. The test tubes and micropipettes are the same as for adults. Neonatal sample requires special barcoding, which is usually not available. Persistence of fetal hemoglobin, bilirubin, and maternal placental steroids affect the analytical assay of several hormones. For microanalysis, the analytical dead space should be less then 50 µL [1,2].

Normative Data and Reference Ranges

Normal levels of many hormones are different in neonates as compared to adults (insulin like growth factor-1 (IGF-1), IGF binding protein-3 (IGFBP-3), 17-hydroxyprogesterone (17OHP), thyroid hormones, phosphorus, alkaline phosphate among others). Generation of age-matched ethnicity-based normative data for all hormone parameters is the need of hour. Although such data are available in certain parts of the globe, these are lacking in the developing world. Such data help in correct decision-making, and give us freedom from over/under-treatment of the neonate and associated complications. However, the generation of normative data is slowed by the cost involved, lack of resources, small size of samples available and lack of social, political and administrative will [2]. In the absence of population- and ethnicity-based normative data and reference ranges, laboratory

frequently depends upon data and ranges supplied by the manufacturer, which may not always be correct for the population evaluated. Hence, laboratories should always develop their own reference ranges. However, a laboratory that does not have access to a large neonatal patient base may not have the resources to determine neonatal reference intervals applicable to its own specific methods and analytical systems. Hence, clinicians should enquire about the source of the reference range(s) when faced with diagnostic dilemma. Apart from assay range, it is important for the clinican to be aware of the assay sensitivity, and intra-assay and inter-assay coefficienct of variation, which all are predictors of assay reliability.

Assay Interference

Maternal steroids persist in the circulation during the neonatal period, which cannot be differentiated by most of the assay platforms leading to labomas. Bioassays were the first hormonal assays, but had a huge limitation with the small size of the neonatal samples. The advent of immunoassays (initially radioimmunoassays (RIAs), which have now largely been replaced by chemiluminescence assays (CLIAs)) have been a breakthrough in hormonal assay systems. Some of the challenges seen with earlier RIAs like Hook's effect are extremely rare with modern CLIAs which huge assay range (*eg.*, Hook's effect does not occur with prolactin levels <20,000 ng/mL) [3]. Hook's effect is a phenomenon seen when the hormone (substrate) levels are very high, which binds to and fills all the antigen binding sites, preventing the desired antigen-subrate-antibody (sandwich) reaction to take place resulting in false negatives or inaccurately low results for the particule hormone/substrate. It was historically seen in single step sandwich immunoassays (immunoassays and nephelometric assays). However, some of the persisting challenges with CLIAs include the interference due to presence of antibodies to assay analytes, autoantibodies and heterophile antibodies, leading to suboptimal to exaggerated results. Free hormonal assays, and assay of hormones at very low levels (pg/mL) remains a challenge with CLIA. High performance liquid chromatography (HPLC), gas chromatography and mass spectroscopy are more robust, but much more costlier and less freely available assay platforms. Presently, they are primarily available at research centers. Liquid chromatography and mass spectroscopy have now become the gold standard for many of the hormonal analytes [1,2].

POTENTIAL CLINICAL SCENARIOS FOR LABOMAS

Neonatal Hypoglycemia

Neonatal hypoglycemia is commonly encountered in neonatal intensive care units. The cut-off for diagnosis ≤45 mg/dL in first 24 hours [5]. Prematurity, infection, high hematocrit, GH deficiency, adrenal insufficiency, metabolic factors and maternal diabetes have all been associated with neonatal hypoglycemia [6]. Whole blood and plasma blood samples can give different glucose values by up to 15 mg/dL. The methods used in glucometers is affected by blood type and hematocrit of the neonate [7]. The values can be spuriously low in neonates with polycythemia. Present day glucometers are calibrated as per the plasma glucose values. Almost all glucometers are calibrated for a blood glucose ranges of 60-160 mg/dL. Reading above and below this range tend to be erroneous [7,8]. Delay in laboratory assessment of blood glucose can lead to a drop in estimated values by 6 to 10 mg/dL/hour. Hence, neonatal hypoglycemia should never be diagnosed by a glucometer reading, and an urgent laboratory confirmation using venous blood should be sought immediately. However, corrective treatment should start immediately after withdrawing the sample for sending to laboratory.

Growth Hormone Deficiency

GH acts through stimulation of hepatic and peripheral IGF-I production and secretion. GH secretion patterns is different between neonates and older children. GH peaks are higher in neonates, and become less pronounced within the first four days of life; the frequency of secretory pulses also halves over the same time period [9]. Even higher GH levels are seen in preterm infants, but the pulsatile pattern of release is similar to the term infant [9]. Kurtoglu, *et al.*[10] demonstrated that the median GH levels significantly decrease from first to fourth postnatal week in appropriate for gestational age neonates, whereas IGF-1 and Insulin-like Growth Factor Binding Protein-3 (IGFBP-3) levels increase significantly in the corresponding period, highlighting the need for week-specific cut-offs for each of these hormone parameters in the neonates [10]. This pattern is reflective of a physiologic GH resistance state at birth, which rapidly reduces over the four weeks of the neonatal period. Sleep is not a stimulus for GH secretion until 3 months of age, but feeding and insulin release stimulate GH secretion at this early stage before sleep entrainment [9].

IGF-I plays a major role in fetal growth, IGF-I levels increase two-to three-fold from 33 weeks gestation to term. Postnatal IGF-I production is involved in both somatic and brain growth, independent of gestational age and caloric intake. Despite our understanding of the GH/IGF-I axis in the fetus and infant, diagnosing GH deficiency in neonatal period remains a challenge. A combination of clinical phenotype, IGF-I levels, IGFBP-3

and GH levels are used [11]. Although, post-stimulation (using various stimulation protocols) GH levels is recommended in older children for diagnosing GHD (>10 µg/L rules out GHD); random GH values are useful in ruling out GHD in first 15 days of life after birth. This is because the basal GH levels are higher in infants and the response to various stimulation tests, especially hypoglycemia is poor in neonates, due to lack of maturation of the counter regulatory response in neonates [12]. Isolated random GH levels above 20 µg/L as per older assay (RIA) and more than 7 µg/L as per newer ELISA/CLIA rules out GH deficiency in neonates [11,13]. If mother is a smoker, we can have spuriously low GH in neonates. IGF-1 level is affected by age, nutrition and ethnicity. However, normative data for GH and IGF-I are limited, and often not available [13].

Congenital Adrenal Hyperplasia (CAH)

In normal children, 17-OHP levels are physiologically high at birth and decrease rapidly during the first few days in the postnatal period [14]. In contrast, in neonates affected with CAH, 17-OHP levels continue to increase progressively in the postnatal period. Because of this, the diagnostic accuracy of 17-OHP measurement is poor in the first two days of birth. Premature, critically ill or stressed neonates have higher levels of 17-OHP than normal term neonates [14]. Laboratories in USA typically use a series of birth weight-adjusted cut-offs for 17-OHP assessment. Use of gestational age-based criteria has improved the positive predictive value of 17-OHP screening in Netherlands and Switzerland. Antenatal corticosteroid treatment (used to induce lung maturation in fetuses at risk for premature birth) might reduce 17-OHP levels. It is recommended that all such infants be retested after several days of life [15]. Liquid chromatography – tandem mass spectroscopy (LC– MS/MS) is the gold standard for 17-OHP assessment. Confirmation of the diagnosis can be done in later stage of life by subjecting the patient to an ACTH-stimulation test. In classical CAH, basal 17-OHP is usually >10 ng/mL. A basal morning 17-OHP <2 ng/mL rules out CAH. Children with basal morning 17-OHP >2 ng/mL need to undergo an ACTH stimulation test, with serum 17-OHP estimation 1 hour after ACTH injection. A stimulated 17-OHP>8 ng/mL is diagnostic of CAH [15]. Cut-off values for screening tests must be empirically derived and vary by laboratory and assay. Patient needs opinion from a endocrinologist before being diagnosed with CAH. If the infant manifests clinical signs of adrenal insufficiency and/or abnormal electrolytes, an endocrinologist should be consulted for appropriate further evaluation and treatment [16]. Treatment in these neonates should not be dalyed be cause of any delay in 17-OHP reporting. The sample can be easily stored for assessment on a later date as 17-OHP is a steroid molecule, and hence is very stable when stored at -20 or -80ºC for long periods. The 17-OHP normative data is unfortunately not available from the developing world, including India. Also, in the neonates, delta-5 steroids like dehydroepiandrostenedionesulphate (DHEAS) and 17-hydroxypregnenolone are high; a result of hyperactive and hyperplastic adrenal fetal zone resulting in a hormonal profile which mimics genetic 3-beta hydroxysteroid dehydrogenase deficiency [3]. Only LC-MS/MS can clearly differentiate among the different types of steroids. However, LC-MS/MS is not yet commonly available in clinical practice in most settings. Hence, biochemical diagnosis of CAH is difficult in the neonatal period and should always be confirmed later in life.

Congenital Hypothyroidism

Congenital hypothyroidism is the commonest treatable cause of mental retardation with incidence of 1 in 2000-4000 newborns [17]. Appropriate initial therapy and follow-up are essential. The protocol for neonatal screening is to measure tetra-iodothyronine (T4) and thyroid stimulating hormone (TSH) at or after 48 hours of life [18]. If only T4 is measured, the false-positive rate is 0.30%; whereas when only TSH is measured, the false-positive rate is 0.05% [18]. Preterm infants have higher false-positive reports. Screening programs use either percentile- based cut-offs (*e.g.*, T4 below 10th centile or TSH above 90th centile) or absolute cut-offs (*e.g.*, TSH >20 mU/L) [18,19]. In proven cases of congenital hypothyrodism, TSH is >50 mU/L is observed in 90% of patients, and T4 £6.5 µg/dL is observed in greater than 75% of patients [19]. In most situations, total T4 is sufficient for diagnosis and monitoring of treatment, but free T4 is a more robust marker as it represents the bioavailable fraction of T4 [20]. Free T4 measurement may be superior and more reliable than T4 estimation in premature or sick newborns, and those with immature liver function, undernutrition, proteinuria, low levels of thyroid binding globulin (TBG) or abnormal protein binding [18,20]. Bacteremia, endotracheal bacterial cultures, persistent ductus arteriosus, necrotizing enterocolitis, cerebral ultrasonography changes, oxygen dependence at 28 days after birth, use of aminophylline, caffeine, dexamethasone, diamorphine, and dopamine are associated with altered TSH, free T4, T4 and T3 levels in premature newborns [21]. Hence, the presence of these comorbidities and use of drugs that interfere with hypothalamic-pituitary-thyroid axis should be taken into account while interpreting thyroid function reports in premature infants. It is always judicious to review the thyroid function again, once the co-morbidities are corrected [21].

In neonates with low total T4 and normal TSH, it is advisable to measure free T4, and if normal, it is likely that the neonate has congenital complete or partial TBG deficiency [20]. On the other hand, if free T4 is low, we should suspect central/secondary hypothyroidism. In neonates with severe neonatal hyperbilirubinemia warranting exchange transfusion, thyroid function should be checked either before the exchange transfusion or at least 3 days after the exchange transfusion [22]. Estimation of T4 and TSH within 3 days of exchange transfusion will lead to false low values of T4 and TSH, leading to a false diagnosis of congenital hypothyroidism, and hence should be avoided [22].

During post-treatment monitoring, the first measurement should preferably be free T4 as total T4 levels will be altered due to alteration in TBG levels. There is, however, controversy regarding the timing of repeat thyroid function tests after the initial screen, as well as the frequency of monitoring required to optimize the outcomes of children who are being treated for congenital hypothyroidism. The incidence of congenital hypothyroidism appears increasing over the last 20 years [17,19]. Whether the increase is real, or is it the result of lowering of screening test cut-offs, changes in the racial/ethnic population, or an increase in preterm births is not clear. It is also unclear whether the additional infants now being detected, including those with mild hypothyroidism and those with "delayed TSH rise" will have permanent or transient hypothyroidism.

There is also uncertainty concerning permanent *vs* transient congenital hypothyroidism during monitoring. Delayed TSH rise is defined as a normal TSH level with low T4 level on a newborn's initial screening, with detection of elevated TSH and persistent low T4 on subsequent screening. Almost 10% of neonates with congenital hypothyroidism cases 'pass' the first test and are detected by an abnormality on the second screening test [20]. Every program that undertakes a second routine test detects an additional 10–15% patients. Infants born preterm or acutely ill term infants are those most at risk for delayed TSH elevation [19,20]. The incidence of delayed TSH elevation is reported to be approximately 1:18,000. The current American Academy of Pediatrics (AAP) guidelines include measurement of thyroid function tests at two weeks if the initial screening shows low T4 and normal TSH in preterm infants, low birth weight infants and sick full-term newborns [20].

Hyperprolactinemia

Routine testing for prolactin should be avoided in neonates. Prolactin levels are normally elevated after birth, and is further complicated by prematurity and stress. Also, untreated hypothyroidism is commonly associated with raised prolactin levels [23]. Hence, prolactin should be tested only when clinically indicated, and prolactin levels should always be interpreted in the context of the thyroid function status of the neonate.

Steroid Hormones Testing

Apart from CAH, steroid hormone testing in neonates is required in patients with disorders of sexual development [24]. Problems with steroid hormone assays in neonates is the cross-reactivity with other circulating structurally homologous steroids due to lack of 100% specificity of the steroid antibodies used in immunoassays [3]. Garagorri, *et al*. [25] demonstrated that in healthy infants from Spain, among the adrenal steroids, except for cortisol, plasma levels of 17-OHP, 11-desoxycortisol, testosterone, DHEAS and androstenedione decreased progressively from birth to 6 months of age. Apart from testosterone and androstenedione (significantly higher in boys), and DHEAS (higher in girls), there was no gender differences among the hormones estimated [25].

Hypovitaminosis D

Un-monitored therapeutic vitamin D supplementation in neonates should be avoided without serum 25-hydroxy-vitamin D testing due to the associated increased risk of hypervitaminosis D [26].

CONCLUSION

Interpretation of hormone assay reports in neonates remain a challenge. The clinical scenario should primarily dictate the treatment formulation, and hormonal assay should only supplement the diagnosis and fine-tune the treatment plans. There is an urgent need to develop normal ranges for most of the hormones in the neonates. Generation of mathematical models to provide clear information of the variability of hormones as per the age of the neonates is required, which can also be more cost effective. It is important to be aware of the pre-analytical challenges, and minimize them. Need for dedicated pediatric laboratories for hormonal assay would always be desirable in the long run, which would minimize the analytical errors.

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