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Reference Intervals of Platelets, Lymphocytes and Cardiac Biomarkers in Umbilical Cord Blood

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Abstract

The umbilical cord blood (UCB) can be used for early detection of neonatal diseases. The UCB can be used for early detection of neonatal diseases. Establishing reference intervals is essential for appropriate interpretation of results of laboratory tests using UCB and for correct medical decisions of pediatricians and neonatologists. The use of proper reference intervals provides reliable information to pediatricians and neonatologists; thus, they could make correct medical decisions for neonates. This chapter discussed reference intervals of platelets, lymphocytes, and cardiac biomarkers in UCB according to the Clinical Laboratory Standards Institute guidelines. Except iatrogenic anemia, thrombocytopenia is the most common hematologic abnormality in neonates. Immature platelet fraction is a novel parameter to estimate megakaryopoiesis and can be useful to understand the mechanism of thrombocytopenia, platelet destruction or bone marrow failure, in neonates. Lymphocyte counts, T cell and B cell, can reflect status of immune system in fetus and neonates. Especially Tregs in UCB may contribute to maintain the immune homeostasis in the fetomaternal relationship, and the presence of Tregs would be essential to prevent immune dysregulation in fetus and neonates. Congenital heart disease or defect is the most common birth defect in newborns. Cardiac biomarkers are essential to evaluate heart function and to give information of myocardial injury, necrosis, or myocardial stretch. There are no current guidelines for their routine use in children.

Keywords: cord blood, reference intervals, platelets, lymphocytes, cardiac markers

1. Introduction

Obtaining admission laboratory studies is necessary to provide appropriate neonatal care. As a general rule, the blood drawn for laboratory testing should not exceed 5% of the total

blood volume per draw; thus, in neonates and infants, a less-than-optimal amount of blood may be available for testing. Approximately 1.5 – 4 mL of blood drawn for admission blood test may cause iatrogenic anemia to neonates especially in extremely low birth weight infants [1, 2]. Umbilical cord blood (UCB) is remained blood in the placenta and attached umbilical cord after the birth of baby. Several studies suggest that UCB could be an alternative source for admission of blood tests in neonates [1, 3, 4]. Especially, very low birth weight infants who typically have greater phlebotomy blood loss on the first day of life than any other day during their hospitalization would benefit most from admission laboratory studies being obtained from UCB [5, 6].

At birth, full-term newborns show relative polycythemia, macrocytosis, and marked polychromasia with nucleated red blood cells (RBCs) [7, 8]. The red cell distribution width (RDW) is elevated, showing anisocytosis, compared with adult standards. Full-term newborns have a high white blood cell (WBC) count with relative transient neutrophilia at birth while soon after birth, neutrophils gradually decrease and lymphocytes become major population in neonate's peripheral blood. This neutrophilia may arise from bone marrow mobilization under stress during labor, and these WBCs show shift-to-left neutrophils such as metamyelocytes, myelocytes, and even circulating blasts [9, 10]. The platelet counts are similar to the older children and adults. Neonatal thrombocytopenia is defined as a platelet count less than $150 \times 10^9/L$ in any neonate of a viable gestational age. Thrombocytopenia is one of the most common hematological abnormalities except iatrogenic anemia in neonates [11, 12].

During the pregnancy, human placenta forms an imperfect barrier, allowing bidirectional passage of soluble antigens and cells between a mother and a baby without any mixing between the two blood supplies [13]. This results in the presence of fetal cells in the maternal circulation, known as fetal microchimerism, and maternal cells in the fetal circulation, known as maternal microchimerism [14]. Maternal microchimerism was first described in 1963 by Rajendra G. Desai who identified maternal leukocytes and platelets in UCB [15]. This bidirectional trafficking of cells begins at seven to 16 weeks, increases steadily after 24 weeks, and reaches a peak at parturition [16]. At delivery, maternal microchimerism has been reported in 42% of normal pregnancies [13]. For this, microchimerism does not occur in all pregnancies, altered maternal-fetal bidirectional passage has been associated with disruption of the fetomaternal interface, and the biologic role of this bidirectional passage is unclear. This passage is implicated in development of the fetal immune system [17]. Substantial numbers of maternal cells cross the placenta to reside in fetal lymph nodes, inducing the immune system, the development of CD4+CD25^{high}FOXP3+ regulatory T cells (Tregs), which suppresses fetal antimaternal immunity.

UCB is a rich source of hematopoietic cells or precursors to blood cells. Since the first UCB stem cell transplantation in 1988 to treat a child with Fanconi's anemia, UCB has been used as an important source of hematopoietic stem cell transplantation [18]. UCB could be collected at birth without any harm to the newborn infant. UCB cells have many theoretical advantages as grafts for stem cell transplantation because of the immaturity of newborn cells and immaturity of the immune system at birth. These properties should decrease the alloreactive potential

of the lymphocytes and should reduce the incidence and severity of graft-versus-host disease after human leukocyte antigen (HLA) -matched or HLA-mismatched transplantation [19]. The recovery rate of colony-forming unit correlated significantly with platelets as well as leukocytes, RBCs, mononuclear cells, CD34⁺ leukocytes, and viable leukocytes [20]. Detection of abnormal levels of platelets, leukocytes, RBCs, mononuclear cells, CD34⁺ leukocytes, and viable leukocytes could be one of UCB screening tests available.

The interpretation of results of laboratory tests includes the comparison between the reported values versus documented reference intervals. The reference intervals are defined as values obtained by measurement of a particular type of quantity on a reference individual who selected for testing on the basis of well-defined criteria who is considered being in healthy state from general population [21]. For these reasons, establishment of reference intervals for blood tests such as complete blood counts and biomarkers in UCB is crucial for clinical laboratory tests. We discuss the reference intervals of platelets, lymphocytes, and cardiac biomarkers in UCB.

2. Establishing reference intervals in umbilical cord blood

The production of health-associated reference values must be implemented in accordance with a well-defined protocol. The Clinical Laboratory Standards Institute (CLSI) offers a protocol for determining reference intervals that meet the minimum requirements for reliability and usefulness related to quantitative clinical laboratory tests [21]. The CLSI suggested a protocol outline for obtaining reference values and establishing reference intervals. First, researchers should establish a list of analytical interferences and sources of biological variability from medical and scientific literatures. Then, they must establish selection and partition criteria and an appropriate questionnaire designed to reveal these criteria in the potential reference individuals. An appropriate written consent should be signed by legal guardians of neonates. Researchers have to categorize the potential reference individuals based on the results of questionnaire and health assessments and exclude individuals based on the exclusion criteria. For UCB, gestational age, birth weight, maternal age, maternal health, and maternal history of medical, smoking, and alcohol consumption are important.

The reference interval is defined as the interval between and including two numbers, an upper and lower reference limit, which are estimated to enclose a specified percentage (usually 95%). For most analytes, the lower and upper reference limits are estimated as the 2.5th and 97.5th percentiles of the distribution of test results for reference populations. To decide on an appropriate number of reference individuals, in consideration of desired confidence limits, the CLSI suggests that a minimum of 120 reference values for 90% confidence limits, 146 observations for 95% confidence limits, and 210 reference values for 99% confidence limits. It is necessary to define whether the sample should be arterial or venous UCB in a manner consistent with the routine practice for patient specimens. Inspection of the reference value data, preparing a histogram, and identifying possible data errors and/or outliers are essential to evaluate the

distribution of data. Furthermore, partitioning into subclasses for separate reference intervals should be considered if appropriate according to gestational age, gender of neonates, and maternal age.

For difficulties of sample obtaining, it is not easy to establish reference intervals of parameters for neonates according to the CLSI guideline. Even published reference intervals using neonates' peripheral blood or UCB are very useful and informative for clinical laboratory tests, physicians should keep in mind that some of published reference intervals did not satisfy the CLSI guideline for sample collection.

3. Platelets

The first morphologically visible platelets appear in the fetal circulation at seven to nine weeks, and the platelet counts reach adult levels before 18th gestational week [2, 22]. The intrauterine thrombocytopenia could diagnose through fetal blood sampling after 18th gestational week [2]. The platelet counts are constant at birth and in neonatal period and compatible to the count in adult. Neonatal thrombocytopenia has been defined traditionally as a platelet count less than $150 \times 10^9/L$. This definition was challenged by recent studies. Large-scale study presented that platelet counts of preterm neonates born before 35 weeks gestation were significantly lower than those that were of late-preterm and preterm infants [23]. Wasiluk [24] reported the platelet count is found to be decreased in preterm and late-preterm newborns. The platelet counts were increasing with completed weeks of gestation and birth weight. Decreased platelet count in preterm could be considered as immaturity of thrombopoiesis and impaired process of megakaryopoiesis characterized by the rapid proliferation of megakaryocyte precursors and full cytoplasmic maturation of megakaryocytes leading to the production of high number of platelets. Levels of thrombopoietin and reticulated platelets (immature platelet fraction, IPF) could reveal the megakaryopoiesis of fetus and neonates.

Except iatrogenic anemia, thrombocytopenia is the most common hematological abnormality in neonates [11]. Incidence of thrombocytopenia is 1–5% in newborns at birth [25–27]. Thrombocytopenia may be caused by feto-maternal and neonatal conditions such as impaired platelet production, consumption and sequestration, and combined mechanisms [28–31]. Platelet transfusion is associated with several risks including infection, transfusion-related acute lung injury, transfusion-associated circulatory overload, alloimmunization, allergic reaction, and other complications. Therefore, platelet should be given when clearly clinically indicated [31, 32]. Reference values for normal platelet counts, especially lower limit, are important to diagnose thrombocytopenia. In particular, there is a need for supplementary parameters in order to evaluate the megakaryopoiesis and bleeding risk [31].

IPF is newly released from fetal liver or the bone marrow and containing high amount of ribonucleic acid (RNA). Thiazole orange, a fluorescent dye, is characterized by binding to nucleic acid, particularly RNA, and flow cytometric analysis of platelets after staining with

Study	Gestational age (week)	Number of participants	Platelet counts ($\times 10^9/L$)	Parameter	Method or instruments	RP% or IPF%	Absolute RP or IPF counts ($\times 10^9/L$)
[67]	36	39	246 \pm 65	RP	Flow cytometry	4 \pm 2.4%	10.5 \pm 8.7
[68]	28 \pm 2.5	37	150–450	RP	Flow cytometry	2.7 \pm 1.6%	NA
[69]	38–41	72	316.96 \pm 60.76	RP	Flow cytometry	1.65 \pm 0.95%	
[70]	36.3 \pm 3.7	456	150–450	IPF	XE-2100, Sysmex	4.3 (95% CI 0.7–7.9)	NA
[34]	39.3 (38.0–41.6)	133	191–392 [†]	IPF	XE-2100, Sysmex	0.7–3.8 [†]	1.94–9.69 [†]
[36]	39.0 (38.0–41.3)	140	174–405 [†]	IPF	XN, Sysmex	1.0–4.4 [†]	2.9–12.8 [†]

IPF, immature platelets; NA, not available; RP, reticulated platelets.

[†] Reference interval.

Note: Data were expressed as mean \pm standard deviation, range, or median (range).

Table 1. Comparison of studies measuring reticulated platelets and immature platelet fraction in healthy newborns.

thiazole orange reflects the activity of megakaryopoiesis in the bone marrow [33]. Measuring IPF of the systemic circulation is a novel parameter to estimate the megakaryopoiesis and can be useful to recognize quickly as having platelet destruction or bone marrow failure in a neonate with low platelet count [34]. Measuring IPF may potentially avoid the need for bone marrow examination. Increased IPF% or normal IPF number (IPF#) is considered in the case of platelet consumption in thrombocytopenia, whereas normal or decreased IPF% and IPF# is considered in the case of bone marrow failure in thrombocytopenia. Today, IPF can be measured on fully automated routine hematology analyzers (XE-2100 and XN modular system; Sysmex, Kobe, Japan) [35]. Establishment of reference intervals for platelet and IPF in neonates is essential for diagnosis of neonatal thrombocytopenia, for facilitating the clinical usefulness of IPF, and for clear indication of transfusion. **Table 1** shows the comparison of studies measuring IPF in healthy subjects. The new automated hematology analyzer, XN modular system, demonstrated remarkable higher and broader reference intervals for platelets and IPF compared with XE-2100 [36]. For these differences, clinical laboratories should establish or verify reference intervals for platelets and IPF according to their own instrument.

4. Lymphocytes

Lymphocytes in UCB are naïve and immature, are enriched in double-negative CD3⁺ cells, and produce fewer cytokines [19]. Lymphocyte counts, T cell and B cell, can reflect status of immune system in fetus and neonates. B- and/or T-cell lymphocytopenia could be noted in some viral infection but also in Wiskott-Aldrich syndrome, X-linked agammaglobulinemia,

and severe combined immunodeficiency [37–40]. To define abnormality of lymphocyte counts, quantitation of the lymphocytes and their subtypes with flow cytometry and establishment of reference interval are necessary.

Circulating T cells in the fetus and neonate are fundamentally different from naïve adult T cells such as containing high concentration of T-cell receptor excision circles (TRECs), high cell turnover, increased susceptibility to apoptosis, and presence of CD25⁺ regulatory T lymphocytes (Tregs), and so on [41]. Natural Tregs originate in the thymus and are specific for self-antigens presented by thymic epithelial cells [42]. Maternal cells commonly cross the placenta and engraft into fetal circulation and tissues in uterus, resulting in maternal microchimerism [17, 43]. Naturally acquired microchimerism can contribute to autoimmune diseases. In particular, maternal microchimerism has been studied in systemic sclerosis, dermatomyositis, and neonatal lupus [43]. Especially, Tregs in UCB may contribute to maintain the immune homeostasis in the fetomaternal relationship, and the presence of Tregs would be essential to prevent immune dysregulation in fetus and neonates [17, 44]. Fetal Tregs are known to regulate fetal immune responses against noninherited maternal alloantigens. During labor, neonatal immune system faces big challenge. The tolerogenic immune state of the semi-allogeneic fetus should switch over to prevent potentially damaging inflammation or infection. In immunosuppressive state, several cells such as helper T cells with a specific cytokine profile, neutrophilic myeloid-derived suppressor cells, erythroid CD71⁺ cells, and Tregs are potential mediators [45, 46].

Infection of newborn and infants is a major healthcare challenge with global mortality in excess of one million lives especially in very low-birth-weight preterm infants [47]. Preterm infants are highly susceptible to invasive infections, which are leading causes of mortality and long-term morbidity. Treg levels and gestational age inversely correlated in several studies [48–50]. Preterm infants have higher Treg levels than full-term newborns. Tregs inhibit antimicrobial immune responses. The T cell immune response in preterm infants is supposed to be dysregulated and affected by prenatal factors including intrauterine inflammation and maternal characteristics. This dysregulation of T cell immunity could lead to ineffective clearance of pathogens [49]. Tregs have two populations (CD31⁺ and CD31⁻), and the ratio alteration of these populations is associated with different intra- and/or extra-uterine milieu. The CD31⁻ Treg levels are significantly higher in UCB of preterm pregnancies associated with inflammation and prenatal lipopolysaccharide exposure. The alteration of homeostatic composition of Tregs subsets related to reduced *de novo* generation of recent thymic emigrants. Tregs may contribute to premature delivery, and *vice versa*. Early-onset septic infants have significantly higher Treg frequencies than infants without early-onset sepsis. The increased Treg level may cause an uncontrolled immunosuppression and therefore results in an increased risk of sepsis for the preterm infants especially for the most vulnerable very low-birth-weight infants (**Figure 1**) [46].

In spite of the growing attention on the importance of Tregs in UCB and neonates, the distribution of Tregs in normal UCB or healthy neonates was not well-known. **Table 2** showed the comparison of studies measuring lymphocyte subsets and Tregs in healthy subjects. Each study showed different values for lymphocyte subsets and Tregs. For these differences, clinical laboratories should establish or verify reference intervals for lymphocyte subsets and Tregs.

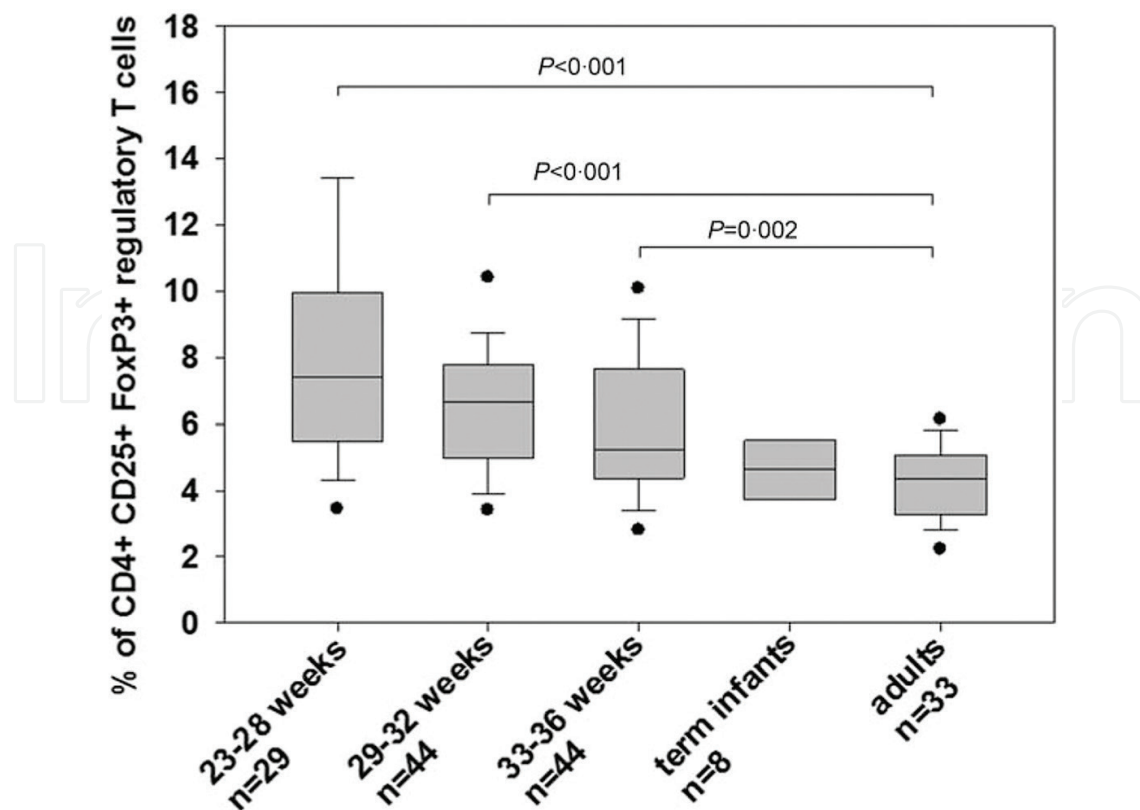


Figure 1. The frequency of regulatory T cells (Tregs) is higher in preterm infants than in term infants. Box-plots [median, interquartile range (IQR), 95% confidence interval (CI)] describe the frequencies of Tregs across groups of different gestational age. Adapted from [46] with permission of John Wiley and Sons, Inc.

Study	Gestational age (week)	Number of participants	Helper T cells (CD3+/CD4+, %)	Cytotoxic T cells (CD3+/CD8+, %)	B cells (CD19+, %)	NK cells (CD3-/CD16+/CD56+, %)	Regulatory T cells (CD4+/CD25 ^{high} /FOXP3+, %)
[71]	Healthy full-term	98	46.7 (40.2–61.9)	16.3 (14.3–21.3)	11.5 (7.6–15.5)	NA	5.2 (3.5–7.0) [¶]
[72]	NA	22	NA	NA	17.2 (13.2–25.4)	NA	NA
[73]	NA	38	44 (34–57) [†]	17 (11–30) [†]	16 (9–23) [†]	16 (6–28) [†]	NA
[44]	38.0–41.3	120	15.40–70.06 [†]	9.65–34.28 [†]	4.50–29.59 [†]	1.42–28.03 [†]	0.35–9.07 [†]
[74]	≥35	18	41 (26–62)	14 (5–37)	10 (3–30)	22 (8–62)	7 (4–13) [¶]
[75]	NA	53	28.9 (11.4–40.3) [†]	11.8 (6.1–18.3)	15.2 (9.3–22.0)	18.2 (8.6–28.2)	16.7 (12.3–23.8) [§]

[†] Reference interval.

[‡] Median values with 10th and 90th percentiles.

[§] CD4+/CD25+.

[¶] CD4+/CD25+/CD127-.

Note: Data were expressed as mean ± standard deviation, range, or median (range).

Table 2. Comparison of studies measuring lymphocyte subsets and Tregs in healthy subjects.

5. Cardiac biomarkers

Congenital heart disease or defect (CHD) is the most common birth defect in newborns [51]. Critical CHDs require intervention or surgery in the first year of life. Echocardiography is a definitive diagnostic tool of CHD and provides hemodynamic and anatomic information of heart. Twenty-five to 30% of children, however, with critical CHD are not detected by fetal echocardiography until after discharge from the birth hospitalization [52]. Cardiac biomarkers are essential to evaluate heart function and give information of myocardial injury, necrosis, or myocardial stretch. Concentrations of cardiac troponins, troponin I (TnI) or troponin T (TnT), are elevated in myocardial necrosis and myocardial infarction. Troponins are also increased in patients with heart failure and myocarditis. Elevated concentration of brain-type natriuretic peptides (BNP) or N-terminal pro-brain natriuretic peptide (NT-proBNP) is related to myocardial stretch and left ventricular dysfunction and can be used for screening and prognosis of heart failure (HF) [53, 54]. Suppression of tumorigenicity 2 (ST2), a member of the interleukin (IL)-1 receptor family, has two isoforms: transmembrane ST2 and soluble ST2 (sST2). IL-33 reduces fibrosis and hypertrophy of myocardium, and preserves ventricular function. The sST2 plays a role as a decoy receptor of IL-33, and binding of IL-33 and sST2 inhibits the beneficial and protective effect of IL-33 on the heart. The concentration of sST2 is elevated in patients with HF and is also associated with the prognosis of acute and chronic HF [55].

Many studies reported that NT-proBNP or BNP was elevated in peripheral blood or UCB with neonates with CHD [56]. A few studies presented the distribution of TnI or TnT in neonates or UCB [57–60]. The distribution and association of sST2 with CHD have not been investigated yet. There are no current guidelines for their routine use in children. The levels of NT-proBNP were significantly increased in UCB of neonates with CHD compared with that in the UCB of control group. In addition, the levels of NT-proBNP were significantly increased in the neonates with tight ventricular outflow tract obstruction without a ventricular septal defect compared with that in the other groups (**Figure 2**). Moreover, there was significant difference between survivors and non-survivors within one-year of birth [61]. Hydrops fetalis is fluid collection in multiple body compartments in fetus because of immune or non-immune mechanism. Congestive heart failure or cardiac dysfunction has been described as one of the major mechanisms for nonimmune hydrops fetalis. The levels of NT-proBNP in cases with hydrops of cardiac origin were higher than those in cases with hydrops of non-cardiac origin. However, levels of TnT did not differ though the causes of hydrops fetalis [62].

The only one study reported 97.5th percentile upper reference limit of NT-proBNP and TnT from healthy neonates, according to the CLSI guideline [21, 63]. In uterus, hemodynamics between placenta and fetal heart can vary with gestational age [64, 65], and reference intervals for cardiac biomarkers in neonates could be different from those in adults. Therefore, establishment of reference intervals is necessary to use cardiac biomarkers in neonates. **Table 3** showed the comparison of sST2, NT-proBNP, high sensitive TnI, and high sensitive TnT in UCB and adults. For these differences between laboratory tests results in UCB and adults, clinical laboratories should establish or verify reference intervals for cardiac biomarkers in UCB. Kim H et al. [66] reported that levels of sST2, NT-proBNP, and high sensitive TnT in UCB were significantly higher than those in adults.

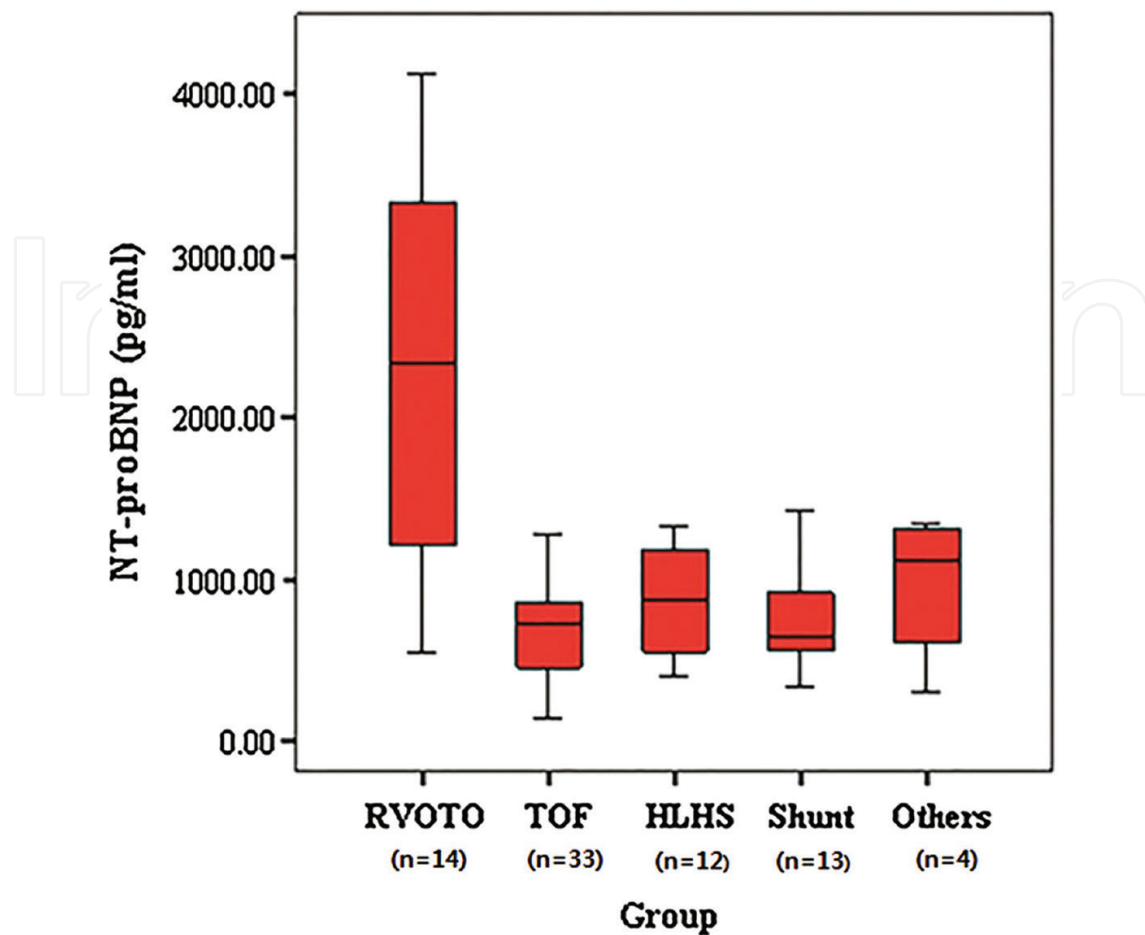


Figure 2. The distribution of NT-proBNP levels in the umbilical cord blood of neonates with cardiac malformations according to the type. Abbreviations: HLHS, hypoplastic left heart syndrome; NT-proBNP, N-terminal pro-brain natriuretic peptide; RVOTO, right ventricular outflow tract obstruction; TOF, tetralogy of Fallot. Adapted from [61] with permission of Springer International Publishing AG.

	97.5 th percentile upper reference limit in UCB from healthy, full-term neonates (90% CI)	Medical decision point for adults (reference)
sST2 (ng/mL)	59.9 (52.7–62.2)	35 [76]
NT-proBNP (pg/mL)	1415.3 (1070.0–2198.0)	300 [77], 125 [78]
hs-TnI (pg/mL)	27.8 (21.v1–30.4)	26.2 [79]
hs-TnT (pg/mL)	86.5 (68.0–99.0)	14 [80]

sST2, soluble suppression of tumorigenicity 2; NT-proBNP, N-terminal pro-brain natriuretic peptide; hs-TnI, high sensitive troponin I; hs-TnT, high sensitive troponin T; UCB, umbilical cord blood.

Table 3. Comparison of sST2, NT-proBNP, high sensitive TnI, and high sensitive TnT in UCB and adults. Adapted from [66] with permission of Walter de Gruyter GmbH.

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