Introduction

Thrombocytopenia is a common haematological abnormality and sometimes associated with severe bleeding. A number of causes may lead to low platelet count`, but two major mechanisms are implicated in the pathogenesis, namely increased peripheral platelet destruction and decreased bone marrow production ***(Monteagudo et al., 2008).***

The incidence of thrombocytopenia in neonates varies significantly, depending on the population studied. Specifically, while the overall incidence of neonatal thrombocytopenia is relatively low (0.7–0.9%), the incidence among neonates admitted to the Neonatal Intensive Care Unit is very high (22–35%) ***(Sola-Visner et al., 2008).***

Fetal studies have shown that the platelet count reaches 150-10^9/ µL by the end of the first trimester of pregnancy, and is maintained at or above this level to term in healthy fetuses. It can therefore be defined as a platelet count > 150-10^9/ µL in any healthy neonate of a viable gestational age. This definition is supported by large population studies that show that >98% of term neonates born to mothers with normal platelet counts have platelets above 150 - 10^9/ µL at birth ***(Roberts, 2008).***

Platelet production, or thrombopoiesis, is a complex process that can be schematically represented as consisting of four main steps. The first step is the production of the

thrombopoietic stimulus, which drives the generation of megakaryocytes and ultimately platelets. Although a number of cytokines and chemokines contribute to this process, thrombopoietin is now widely recognized as the most potent known stimulator of platelet production. Thrombopoietin mostly acts by promoting the proliferation of megakaryocyte progenitors (the cells that multiply and give rise to megakaryocytes), and the maturation of the megakaryocytes ***(Sallmon et al., 2009).***

Reticulated platelets are the youngest forms of circulating platelets that contain messenger RNA residual. It has been observed in animal models that mRNA is unstable, degrades within 24 h, and disappears in parallel with the aging of platelets. Reticulated platelets are functionally active and are the platelet equivalent of red cell reticulocytes ***(Pons et al., 2010).***

Clinical conditions often associated with neonatal thrombocytopenia include sepsis, disseminated intravascular coagulation, necrotizing enterocolitis, asphyxia, exchange transfusion, genetic syndromes, drug effects, maternal hypertension, and maternal antiplatelet antibodies ***(Peterec et al., 1996).***

Reticulated platelets with a high ribonucleic acid content are considered to be newly synthesised cells. Direct measurement of reticulated platelets by flow cytometry may give an estimate of platelet production and the quantity of young platelets, reflecting the activity of thrombopoiesis. A fluorescent dye, thiazole orange, that permeates cell membranes

and binds to intracellular nucleic acids, has been used in the analysis of reticulated platelets ***(Joutsi-Korhonen et al., 2000).***

Determination of reticulated platelets by flow cytometry assessed indirectly megakaryocytic activity. It is useful in patients with thrombocytopenia, showing marked increase in the percentage of reticulated platelets in thrombocytopenia with increased platelet turnover. As immune thrombocytopenia or disseminated intravascular coagulopathy ***(Pons el al., 2010).***

Aim of the Work

To evaluate the diagnostic usefulness of reticulated platelets in neonatal thrombocytopenic sepsis, their prognostic value and value in decrease the need for platelet transfusion.

Megakaryopoiesis and Thrombopoiesis

Hematopoiesis is a complex process that takes place in the yolk sac in the first few weeks then occurs in liver and spleen in the period from 6 weeks until 6-7 months of life. Liver and spleen continue to produce blood cells until 2 weeks after birth ***(Stohlawetz et al., 2001).***

After birth, the major site of hematopoietic activity shifts gradually from the liver and spleen to the bone marrow cavities of nearly all bones of the axial and appendicular skeleton. By early adulthood, the long bones no longer bear red marrow and the primary sites of red marrow are confined to the sternum, ribs, vertebrae and pelvis ***(Emerson et al., 2002).***

The process of megakaryopoiesis and platelet production is complex, with the potential for regulation at multiple stages.

The megakaryocyte development is arbitrarily divided into four stages. The major criteria differentiating these stages are the quality and quantity of the cytoplasm and the size, lobulation, and chromatin pattern of the nucleus.

Table (1): Maturation Stages of Megakaryocytes.

Term	Size (µM)	Morphology
Megakaryoblast (stage I)	>10	Lobed nucleus, basophilic cytoplasm
Basophilic megakaryocyte (stage II)	>20	Horseshoe-shaped nucleus, basophilic cytoplasm, azurophilic granules around centrosome
Granular megakaryocyte (stage III)	>25–50	Large multilobed nucleus, acidophilic cytoplasm, numerous azurophilic granules
Mature megakaryocyte (stage IV)	>25–50	Pyknotic nucleus, groups of 10–12 azurophilic granules

(Junmei et al., 2010).

Megakaryoblasts are not morphologically recognizable as megakaryocytes. They are thought to represent a transitional stage of development in megakaryopoiesis, bridging the gap between progenitor cells and morphologically identifiable megakaryocytes. Megakaryoblasts is the first cells to increase in number after induction of thrombocytopenia and to decrease under conditions of thrombocytosis ***(Long et al., 2000).***

Immature MKs (alternatively designated stage II MKs or promegakaryocytes) have a larger size and cytoplasm-to-nucleus ratio than megakaryoblasts. Their single, multiple lobes containing nucleus have a horseshoe shape. In addition, a scarce amount of azurophilic granules is dispersed in their cytoplasm, which contains numerous mitochondria. Eventually, cells with a large nucleus are generated and are called mature MKs (alternatively designated stage III MKs, or granular megakaryocytes). Their cytoplasm is acidophilic and harbors a sizeable amount of azurophilic granules. The hallmark of Stage IV MKs is giving birth to platelets ***(Wickrema et al., 2009).***

MKs are large cells with a median diameter size of 30–70 m (range 20–100 m), and a single large polyploid nucleus that is multi segmented with coarse-grained chromatin. The cytoplasm of MKs can be divided into three zones. The perinuclear territory remains attached to the nucleus after release of platelets and contains several organelles, such as ER, Golgi apparatus, and centrioles. The intermediate zone contains the demarcation membrane system (DMS), which plays a role in the biogenesis of platelets. The DMS consists of an extensive network of vesicles and tubules and serves as the reservoir of proplatelet formation.

It is estimated that a single MK cell can shed approximately 10^9 platelets. Finally, the marginal zone involves the megakaryocytic cell membrane and is composed mainly of cytoskeletal filaments ***(Junt et al., 2007)***.

Mechanisms of platelets production from megakaryocytes:

1. It was believed that demarcation membranes, internal membranes of the MK, determine platelet territories corresponding to future platelets, which would be liberated via cytoplasmic fragmentation ***(Zucker et al., 2000).***
2. **Proplatelet model:** Megakaryocytes send pseudopodia out into the bone marrow sinusoids and then platelets and pro platelets bud off. Pro platelets are elongated strands of megakaryocyte cytoplasm that are larger than normal platelets and later fragment into a number of platelets ***(Saito et al., 2005).***
3. **Pulmonary platelets production model**: Intact mega-karyocytes reach the pulmonary capillaries and they release platelets by fragmentation of their cytoplasm at this site. Ninety eight percent of megakaryocytes leaving the lung are devoid of cytoplasm ***(Zucker et al., 2000).***

When platelets released from the bone marrow into the peripheral circulation they contain residual RNA which is subsequently degraded as the cells circulate. This young reticulated platelet appears normally in the peripheral blood at low level up to 4.5% of total platelets ***(Guthikonda et al., 2008).***

Neonatal Thrombocytopenia

Thrombocytopenia is a common hematological abnormality and sometimes associated with severe bleeding. A number of causes may lead to low platelet count, but two major mechanisms are implicated in the pathogenesis, namely increased peripheral platelet destruction and decreased bone marrow Production ***(Monteagudo et al., 2008).***

Definition:

During fetal life, the platelet count progressively increases and reaches a level of approximately 150-10^9/L by the end of the first trimester. Thus, healthy fetuses and neonates at gestational age's ≥22 weeks have platelet counts within the normal range for adults, and therefore neonatal thrombocytopenia is defined as a platelet count < 150-10^9/L regardless of gestational age **(*Sola-Visner et al., 2008).***

Incidence:

At birth:

Previous studies report a prevalence of thrombocytopenia of 1–5% of all newborns. However, the prevalence varies depending upon the population studied ***(Roberts et al., 2008).***

In NICU patients:

Thrombocytopenia develops in 22–35% of all babies admitted to NICU and in up to 50% of those admitted to NICU who require intensive care.A considerable proportion (20%) of

these episodes of thrombocytopenia is severe. This means that 8% of preterm and 6% of all neonates admitted to an NICU have sever thrombocytopenia and are at increased risk of hemorrhage, presenting a common management problem ***(Roberts and Murray, 2003).***

Thrombocytopenia severity grading:

Thrombocytopenia severity was graded as follows:

Platelets <25 = Grade Four Thrombocytopenia
Platelets 25-50 = Grade Three Thrombocytopenia
Platelets 51-75 = Grade Two Thrombocytopenia
Platelets 76-139 = Grade One Thrombocytopenia

This is in accordance with the National Cancer Institute common terminology criteria ***(Jenifer et al., 2010).***

Causes of neonatal thrombocytopenia (NT):

NT usually presents in one of two clinical patterns, which reflect the most common causes: early NT (within 72 h of birth) or late NT (after 72 h of life). Clinically, the most important cause of severe early NT is NAIT. However, NAIT accounts for only a small proportion (<5%) of early NT overall ***(Roberts and Murray, 2008).***

Table (2): Comparison of natural history of early and late onset thrombocytopenia in neonates.

	Early	Late
Degree of Thrombocytopenia	Mild to moderate (platelet nadir rarely < 50-109/L)	Sever (platelet nadir frequently <50-109/L)
Onset and Progression	Evolves slowly over several days	Rapid onset and progression over 24-28 hours
Associated with	Complicated pregnancies (Pre-eclampsia, IUGR, maternal diabetes)	Sepsis and NEC
Management	Rarely requires specific Treatment	Muliple platelet transfusions often required
Mechanisms	Impaired platelet production	Combined platelet consumption and impaired production

(Roberts and Murray, 2003)

Many clinical conditions are associated with neonatal thrombocytopenia (NT) but, until recently, almost half of all cases were classified as idiopathic. However, recent studies detailing the natural history of NT have identified reduced platelet production as the main underlying mechanism of many idiopathic cases. This has led to newer classifications of NT based on the timing of onset of thrombocytopenia **(Table 3).**

Table (3): Classification of fetal and neonatal thrombocytopenia.

	Condition
Fetal	➢ Alloimmune ➢ Congenital infection (e.g. CMV, toxoplasma, rubella, HIV) ➢ Aneuploidy (e.g. trisomies 18, 13, 21, or triploidy) ➢ Autoimmune (e.g. ITP, SLE) ➢ Severe Rhesus disease ➢ Congenital/inherited (e.g. WiskotteAldrich syndrome)
Early-onset neonatal (<72 h)	➢ Placental insufficiency (e.g. PET, IUGR, diabetes) ➢ Perinatal asphyxia, Perinatal infection (e.g. Escherichia coli, Haemophilus influenzae, GBS) ➢ DIC ➢ Alloimmune ➢ Autoimmune (e.g. ITP, SLE) ➢ Congenital infection (e.g. CMV, toxoplasma, rubella, HIV) ➢ Thrombosis (e.g. aortic, renal vein) ➢ Bone marrow replacement (e.g. congenital leukaemia) ➢ KasabacheMerritt syndrome ➢ Metabolic disease (e.g. proprionic and methylmalonic acidaemia) ➢ Congenital/inherited (e.g. TAR, CAMT)
Late-onset neonatal (>72 h)	➢ Late-onset sepsis ➢ NEC ➢ Congenital infection (e.g. CMV, toxoplasma, rubella, HIV) ➢ Autoimmune ➢ KasabacheMerritt syndrome ➢ Metabolic disease (e.g. proprionic and methylmalonic acidaemia) ➢ Congenital/inherited (e.g. TAR, CAMT)

(Roberts and Murray, 2008)

Acquired thrombocytopenia:

Non-immune-mediated thrombocytopenia:

- ***Chronic fetal hypoxia***

Thrombocytopenia is the most common hematological abnormality encountered in hypoxic neonates admitted in the neonatal intensive care unit ***(Murray, 2002).***

Most frequently thrombocytopenia is seen in neonates exposed to chronic hypoxia as observed in maternal diabetes, intrauterine growth restriction or HELLP syndrome of the mother. Thrombocytopenia is rarely severe and typically self-limiting ***(Roberts and Murray, 2008)***.

Often additional hematological changes as poly-cythaemia or neutropenia are observed in those neonates. Megakaryopoesis has been shown to be substantially impaired in thrombocytopenic neonates exposed to chronic fetal hypoxia ***(Watts et al., 1999)***.

- ***Necrotizing enterocolitis***

Necrotizing enterocolitis is a common and serious gastrointestinal disorder that predominately affects premature infants. 90% of neonates with NEC develop late onset thrombo-cytopenia. Thrombocytopenia is often severe (<50 _109/L) and associated with bleeding. Many patients require multiple platelet transfusions. Both the severity of thrombocytopenia and the number of platelet transfusions required predict outcome in NEC ***(Kenton et al., 2005).***

In the early stages of NEC, declining platelet counts correlate with the presence of necrotic bowel and worsening disease. The primary mechanism of thrombocytopenia appears to be platelet destruction, although this is not caused by DIC in most cases ***(Sola et al., 2000).***

- ***Congenital infections***

Congenitally acquired infections, including rubella, CMV, herpes virus, enterovirus, or HIV can cause fetal and neonatal thrombocytopenia. Most of the cases platelets are only moderately reduced and platelet numbers normalize within the first weeks of life. Severe bleeding is rare. In CMV-infected neonates thrombocytopenia is especially common with a prevalence of 36% In affected infants additional symptoms such as low birth weight, microcephaly, HSM, intracerebral calcification or chorioretinitis are often present also and lead to the diagnosis ***(Holzhauer and Zieger, 2011).***

- ***Neonatal infections***

Neonatal sepsis (NS) is a clinical syndrome of systemic illness accompanied by bacteremia occurring in the first month of life ***(Badrawi et al., 2001)***. Early-onset sepsis is defined as the onset of symptoms within the first days of life. There is variability of the age at onset, with some experts defining early-onset sepsis as bloodstream infection at ≤72 hours of age ***(Bizzarro et al., 2008)***. Late-onset sepsis is defined as the onset of symptoms after the first days of life. Similar to early-onset sepsis, there is variability in its definition ranging from an onset at >72 hours or ≥7 days of age ***(Pickering and Baker, 2012).***

Early-onset infection is usually due to vertical transmission by ascending contaminated amniotic fluid or during vaginal delivery from bacteria colonizing or infecting the mother's lower genital tract. As a result, the risk for sepsis increases from 1 to 4 percent in neonates born to mothers with chorioamnionitis ***(Polin, 2012).***

Late-onset sepsis is uncommonly associated with maternal obstetrical complications. Risk factors can include use of forceps during delivery or electrodes placed for intrauterine monitoring, which penetrate the neonatal defensive epithelial barriers of the skin and mucosa. Metabolic factors, including hypoxia, acidosis, hypothermia, and inherited metabolic disorders are likely to contribute to risk for and severity of neonatal sepsis. These factors are thought to disrupt the neonate's host defenses ***(Nizet and Klein, 2010).***

Late onset thrombocytopenia is most often caused by neonatal acquired infections. 55-65% of neonates with bacterial sepsis do have platelet counts <100_109/L. Thrombocytopenia may develop rapidly, especially in very preterm neonates, and is often severe.Sepsis due to Gram-negative bacteria results in particularly severe thrombocytopenia. DIC with increased platelet consumption is one of the major mechanisms resulting in low platelet numbers in neonatal infection ***(Holzhauer and Ba Zieger, 2011).***

The overall incidence of neonatal sepsis ranges from 1 to 5 cases per 1000 live births. The estimated incidence is lower in term infants, with a reported rate of 1 to 2 cases per 1000 live births ***(Bailit, 2010).***

Currently, Group B streptococcus and Escherichia coli are the most common causes of both early and late-onset sepsis. The incidence of early-onset GBS has declined by 80 percent with the use of intrapartum antibiotic prophylaxis, however, GBS and *E.coli* continue to account for approximately two-thirds of early-onset infection ***(Bizzarro, 2005).***

The morbidity and mortality from NS continues to be a major problem ***(Abdel-Hady and Zaki, 2003)***. Fungal infections are prevalent among very low birth weight infants and are associated with significant morbidity and mortality ***(Kaufman, 2004).*** Candida species rank second to fourth as the most frequent cause of late onset sepsis in VLBW ***(Yalaz et al., 2006).***