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## **Role of Thrombopoietin in Megakaryopoiesis and Thrombopoiesis with Relation to Platelets Ultrastructure**

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Thrombopoietin (TPO) is a cytokine that has been isolated and identified as the primary growth factor responsible for regulation of megakaryopoiesis and thrombopoiesis. The aim of this work was to study thrombopoietin (TPO) level in normal adults and children as well as its level estimation in quantitative platelet disorders. The correlation between TPO levels and platelet count was also studied. The correlation between TPO levels and ultrastructure changes of platelets was studied as well. The present study was conducted on 100 persons. They were 40 patients selected from the outpatient clinic of National Cancer Institute, Cairo University and they had quantitative platelet disorders in the form of thrombocytopenia and thrombocytosis. The remaining 60 cases were apparently healthy individuals. Assessment of TPO assay to all the studied groups was done by using ELISA method. Examination by Transmission Electron Microscopy (TEM) has been done for the circulating blood platelets of selected cases of quantitative platelet disorders as well as for some normal control cases. Significant differences were found between TPO levels in children and neonates groups as compared to normal adult control. Highly significant inverse correlation between TPO and platelet counts in adult control group. Comparison between TPO means was done in three subgroups as regards megakaryocyte frequency (normal MK1, decreased MK 2, increased MK 3) by using ANOVA test. ANOVA revealed significant statistical difference between TPO means of normal (MK1, n=19), TPO means of decreased megakaryocyte frequency (MK2, n = 10) and TPO means of increased megakaryocyte frequency (MK3, n =11). It was observed that high values of TPO levels were accompanied by decreased megakaryocyte frequency and lower TPO values accompanied by increased megakaryocyte frequency. Therefore, TPO levels are inversely related to megakaryocyte frequency. The ultrastructure study of circulating blood platelets was done for some selected and representative cases from thrombocytopenia group and thrombocytosis group as well as normal control group. It was observed that normal platelet morphology seen by E.M. was accompanied by normal TPO levels. While, abnormalities seen in platelets of thrombocytopenia were accompanied by abnormal high TPO levels. In thrombocytosis cases, abnormalities seen in platelets were accompanied by relatively lower TPO levels than that of normal cases.

**Key words:** Thrombopoietin (TPO), platelets, thrombocytopenia, thrombocytosis

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## INTRODUCTION

Human Thrombopoietin (TPO), the main stimulus for thrombopoiesis is produced primarily in the liver (Rios *et al.*, 2005). It is encoded by a single gene located on chromosome 3q 27-28, a region of the long arm of chromosome 3. The mapping of the TPO gene is particularly interesting as structural abnormalities of the long arm of chromosome 3 have been associated with increased bone marrow megakaryocytes (BM MK) and elevated platelet counts in patient with acute non lymphocytic leukemia (Ballaum, 1998).

Thrombocytopenia may be due to deficient platelet production like in: Aplastic Anemia (AA), Thrombocytopenia associated with acute leukemia, Myelodysplastic Syndrome (MDS), Chemotherapy-Induced Thrombocytopenia or Congenital megakaryocytic thrombocytopenia. Moreover, thrombocytopenia may be due to increased platelet destruction: Autoimmune Thrombocytopenic Purpura (AITP), Disseminated Intravascular Coagulopathy (DIC) and Thrombotic Thrombocytopenic Purpura (TTP) or Preeclampsia. Other miscellaneous causes of thrombocytopenia are thrombocytopenia associated with liver diseases, thrombocytopenia associated with renal failure, Human Immunodeficiency Virus (HIV) associated thrombocytopenia (Sundell and Koka, 2006), thrombocytopenia associated with fulminant meningococcal septicemia, neonatal thrombocytopenia and juvenile cyclic megakaryocytic thrombocytopenia (Bruin *et al.*, 2005).

Thrombocytosis can be divided into two categories. One group, termed clonal or primary thrombocytosis, occurs in the context of the chronic myeloproliferative disorders, including Essential Thrombocytopenia (ET), idiopathic myelofibrosis, polycythemia vera and chronic myeloid leukemia. The other group, called reactive or secondary thrombocytosis, occurs in various inflammatory states, neoplastic diseases, iron deficiency anemia and after splenectomy. The distinction between these two groups is clinically relevant because thrombohemorrhagic complications are more common in clonal thrombocytosis (Schafer, 2002).

Measurement of blood TPO levels may be used to distinguish between clonal and reactive Thrombocytosis. Some studies showed that blood TPO level were significantly higher in patients with clonal thrombocytosis than in patients with reactive thrombocytosis (Hou *et al.*, 1998; Wang *et al.*, 1998).

The aim of this research is to study thrombopoietin (TPO) level in normal adults, children and newborn as well as its level estimation in quantitative platelet disorders.

The correlation between TPO levels and platelet count were also studied. The correlation between TPO levels and ultra-structure of platelets were studied as well.

## MATERIALS AND METHODS

The present study was done on 100 persons, 40 patients of them were selected from the outpatient clinic of National cancer institute, Cairo University. The other 60 person were taken as normal subject. The studied cases were divided into 5 groups.

Blood samples were aspirated from all cases under completed aseptic conditions using vacuum tube (Vacutainer, Becton Dickinson, Meylan cedex, France). Plasma samples were collected on ice using EDTA as an anticoagulant. Those samples were processed for complete blood pictures including platelet counts and for TPO assays as well. For TPO assay, a rapid separation of plasma by centrifugation at 1000x g for 10 min. After collection of samples to ensure optimal recovery and minimal platelet contamination. The samples were stored in cryotubes (Nunc, Roskild, Denmark) at -20°C.

The following laboratory investigations were done:

- Complete blood count including platelet count (Statland *et al.*, 1998).
- Bleeding time (Channing and Levin, 1990).
- Bone Marrow Examination (Islam, 1991).
- Assessment of TPO Assay: by ELISA

Serum and plasma samples were evaluated for the presence of TPO in this assay. All platelet-poor plasma samples measured less than the lowest TPO standard, 31.2 pg mL<sup>-1</sup>. The normal range is ND-136 pg mL<sup>-1</sup> with a mean of 67.1 pg mL<sup>-1</sup> for serum sample (n = 100) (Miyazaki, 1996).

- Preparation for examination of Transmission Electron Microscopy (TEM): The preparation of the samples for E.M procedure was carried out according to the method of White (1983): 10 mL of venous blood was obtained in 20 mL plastic syringes. A 19-gauge needle is routinely employed. The collected blood samples were put on heparin as an anticoagulant.

In this study TEM has been used for circulating platelet disorders as well as for some normal control cases. It was performed in E.M unit, Clinical pathology department, National Cancer Institute, Cairo University, using JEOL 100S EM transmission electron microscopy. This TEM has a magnification power of more than 250,000 X and a resolution of better than 0.5 nm.

**RESULTS**

The present study was conducted on 100 persons. They were 40 adult patients having quantitative platelet disorders in the form of either thrombocytopenia or thrombocytosis and 20 normal adult as control. The remaining 40 cases were apparently healthy children and neonates. In this study, correlation between TPO levels and ultra structure of platelets in some selected cases of quantitative platelets disorders was done. Then the changes detected in these cases were compared by normal control cases.

Bone marrow aspiration was done for only pathological groups (thrombocytopenia and thrombocytosis groups) and that to determine the degree of megakaryocyte frequency in Bone marrow in these cases. Megakaryocyte frequency was subsequently divided into three categories, which were normal megakaryocyte frequency (MK1), decreased megakaryocyte frequency (MK2) and increased megakaryocyte frequency (MK3).

Table 1 shows significant differences between TPO levels in children and neonates groups as compared to normal adult control ( $p = 0.00$ ). TPO mean in neonate group ( $207.59 \text{ pg mL}^{-1}$ ) was the highest value, which tended to decrease when the age is increased to be the lowest value in adult group ( $82.53 \text{ pg mL}^{-1}$ ).

For sex variation, comparisons of TPO means in all different age groups comparing males and females by using student's t test showed no significant differences between TPO levels of males and females in different age groups ( $p = 0.71$  in control group,  $p = 0.52$  in children group and  $p = 0.61$  in neonate group).

Table 2 revealed highly significant inverse correlation between TPO and platelet count in adult control group ( $r = -0.97$  and  $p = 0.00$ ). There was insignificant direct correlation between TPO mean and platelet counts in children group ( $r = 0.22$ ,  $p = 0.35$ ). There was highly significant inverse correlation between TPO levels and platelet counts in neonate group ( $r = -0.78$ ,  $p = 0.00$ ). There was significant inverse correlation between TPO and platelet count in thrombocytopenia group ( $r = -0.54$ ,  $p = 0.01$ ). Lastly, there was insignificant inverse correlation between TPO and platelet count in thrombocytosis group ( $r = -0.27$ ,  $p = 0.24$ ).

Figure 1 shows significant inverse correlation between TPO values and Platelet Counts (PC) in adult control group.

Table 3 demonstrate comparison between TPO means in both thrombocytopenia and thrombocytosis groups as compared to normal adult control group by

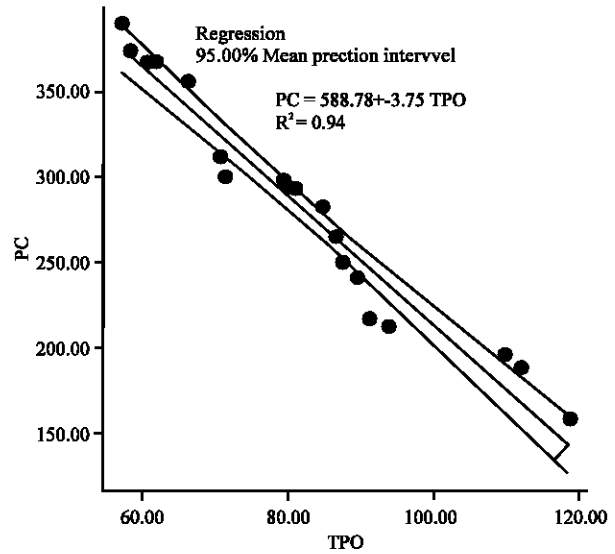


Fig. 1: Regression line of TPO and platelet count in adult group

Table 1: Statistical comparison of TPO means in different age groups

Group	TPO ( $\text{pg mL}^{-1}$ ) Mean $\pm$ SD	F	p-value
Adult	82.53 $\pm$ 17.68	217.72	0.001
Children	117.42 $\pm$ 20.49*		
Neonates	207.59 $\pm$ 20.37*		

N = 20,  $p\text{-value} \leq 0.05$  is considered significant, \*Significant

Table 2: Statistical correlation between TPO and platelet count in all studied groups

Group	r	p-value
Adult	-0.97 **	0.00
Children	0.222 †	0.35
Neonates	-0.78 **	0.00
Thrombocytopenia	-0.54 *	0.01
Thrombocytosis	-0.274 †	0.24

N = 20  $p\text{-value} \leq 0.05$  is considered significant, \*\*Highly significant \*Significant, † Non Significant

Table 3: Analysis of variance comparing megakaryocyte frequencies in relation to their TPO means

MK Freq.	Code	No.	TPO Means ( $\text{pg mL}^{-1}$ ) $\pm$ SD	F	p-value
Normal	MK 1	19	52.12 $\pm$ 12.76	30.67	0.00
Decreased	MK 2	10	875.91 $\pm$ 589.13*		
Increased	MK 3	11	48.94 $\pm$ 9.97*		

$p\text{-value} \leq 0.05$  is considered significant, \*Significant

using ANOVA. ANOVA revealed significant statistical difference between TPO means in these groups and control ( $F = 9.28$ ,  $p = 0.00$ ).

Table 4 show comparison of TPO means in megakaryocyte frequency subgroups (normal (MK1), decreased (MK 2) and increased (MK 3)), by using ANOVA test. ANOVA revealed significant statistical difference between TPO means ( $52.12 \text{ pg mL}^{-1}$ ) of normal (MK1,  $n = 19$ ), TPO means ( $875.91 \text{ pg mL}^{-1}$ ) of decreased

Table 4: Analysis of variance for TPO means in thrombocytopenic and thrombocytosis groups as compared to control

Group	TPO Means (pg \ml)±SD	F	p-value
Control	82.53±17.68	9.28	0.00
Thrombocytopenia	464.07±585.08*		
Thrombocytosis	49.91±11.12*		

N = 20, p-value ≤ 0.05 is considered significant, \*Significant p-value

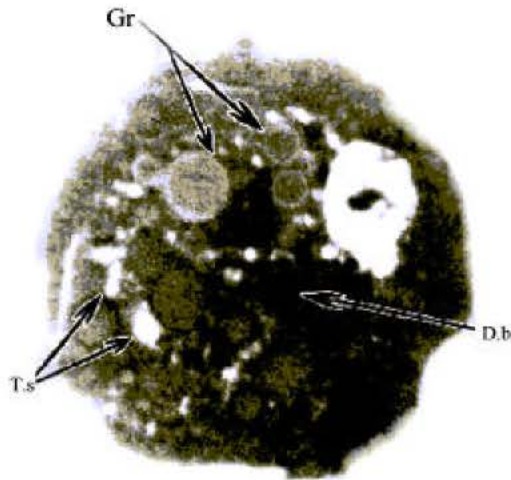


Fig. 2: Electron micrograph showing normal blood platelet with dense Tubular System (T.s) scattered around the  $\alpha$ -granules (Gr). A Dense Bodies (D.b), many granules are present (X 5400)

megakaryocyte frequency (MK2, n = 10) and TPO means ( $48.94 \text{ pg mL}^{-1}$ ) of increased (MK3, n = 11), ( $F = 30.67$ ,  $p = 0.00$ ).

It was observed that high values of TPO levels accompanied by decreased megakaryocyte frequency and lower TPO values accompanied by increased megakaryocyte frequency. Therefore, TPO levels are inversely related to megakaryocyte frequency.

**Results of electro-microscopic study:** The present study included 100 cases. The ultra structure study was done for some selected and representative cases from thrombocytopenia group and thrombocytosis group as well as normal control group.

Normal blood platelet show dense tubular system scattered around the granules, dense body and many granules. The electron density of the  $\alpha$ -granules matrix substance is appreciably less, however than that of the dense granule.  $\alpha$ -granules are variable in size and shape but usually small and round or oval Fig. 2. Thrombocytopenia shows an abnormal blood platelet with shape changes. The platelet organelles (dense bodies, mitochondria,  $\alpha$ -granules and lysosomes) are usually collected in one area leaving other parts of platelet devoid of granules Fig. 3 a giant sized platelets with marked

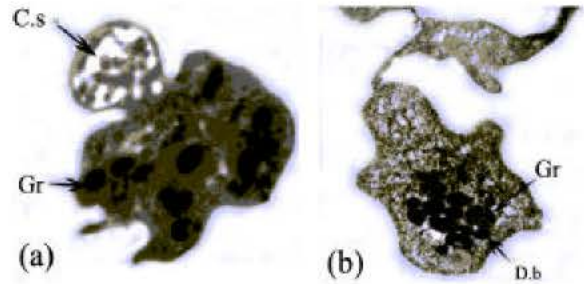


Fig. 3: Electron micrograph of: (a) a case of thrombocytopenia showing an abnormal shaped platelet with reduced numbers of Granules (Gr) and other organelles. Dilated channels of open Canalicular System (CS) are present (X 6200). (b) abnormal platelet from a case of thrombocytopenia, where the platelet organelles and lysosomes are collected in one area leaving other parts devoid organelles. The adjacent platelet is completely devoid of granules (X 9000)

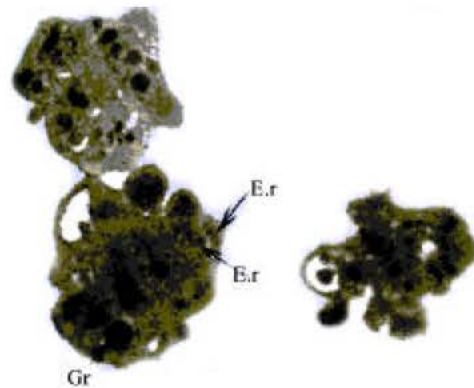


Fig. 4: Electron micrograph from a case of thrombocytosis showing an abnormal platelet with abnormal large granules (Gr) and widening of the endoplasmic reticulum (X 3600)

reduction in granule contents may also be present in cases with thrombocytopenia. In thrombocytosis abnormally increased blood platelets show significant decrease in the lysosomes and  $\alpha$ -granules. The platelets show increase in the demarcation membrane with hypertrophy of the dense tubular system. These may also show abnormal large granules and widening of the endoplasmic reticulum Fig. 4.

It was observed that normal platelet morphology seen by EM was accompanied by normal TPO levels. While, abnormalities seen in platelets of thrombocytopenia were accompanied by abnormal high TPO levels. In thrombocytosis cases, abnormalities seen in platelets were accompanied by relatively lower TPO levels than that of normal cases.

## DISCUSSION

TPO is a haemopoietic growth factor that stimulates development of megakaryocyte precursors, megakaryocytes and platelets (Rios *et al.*, 2005). Measurement of TPO levels may have utility by enhancing the clinician's ability to differentiate thrombocytopenia caused by decreased megakaryopoiesis from that caused by increased platelet destruction or ineffective thrombopoiesis. In some cases, TPO measurement might obviate a marrow aspiration to evaluate megakaryopoiesis (Kuefer *et al.*, 1998).

This study aims to focus on thrombopoietin levels in different normal age groups (neonates, children and adults), also on thrombopoietin levels in quantitative platelet disorders. Comparison between TPO levels in different age groups including neonates, children and adults were carried out using ANOVA. This was done to evaluate whether TPO levels in these different age groups differs or not. There was significant difference in TPO levels among these groups. Plasma TPO values were distributed differently between children and adults and high levels of TPO were generally found during the neonatal period. Mean TPO levels in children group were higher than adult levels. TPO mean in neonatal period was the highest TPO levels and significantly different from those of adult control group.

In agreement with this result, Ishiguro *et al.* (1999) had reported that serum TPO values were distributed differently between children and adults and that high levels of TPO were generally found during the neonatal period. They have analyzed TPO values by dividing the subjects into 11 age groups. Mean TPO levels in any group of children ( $1.24\text{-}5.92\text{ fmol mL}^{-1}$ ) were significantly higher than adult levels ( $p\text{ value}<0.001$ ). Cord blood contained high levels of TPO and the highest mean level was found 2 days after birth. TPO levels from birth to 1 month of age were significantly higher than those in the older subjects ( $4.24\pm 1.58\text{ fmol mL}^{-1}$  versus  $1.63\pm 0.79\text{ fmol mL}^{-1}$ ,  $p<0.001$ ). They became stable from 2 months to 4 years of age ( $1.97\text{-}2.23\text{ fmol mL}^{-1}$ ) and then generally decreased by 11-15 years of age (Ishiguro *et al.*, 1999).

Also, Sainio *et al.* (2000) had compared TPO levels in cord blood at term with those of adult controls and they found the significantly higher difference in all cases ( $p<0.001$ ). TPO levels in adult controls  $28\text{ pg mL}^{-1}$  ( $6\text{-}60\text{ pg mL}^{-1}$ ); while in newborn controls,  $76\text{ pg mL}^{-1}$  ( $55\text{-}178\text{ pg mL}^{-1}$ ).

The exact mechanisms of elevated TPO levels during neonatal period remain under investigation. The elevation of blood TPO levels might be explained by an increased

rate of TPO production, decreased TPO clearance and or reduced c-Mpl mass on megakaryocyte and platelets. Megakaryocyte cultured from cord blood have one tenth the c-Mpl compared with adult receptors (Kuwaki *et al.*, 1999).

In this study, statistical comparison of TPO means between males and females in different normal age groups was done using student's t-test. It revealed insignificant relation between TPO means in males and females. Jing *et al.* (2002) reported the same results in patients with AA. The mean plasma TPO level in male AA patients ( $842\pm 408\text{ pg mL}^{-1}$ ) was not different from that in female patients ( $724\pm 382\text{ pg mL}^{-1}$ ) ( $p>0.05$ ).

Moreover, there was significant inverse correlation between TPO levels and platelet counts in adult control group, neonate group and thrombocytopenia group. Mean platelet counts in adult control group was highly significantly correlated to TPO levels and there was inverse relation between them. Mean platelet counts in neonates was significantly correlated to TPO levels of this group and there was inverse relation between them. There was significant inverse correlation between platelet counts and TPO in thrombocytopenia group.

Matondang *et al.* (2004) had reported the same results in which there was significant negative correlation between the TPO level and the platelet count in the normal individuals and the dengue fever patients (thrombocytopenic group).

For the relationship between TPO levels and platelet count during neonatal period, the study done by Sainio *et al.* (2000) came in the same line with this present study. They had reported that TPO levels correlated inversely with platelet counts in normal babies at term ( $r = 0.53$ ,  $p = 0.006$ ).

Also, Hsu *et al.* (2002) had studied TPO levels in patients with AML/MSD before and after chemotherapy. TPO levels were significantly higher in all patients than in normal subjects ( $p<0.05$ ). Platelet counts were significantly lower in the AML/MSD patients before ( $71.2\pm 11.6\times 10^9\text{ L p}^{-1}<0.05$ ) and during chemotherapy ( $47.2\pm 6.1\times 10^9\text{ L p}^{-1}<0.05$ ) as compared to normal control subjects ( $240\pm 47\times 10^9\text{ L}^{-1}$ ), but subsequently restored to normal levels during complete remission ( $181.4\pm 26.3\times 10^9\text{ L}^{-1}$ ). Correlation analysis showed an inverse correlation between platelet counts and TPO levels in AML/MDS patients during diagnosis ( $r = 0.277$ ,  $p<0.05$ ), chemotherapy ( $r = -0.402$ ,  $p<0.05$ ) and in complete remission ( $r = -0.512$ ,  $p<0.05$ ).

In contrary to the present study, Ishiguro *et al.* (1999) had reported that the relationship between serum TPO levels and the platelet counts was not significant in all subjects ( $r = 0.27$ ,  $n = 243$ ), in children alone ( $r = 0.12$ ,  $n = 214$ ), or in any age groups.

From the analysis of the data obtained from thrombocytopenic group in the present study, it was observed that TPO values were not constantly high in all cases. About 8 cases from thrombocytopenic group were giving high values of TPO and the rest of patients were being within normal range. This may be explained by the study of Nagata *et al.* (1997) as they had reported that platelet count is not exactly inversely proportional to serum TPO level. During acute thrombocytopenia, serum TPO level transiently increased a few hours after anti-platelet monoclonal antibody administration in mice and returned to basal level just when matured megakaryocyte accumulated in bone marrow but the platelet count was still low. Elevated TPO levels in thrombocytopenia are likely due to decreased platelet and megakaryocyte masses that directly regulate TPO levels by receptor-mediated absorption (Cohen-Solal *et al.* (1996).

Another explanation for increased TPO levels during thrombocytopenia came in the study of Jing *et al.* (2002) who studied TPO levels in patients with AA. The increased concentration could be explained by up-regulation of TPO production due to a feedback mechanism, decreased elimination, or decreased utilization of TPO. Their results also showed a significant inverse correlation between TPO levels and platelet counts in AA patients, supporting the feedback mechanism of TPO production by platelets.

In this study, TPO did not significantly correlate with platelet counts in thrombocytosis. Also, Nagata *et al.* (1997) had studied the effect of thrombocytosis in TPO transcriptional levels in liver, kidney, bone marrow and spleen. They had found that seven days after the first injection of recombinant mouse TPO into mice, the platelet count in circulation increased about two-fold above the basal level ( $3.0 \times 10^6 \text{ mm}^{-3}$ ). TPO transcription levels decreased slightly in liver and kidney, but they remained high even in thrombocytosis. The levels of TPO transcripts in bone marrow and spleen were constant during thrombocytosis. Therefore, thrombocytosis apparently does not affect the TPO transcription.

The TPO means in both thrombocytopenia and thrombocytosis groups were compared to normal control group. The statistical analysis revealed that there was significant difference between TPO means in these pathological groups as compared to normal control group. In agreement with this result, Tahara *et al.* (1996) had reported that there was higher serum TPO levels in studied cases of ALL (mean platelet count  $103 \pm 99 \times 10^9 \text{ L}^{-1}$ ) than normal ( $p = 0.016$ ). Their serum TPO levels ranged from 4.04 to 18.53  $\text{fmol mL}^{-1}$  with a mean of  $10.360 \pm 5.57 \text{ fmol mL}^{-1}$ . Similarly, the mean serum TPO

levels of  $18.53 \pm 12.37 \text{ fmol mL}^{-1}$  in seven patients with AA (mean platelet count  $56 \pm 58 \times 10^9 \text{ L}^{-1}$ ) was significantly higher than normal ( $p < 0.01$ ).

Significant inverse correlation was found between TPO levels and megakaryocyte frequency. So, patients with decreased megakaryocytes had markedly increased TPO levels compared to normal megakaryocytes. Their levels were significantly different from those of patients with increased megakaryocytes.

In agreement with this result, Kuefer *et al.* (1998) had reported that patients with decreased megakaryocytes had markedly increased TPO levels (median 1419  $\text{pg mL}^{-1}$ , range 1076 to 2457  $\text{pg mL}^{-1}$ ) compared to normal controls (median 102  $\text{pg mL}^{-1}$ , range 51 to 333  $\text{pg mL}^{-1}$ ). Their levels were significantly different ( $p = 0.03$ ) from those of patients with increased megakaryocytes. Patients with chemotherapy-induced depression of megakaryopoiesis had a marked increase in TPO levels with a subsequent decrease in TPO levels when megakaryopoiesis recovered. They had concluded that TPO levels were inversely related to megakaryocyte frequency.

In another study, Nagasawa *et al.* (1998) counted the number of megakaryocyte in bone marrow of the patients with diffuse large cell lymphoma before chemotherapy and after chemotherapy, then comparing them to normal subjects. They found that the number of megakaryocyte before chemotherapy was increased to  $7.5 \text{ mm}^{-2}$  in the clot section, compared with that of normal subjects ( $3.4 \pm 1.8 \text{ mm}^{-2}$   $n = 15$ ). The numbers of megakaryocyte on days 4, 7, 14, 20 of chemotherapy were 3.6, 0.2, 3.9 and  $6.6 \text{ mm}^{-2}$  in the clot section. They had also found that the TPO level started to increase on day 4 in accord with the decrease of megakaryocytes, despite the increased platelet count in response to dexamethasone. On days 7-20 a clear reciprocal relationship was observed between the TPO levels and megakaryocyte numbers as well as platelet counts.

Several qualitative and quantitative abnormalities had been found in ultra structure of platelets obtained from patients with both thrombocytopenia and thrombocytosis. These included shape changes, reduced numbers of granules, dilated channels of open canalicular system, complete absence of granular contents, abnormal shaped granules, significant decrease in the lysosomes, increase in the demarcation membrane, hypertrophy of the dense tubular system and widening of the endoplasmic reticulum.

In agreement with this result, Raman *et al.* (1989) had found several qualitative and quantitative abnormalities in ultra-structure of platelets in patients with MPD when compared with 25 controls. These included a decrease in

the number of platelet granules, an increase in the number of platelet granules, abnormally shaped granules, dense tubular disarray, hypertrophy of dense tubules, increased glycogen and the presence of cytoplasmic crystalloid structures. They found that the degree of the type of abnormality seen could not correlate with the clinical course in general. Also, there was no pattern in the type of ultra structural abnormalities seen in different disease categories (PV, CML, MF, MDS and secondary thrombocytosis ST).

Decreased granular content can be caused by production of empty dense granule membranes, the empty sack syndrome, (Mc Nicol *et al.*, 1994) or can result from slow spontaneous leakage of granular contents as can occur in Chediak-Higashi syndrome (Apitz-Castro *et al.*, 1989).

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