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Platelet dysfunction in patients with MDS and ITP

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List of abbreviations

MDS	Myelodysplastic syndrome
ITP	Idiopathic thrombocytopenic purpura
TxA_2	thromboxane A_2
TxB_2	thromboxane B_2
PRP	platelet-rich plasma
PPP	platelet-poor plasma
LTA	light transmission aggregometry
AA	arachidonic acid
ADP	adenosine diphosphate
PGE_1	prostanglandin E_1
ASA	acetylsalicylic acid
EPI	epinephrine
GPCR	G protein-coupled receptors
Gp	glycoprotein
cAMP	cyclic adenosine monophosphate
CRP	collagen reactive peptide
SRP	synthetic liquid reactive peptide

Summary

Background. Hemorrhagic complications are frequent and among the leading causes of death in patients with myelodysplastic syndromes (MDS). Not only thrombocytopenia, but also platelet function abnormalities contribute to bleeding tendency of MDS patients. However, no published studies comprehensively evaluated several parameters of platelet function, including the measurement of the platelet levels of cyclic AMP (cAMP), which is an effective inhibitor of platelet function. In this case-control study, we aimed to comprehensively assess in vitro platelet function of patients with MDS of any IPSS risk category.

Methods. Patients diagnosed as MDS according to WHO guidelines and two control groups, primary Immune ThrombocytoPenia (ITP) patients and healthy subjects were consecutively recruited. Platelet aggregation and secretion in platelet rich plasma (PRP) with platelet count >150x10^9/L were induced by adenosine diphosphate (ADP) (2µM), epinephrine (5µM), thrombin receptor-activating peptide (TRAP) (10µM), synthetic reactive peptide (SRP) (4 ng/mL) (Biodata, USA), collagen reactive peptide (CRP) 0.1µg/mL (Farnardale lab, UK), Horm collagen (2µg/mL) and arachidonic acid (AA) (1 mM) and measured by lumi-aggregometry. In addition, the effect of Prostaglandin E1 (PGE1) (0.1µM) was tested in collagen-induced platelet aggregation. Intraplatelet cAMP content was measured by an ELISA assay (Cayman, USA) in extracts of PRP containing theophylline (1 mM), incubated with PGE1 (1 or 0.1 µM) or Tyrode's buffer alone for 2 minutes. Differences among the three groups were analyzed by Kruskal-Wallis and Dunn's multiple comparisons test. Correlation was studied by Spearman's rank correlation analysis.

Results. 32 MDS (male=22), 26 ITP (male=12) patients, and 54 healthy subjects (male=26) were enrolled. Platelet aggregation measured in PRP samples induced by all agonists was significantly lower in MDS patients than in both control groups, but there was no difference between ITP and healthy subjects. The platelet aggregates formation induced by collagen was significantly decreased in presence of PGE1 (0.1uM) in all groups. The extent of decrease was significantly higher in MDS patients than in the other two groups. MDS and ITP patients showed abnormal ATP release measured during aggregation induced by all agonists compared to controls. Intraplatelet cAMP production induced by PGE1 (0.1uM and 1 uM) were similar in MDS patients, ITP patients and healthy controls. However, when calculated as the ratio of cAMP increase after PGE1 stimulation, both MDS and ITP groups showed significantly lower increase ratio than healthy subjects.

Conclusion: The platelets dysfunction in MDS and ITP groups showed despaired aggregation and secretion, decreased intra-platelets granules contents, but with normal platelet surface receptors expression. Lower total cAMP level, and lower increase of cAMP ratiao (PRP – PPP)/PPP after adding of extra PGE1, may suggest that there could be dysfunctional maturation of platelets.

1.Introduction

1.1 Platelet

1.1.1 Structure of platelets

Human blood platelets are small, anucleated cells that play a critical role in hemostasis and thrombosis. Human platelets normally survive for approximately 10 days in the circulation. Normal human platelets are small and discoid in shape, the size is about 0.5 × 3.0 μm, have a mean volume of 7–11 fL, and normally circulate in relatively high numbers between 150 and 400 × 10^9/L.

1.1.2 Platelet function in primary haemostasis

Their small disc shape enables platelets to marginate toward the edge of vessels so that the majority circulate adjacent to the vascular endothelial cells that line all blood vessels. Upon detection of vessel wall damage, they undergo rapid but controlled adhesion, activation, and aggregation to form a hemostatic plug and thus rapidly prevent blood loss. Endothelial cells produce a number of potent antiplatelet substances (e.g. nitric oxide, prostacyclin and CD39) that normally inhibit vessel wall–platelet interactions. Vessel wall damage exposes highly adhesive substrates [e.g. P selectin, Von Willebrand factor (VWF), collagen, and other extracellular matrix components], which result in a sequence of stepwise events resulting in the formation of a hemostatic plug[1]:

1. *Platelet adhesion*. The process of platelet adhesion involves the interaction of receptors on the platelet membrane glycoprotein (GP) VI, GPIbα and GPIIb/IIIa, with its ligands in the like collagen, and VonWillebrand factor (vWF). As results of these interactions, a platelet activation process takes place that includes participation of one or more signal transduction pathways.

2. *Platelet aggregation and platelet procoagulant activity*. Platelet activation triggers the synthesis and release of several autocrine and paracrine mediators, including adenosine diphosphate (ADP), thrombin, epinephrine, and thromboxane A2 (TXA2). ADP, TXA2 and thrombin activate platelets through their interaction with G protein-

coupled receptors (GPCR), which activate multiple G protein–mediated signaling pathways to induce platelet-shape change, degranulation and integrin activation. Some GPCR are associated with G proteins (Gs) that stimulate adenilyly cyclase, thereby increasing the levels of cyclic AMP (cAMP), which inhibits platelet function: prostacuyclin and prostaglandin E1 are among the ligands that bind to Gs coupled receptors.

Activated platelets also express negatively charged phospholipids on their surface, facilitating the local generation of thrombin, which not only further activates platelets, but also stabilizes the platelet plug through fibrin formation. In this manner, platelets contribute to sealing any areas of vessel wall damage.

1.2 Myelodysplastic syndromes

Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal disorders of the hematopoietic stem cell (HSC), characterized by peripheral cytopenias despite increased hematopoïetic precursors, resulting in anemia, neutropenia or thrombocytopenia. Patients are at increased risk of infection and bleeding. About 30% of MDS patients develop acute myeloid leukemia. Among the cytopenias, thrombocytopenia is a serious complication of ineffective hematopoiesis and is associated with worse overall survival.

MDS is thought to result from the accumulation of genetic or epigenetic (such as promoter hypermethylation) lesions, occurring initially in an immature progenitor and leading to a proliferative advantage of the MDS clone over normal immature progenitors. Age-induced genetic, epigenetic, and immune-mediated changes in haemopoietic stem cells (HSC) lead to oligoclonal expansion of myelodysplastic stem cells, with defective differentiation, characterised by increased apoptosis of erythroid and myeloid progenitors[2]. Microenvironmental changes and immune deregulation contribute to this differentiation defect. MDS progenitors display abnormal terminal

differentiation and increased susceptibility to apoptosis, which may explain the clinical consequences of blast accumulation and peripheral cytopenias[3].

The median age at diagnosis is approximately 70 years. The incidence is 4 to 5 per 100,000 persons per year. The etiology is generally unknown, the role of exposure to environmental chemical and physical mutagens is sometimes suspected. In 15 to 20% of cases, however, MDS are secondary (sMDS) to chemotherapy and/or radiotherapy for a prior illness. More rarely, they are secondary to exposure to benzene or other aromatic hydrocarbons, or products used in agriculture.

Presence of dysplasia is the first key criterion for diagnosis and prognosis of MDS. A given lineage is considered dysplastic if two or more dysplastic features are found in > 10% cells. Multilineage dysplasia (MD) is defined as the coexistence of dysplasias in two or more lineages. Blast cells excess is observed in about 40-60% of cases.

Myeloblasts are consensually defined by a high nuclear/cytoplasmic ratio and diffuse chromatin pattern, can be "agranular" or "granular". MDS have received a variety of nomenclatures, until the first international classification by the French American British (FAB) group in 1982. This classification has been refined in 2001 then in 2008 by a WHO expert committee[4], integrating novel prognostic factors in MDS, such as multilineage dysplasia. The marrow blast threshold of AML was lowered from 30% to 20%. This classification also designed the term MDS/MPN (Myeloproliferative Neoplasm) to regroup a heterogeneous set of rare entities including chronic myelomonocytic leukemia (CMML), previously considered as a MDS.

Table 1.1. WHO classification for MDS

Subtype	Peripheral blood	Bone marrow	Relative Proportion
Refractory cytopenia with unilineage dysplasia (RCUD)			
Refractory anaemia (RA) Anaemia, <1% blasts	Unilineage erythroid dysplasia (in >10% cells), <5% blasts	10–20	
Refractory neutropenia (RN) Neutropenia, <1% blasts	Unilineage granulocytic dysplasia, <5% blasts	<1	
Refractory thrombocytopenia	Thrombocytopenia, <1% blasts	Unilineage megakaryocytic dysplasia, <5% blasts	<1%
Refractory anaemia with ring sideroblasts (RARS)	Anaemia, no blasts	Unilineage erythroid dysplasia, >15% erythroid precursors are ring sideroblasts, <5% blasts	3–10%
Refractory cytopenia with multilineage dysplasia (RCMD)	Cytopenia(s), <1% blasts. No Auer rods	Multilineage dysplasia +/. ring sideroblasts, <5% blasts. No Auer rods	30%
Refractory anaemia with excess blasts type 1 (RAEB-1)	Cytopenia(s), 2–9% blasts. No Auer rods	Unilineage or multilineage dysplasia, 5–9% blasts. No Auer rods	40%
Refractory anaemia with excess blasts type 2 (RAEB-2)	Cytopenia(s), 5–19% blasts +/. Auer rods	Unilineage or multilineage dysplasia, 10–19% blasts. +/. Auer rods	
Myelodysplastic syndrome associated with isolated del(5q)	Anaemia Normal or high platelet count, <1% blasts	5q31 deletion Anaemia, hypolobulated megakaryocytes, <5% blasts	<5%

Myelodysplastic syndrome, unclassifiable (MDS-U)	Cytopenia(s), <1% blasts	Does not fit in other groups	Rare
Childhood myelodysplastic syndrome	Pancytopenia	<5% blasts	<1%

BM biopsy first allows objective evaluation of BM cellularity, which physiologically declines with age. Application of a standardized age correction to cellularity brings the incidence of "hypoplastic" MDS from 29% to 7%[5]. Hypoplastic MDS raises the question of the differential diagnosis with aplastic anemia (AA). Features of MDS are the presence of circulating myeloblasts, megakaryocytic or granulocytic dysplasia. Other MDS criteria include abnormal sideroblasts, presence of two or more blast cell clusters. Clusters (3-5 cells) or aggregates (> 5 cells) of blasts cells away from endosteal or vascular niches, in the central portion of the BM have been dubbed "abnormally localized immature myeloid progenitors" (ALIP). ALIP have been proposed as diagnostic and prognostic markers.

Immunohistochemistry with a CD34 antibody marks immature hematopoietic progenitors and megakaryocytes, and can be used to asses the blast percentage. However, some MDS have CD34- blasts: in those cases, CD117 has been proposed as a surrogate marker. Some authors have proposed that the presence of CD34+ cell clusters may better reflect prognosis than CD34+ cell percentage.

Prognostic evaluation in MDS still largely relies on an International Prognostic Scoring System (IPSS) established on the basis of an international cohort of patients (IMRAW cohort) treated symptomatically and recently revised (IPSS-R)[6]. IPSS relies on number of cytopenias, marrow blast percentage and cytogenetic. Patients are regrouped into four risk categories (low, intermediate 1 and 2, and high). IPSS categories are often regrouped into lower-risk MDS (IPSS low and intermediate-1), and higher-risk MDS (IPSS intermediate-2 and high). Lower-risk MDS are patients with prolonged survival where the main objectives are to cope with chronic cytopenias notably anaemia, and to defer ASCT. On the other hand the treatment in higher-risk MDS should alter disease history and prolong survival. The approval of azacytidine in higher-risk underscores the importance of IPSS evaluation in all patients at diagnosis.

According to the IPSS-R, 27% of the lower-risk MDS patients of the original IPSS are

reclassified as having a higher risk and they potentially need a more intensive treatment. Conversely18% of high-risk MDS patients, as defined by the original IPSS, are reclassified as low risk by the IPSS-R.

Table 1. 2. Comparation of items in IPSS with Revised IPSS (R-IPSS)

IPSS			**R-IPSS**		
Parameter	**Score**		**Parameter**	**Score**	
Blastes			***Blastes***		
<5%	0		=<2%	0	
5-10%	0.5		2-5%	0.5	
15-20%	1.5		5-10%	1.5	
21-30%	2		>10%	2	
Cytogenetics			***Cytogenetics***		
Good	0		Very good	0	
Intermediate	0.5		Good	0.5	
Poor	2		Intermediate	2	
			Poor	3	
			Very poor	4	
			Hb		
			8-10	1	
			<8	1.5	
			ANC		
			<0.8	0.5	
			Platelts		
			50-100	0.5	
			<50	1	
Risk groups	***score***	***OS***	***Risk groups***	***score***	***OS***
Low	0	5.7	Very low	=<1.5	8.8
Int-1	0.5-1	3.5	Low	1.5-3	5.3
Int-2	1.5-2	1.2	Int	3-4.5	3.
High	>2	0.4	High	4.5-6	1.6
			Very high	>6	0.8

*OS: Overall Survival ANC: Absolute Neutrophil Count,

Table 1.3. Karyotype (IPSS-R)

	Proportion of patients (%)	Karyotype	Median survival (years)	Time to 25% AML evolution (years)
Very good	4%	-Y, del(11q)	5.4	NR
Good	72%	Normal, del(5q), del(12p), del(20q), double including del(5q)	4.8	9.4
Intermediate	13%	del(7q), +8, +19, i(17q), any other single or double independent clones	2.7	2.5
Poor	4%	-7, inv(3)/t(3q)/del(3q) double including -7/ del(7q) Complex: 3 abnormalities	1.5	1.7
Very poor	7%	Complex > 3 abnormalities	0.7	0.7

Treatment

Treatment varies from symptomatic treatment of cytopenias, especially by transfusions for anemia, to allogeneic stem-cell transplantation. Treatment of patients with lower-risk myelodysplastic syndromes includes growth factors and lenalidomide. Higher-risk patients are treated with hypomethylating agents and allogeneic stem-cell

transplantation. [7]

Table1.4. Treatment

Summary of Treatments for Thrombocytopenia in Myelodysplastic Syndromes	
Regimen class/drug category	
Platelet focused	Drug name
Androgens	Danazol
Thrombopoietic growth factors	IL-11; IL-6; PEG-rHuMGDF
General	
Differentiation agents	Azacitidine; decitabine
Anti-TNFa factors	Amifostine; etanercept; pentoxifylline
Hematopoietic growth factors	IL-3
Immunosuppressive agents	Cyclosporine; ATG
Retinoids	ATRA; 13-cis retinoic acid
Angiogenic inhibitors	Arsenic trioxide; sirolimus
Immunomodulatory agents	Lenalidomide; thalidomide
Chemotherapy	Cytosine arabinoside; Combination of topotecan, cytarabine, and idarubicin
Farnesyl-transferase inhibitors	Tipifarnib; lofarnib
Vitamin D compounds	Calcitriol
Vitamin K2	Menatetrenone

1.3 Immune thrombocytopenia

Immune thrombocytopenia (ITP) is defined as isolated low platelet count (thrombocytopenia) with normal bone marrow and the absence of other causes of thrombocytopenia such as systemic lupus erythematosus, malignancy, and DIC. It causes an increased tendency to bleed[8].

The incidence of ITP is estimated at 50–100 new cases per million per year, with children accounting for half of that amount. At least 70 percent of childhood cases will end up in remission within six months, even without treatment. Moreover, a third of the remaining chronic cases will usually remit during follow-up observation, and another third will end up with only mild thrombocytopenia (defined as a platelet count above 50*10^6/㎖).

ITP is usually chronic in adults and the probability of durable remission is 20–40 percent. The male to female ratio in the adult group varies from 1:1.2 to 1:1.7 in most age ranges and tends to widen with age (childhood cases are roughly equal for both sexes) and the median age at the diagnosis is about 60 years.[9]

In approximately 60 percent of cases, antibodies against platelets can be detected. Most often these antibodies are against platelet membrane glycoproteins IIb-IIIa or Ib-IX, and are of the immunoglobulin G (IgG) type.

In mild cases, only careful observation is required. In contrast, severe cases, especially if accompanied by bleeding manifestations, need treatment with corticosteroids, intravenous immunoglobulin, anti-D immunoglobulin, or other immunosuppressive drugs. Refractory ITP (not responsive to conventional treatment) may require splenectomy.

1.4 Measurement methods for evaluating platelet function

1.4.1Lumi-aggregometry measurement for platelet aggregation and secretion

Of all the functional responses of platelets, aggregation is probably the mostly widely investigated. Turbidimetric aggregometry in its modern form was initially described in the 1960s. Light is passed through a stirred turbid suspension of platelets[9]; the presence of the platelets in suspension causes the light to be scattered such that a reduced proportion of it passes directly through the platelet suspension unobstructed. The amount of transmitted light is recorded and provides a measure of the optical density of the platelet suspension. On addition of a proaggregatory stimulus, as the platelets aggregate, the optical density of the suspension is reduced. A typical curve for platelet aggregation recorded by LTA method showed in Figure 1[10].

The unstimulated suspension of platelet-rich plasma (PRP) has a relatively high optical density (OD). Following addition of the agonist (A) the platelets aggregate, allowing

more light to pass through the suspension of platelets and resulting in a reduction in the optical density. Autologous platelet-poor plasma (PPP) provides the measured optical density equivalent to 100% aggregation as indicated by the calibration mark. The transient increase in optical density that is typically observed following addition of the agonist is commonly attributed to the phenomenon of platelet shape change.

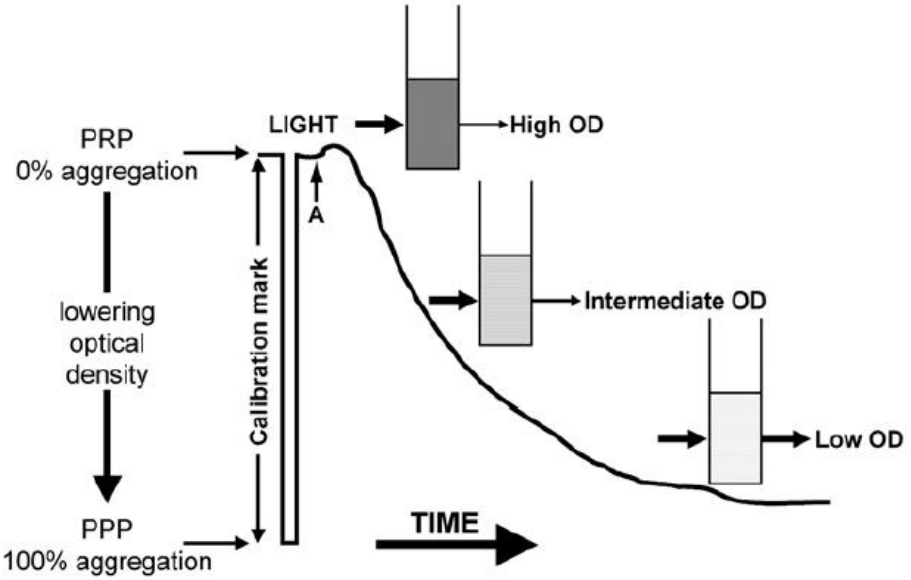

Figure1.1. Diagram of a typical turbidimetric aggregometry trace

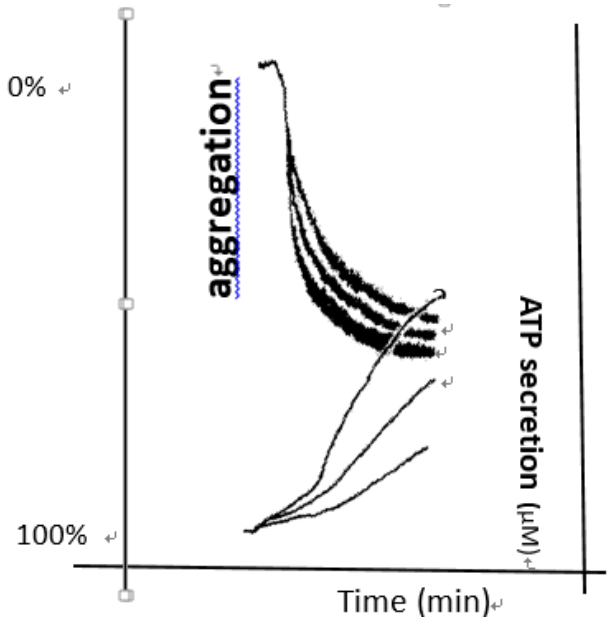

Figure1.2. Diagram of a typical lumi- aggregometry tracing:
the upper tracings refer to platelet aggregation,

while the lower tracing refer to platelet secretion of ATP.

During platelet aggregation, contents of platelet granules, such as ATP, can be secreted into the extracellular medium. In order to detect secreted ATP, the firefly luciferin-luciferase system is used, because it produces a light output proportional to the ATP concentration in the extracellular medium.[11]. The firefly luciferin-luciferase system enables continuous monitoring of the ATP concentration, in parallel with platelet aggregation in the lumiaggregometer instrument, according to the following formula:

$$ATP + D\text{-}luciferin + O2 \rightarrow AMP + PPi + oxyluciferin + CO2 + light \quad \textbf{(1.1)}$$

Studies on platelet aggregation and secretion in MDS patients

According to literature, patients with MDS have no specific disorders of blood coagulation or fibrinolysis, but their bleeding time may be prolonged. There have been many reports of abnormalities of platelet function in MDS patients, the most common being reduced aggregation when upon stimulation by epinephrine or collagen. Individual patients are affected to variable degrees, and it is possible that various subpopulations of normal and abnormal cells coexist during the course of illness. The following table shows a summary of studies on platelet aggregation in MDS patients.

Table1.5. Studies of platelet aggregation in myelodysplastic syndromes

No. Abnormal / No. Tested					Ref.
ADP	Epinephrine	Collagen	Arach.acid	Ristocetin	
0/2	2/2	1/2	-	-	Caen et al[11]
1/1	-	1/1	-	-	Sultan et al[12]
6/6	6/6	6/6	-	-	Rahman et al[13]
2/2	-	2/2	1/1	-	Russell et al[14]
7/14	12/14	12/14	-	7/14	Lintula et al[15]
2/3	-	2/3	-	2/3	Tricot et al[16]
6/6	6/6	4/6	-	-	Stuart[17]
0/17	-	7/17	-	0/17	Pamphilon[18]
5/18	9/18	11/18	3/18	7/18	Lintula et al
13/23	15/23	4/23	11/23	5/23	Aliza Zeidman[19]
		5/8			Blockmans

					D[20]
TOTAL					
29/69 (42%)	35/46(76%)	46/69(67%)	4/19(21%)	16/52(31%)	

These studies studied small patient populations and never measured platelet aggregation and secretion simultaneously.

1.4.2 Platelet granules contents measurement

In the resting state, platelet α-granules and dense granules are distributed throughout the platelet cytoplasm[21]. When platelets are activated, centralization of granules occurs. α-granules secrete fibrinogen and VWF, adhesive proteins that mediate platelet-platelet and platelet-endothelial interactions. Components of the VWF receptor complex (GPIbα-IX-V), the major receptor for fibrinogen (integrin αIIbβ3), and the collagen receptor (GPVI) are stored in α-granules. Although these receptors are also constitutively expressed on the platelet plasma membrane, an estimated one-half to two-thirds of αIIbβ3 and one-third or more of GPVI reside in α-granule membranes in resting platelets and are expressed following activation. Platelet α-granules contain a number of coagulation factors and co-factors that participate in the coagulation process. Factors V, XI, and XIII each localize in α-granules and are secreted upon platelet activation. Platelet factor 4 (PF4) is a small cytokine belonging to the CXC chemokine family that is also known as chemokine (C-X-C motif) ligand 4 (CXCL4). This chemokine is released from alpha-granules of activated platelets during platelet aggregation, and promotes blood coagulation by moderating the effects of heparin-like molecules.

δ-granules contain several molecules: ATP and ADP, serotonin, calcium, inorganic polyphosphate Platelets are the main storage site for serotonin in the human body. Normal platelets avidly take up serotonin from the blood stream and store it in the δ-granules, where it is protected from the action of mitochondrial monoamino oxidases.

Patient with δ-Storage Pool Deficiency (δ-SPD) are characterized by mild to moderate bleeding diathesis (mucocutaneous bleeding, epistaxis, menorrhagia, easy brusing)[22], mildly to moderately prolonged bleeding time, abnormal platelet aggregation and secretion induced by several platelet agonists, and decreased content of δ-granules in

platelets and megakaryocytes. The platelet count is generally normal, although it may be slightly decreased in 20-40% of cases. The storage pool contains more ADP than ATP, while the opposite is true for the metabolic pool. The normal ratio between total concentrations of ATP and ADP is 2.5:1: as a consequence this ratio is higher in patients with δ-SPD.

In order to measure ADP, it is possible to use the luciferine-lucipherase reagent, which measures ATP (see above), in the presence of pyruvate kinase, which catalyzes the transfer of a phosphate group from phosphoenolpyruvate (PEP) to ADP, yielding one molecule of pyruvate and one molecule of ATP.

$$PEP + ADP \rightarrow Pyruvate + ATP \qquad \textbf{(1.2)}$$

Figure 1.3 shows a typical tracing of nucleotide measurements in a platelet sample. The first peak is generated by ATP in the sample, the second peak refers to an internal standard of ATP is added; the third peak is greeted by the appearance of ATO resulting from the conversion of ADP in the presence of PEP+PK. The last peak refers to additional Standard ATP added to the sample.

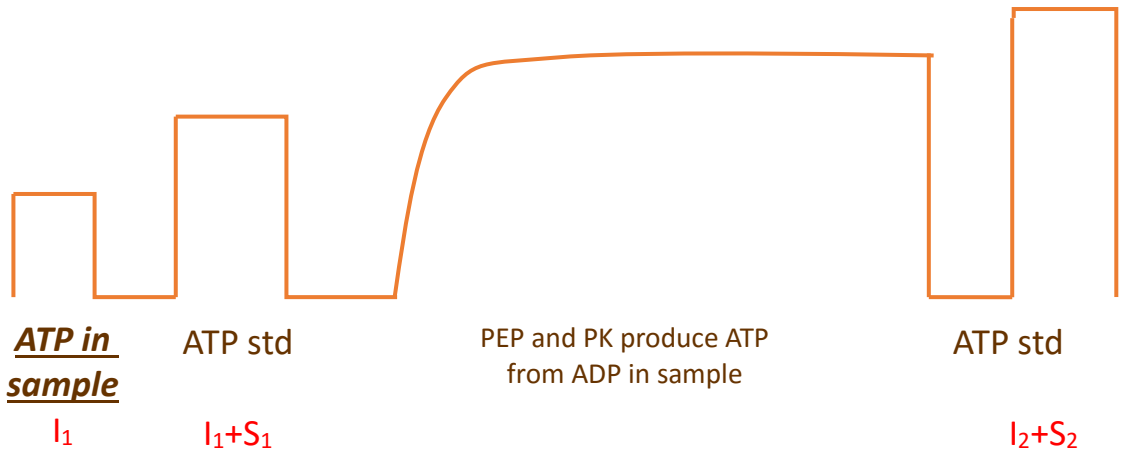

Figure1.3 Tracings obtained during the measurement of ATP and ADP in platelet samples.

1.4.3 Flow cytometry

Flow cytometry allows the analysis of multiple antigens in a single tube at a single cell level. Flow cytometry allows the rapid analysis of many thousands of individual cells per second as they pass through the focused beam of a laser light source. Reflected or refracted light is detected by photodiodes or photomultiplier tubes and translated into an electronic signal, which represents a measure of the cells' size and granularity, respectively. This allows discrimination of different cell types with distinct morphological characteristics within a complex mixture such as whole blood, thus enabling analysis of the platelets.

Flow cytometry is an accurate method for detecting and enumerating constitutively expressed platelet membrane glycoproteins[23]. The method can form part of a routine laboratory screening program for the diagnosis of patients with congenital platelet defects, to give a clear diagnosis of patients with severe, homozygous defects. Levels of receptors can also be measured in relation to gene polymorphisms and platelet function. The constitutively expressed platelet receptors can be measured in this way, provided they are present at a high enough density to reach the level of detection by flow cytometry.

The most important antigens for platelets are the VWF binding receptor glycoprotein (GP) complex Ibα/β-IX-V (CD42a-d), the collagen binding receptor GPVI, and the platelet aggregation receptor alpha2b-beta3 (CD41/CD61)[24]. CD41 and CD61 are both or separately aberrant in Glanzmann Thrombasthenia (GT), an autosomal inherited platelet disorder. GPIX (CD42a) or GPIbα (CD42b) can be aberrant in the bleeding disorders Bernard-Soulier syndrome. Aberrant expression of CD62P has been reported in patients with α-storage pool disease (α-SPD).

Measurement of glycoproteins on platelets can be carried out by single-color analysis in which the platelets are identified solely on the basis of their forward scatter (FS, a measure of cell size) and side scatter (SS, a measure of cell granularity), then analyzed for antigen expression.

However, to ensure that all platelets are identified, and more importantly, to ensure that

any platelet-sized particles derived from other cells are eliminated from the analysis, a two-color approach can be used. In this case the sample is co-labeled with an antibody to a highly expressed, platelet-specific antigen and an antibody to the antigen being studied. The two antibodies are labeled with different fluorophores.

Fluorescence measurement can be expressed either as the percentage of positive cells above a threshold set with an appropriate negative control, or as the mean or median fluorescence intensity (MFI) of a population of cells; the median is the more appropriate measure for an antigen that shows a non-Gaussian, or heterogeneous, distribution on the platelet population. Percentage positive data are used primarily to measure increased expression of an antigen that is not normally present on the platelet surface (e.g., an activation antigen), or to discriminate between populations of cells, whereas MFI is used to quantify the level of expression of an antigen that is constitutively expressed.

1.4.4 Platelet TxA2 pathway

Platelets synthesize prostaglandins when activated by thrombin or collagen, which promote liberation of arachidonic acid (AA) from platelet phospholipids by the enzyme, phospholiphase A2 and the subsequent synthesis of prostaglandin G2 (PGG2) AND PGH2 from AA by the enzyme cyclo-oxygenase[25]. PGH2 is the substrate for several synthases that generate a range of bioactive prostanoids such as PGD2, PGE2, PGI2 and TxA2. While almost all human tissue is capable of generating PGH2, its metabolite is tissue-specific and depends on the presence of tissue-specific enzyme. In platelets, thromboxane synthase (TXS) is responsible for the conversion of PGH2 into TxA2, a known strong platelet agonist (Figure 1).

Once produced, TxA2 has a short half-life (30 seconds) and acts on neighboring cells via autocrine or paracrine systems. TxA2 undergoes rapid non-enzymatic hydrolysis to the inactive TxB2. The synthesis of the endoperoxides and TxA2 during platelet activation leads to secretion of the contents of the dense granules.. TxA2 exerts its action through the specific G protein-coupled TxA2 receptor (TP). The TP receptor is a member of the G-protein-coupled receptor superfamily, which in turn regulates several effectors,

including phospholipase C, guanine nucleotide exchange factor of the small G protein Rho (RhoGEF). TP is expressed in various tissues such as platelets, endothelial cells, smooth muscle cells, monocytes, macrophages, kidney, heart and spleen cells.

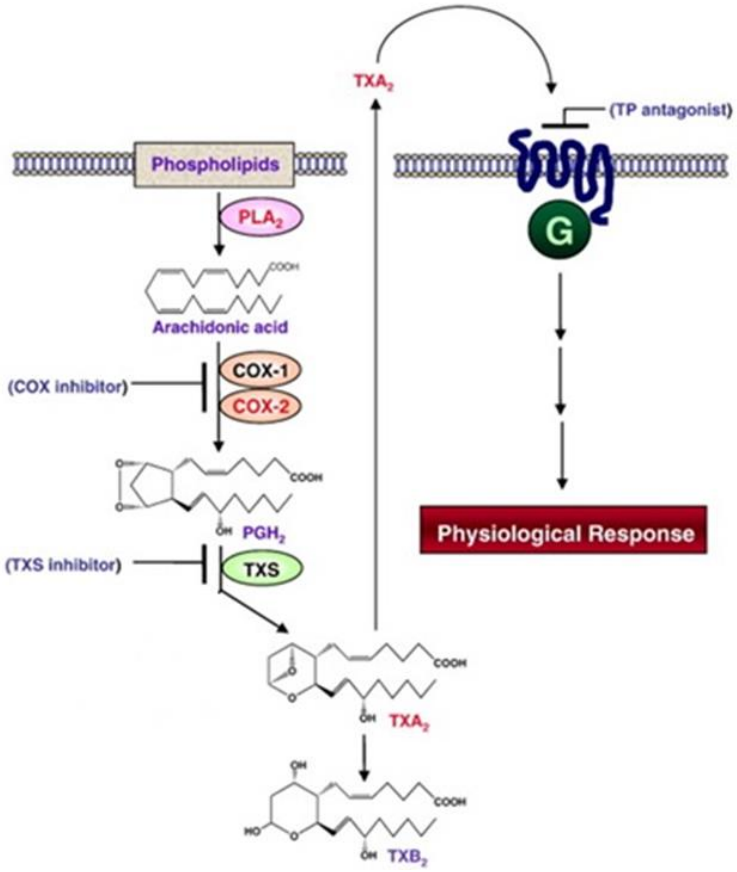

Figure 1.4. The arachidonic acid, prostaglandins and thromboxane pathway Thromboxane pathway. PLA_2, phospholipase A_2; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; TXS, thromboxane synthase; PGH2, prostaglandin H2; TxA_2, thromboxane A_2; TXB_2, thromboxane B_2.

1.4.5 cyclic AMP

The activation of blood platelets at sites of vascular injury is critical to haemostasis and prevention of blood loss, but, if unregulated, can lead to pathological thrombosis. To moderate excessive activation and return platelets to their quiescent state after transient activation, platelets are inhibited by endothelium-derived nitric oxide (NO) and PGI2

(prostaglandin I2, also known as prostacyclin). NO and PGI2 regulate multiple aspects of platelet function by triggering cGMP-dependent and cAMP-dependent signaling pathways respectively. Elevated cAMP is associated with reduced platelet aggregation and diminished accrual of platelets at sites of vascular injury[26].

ACs (adenylate cyclases) belong to a family of ubiquitously expressed transmembrane proteins that synthesize cAMP upon activation. In platelets, ligation of GPCRs by some prostaglandins aor adenosine leads to AC-mediated elevation of intracellular cAMP. The foremost effector of cAMP in platelets is PKA (protein kinase A), which can inhibit platelet function by targeting several pathways of platelet activation. s[27]. cAMP is a critical intracellular second messenger provided with strong inhibitory activity on platelet functions[28].

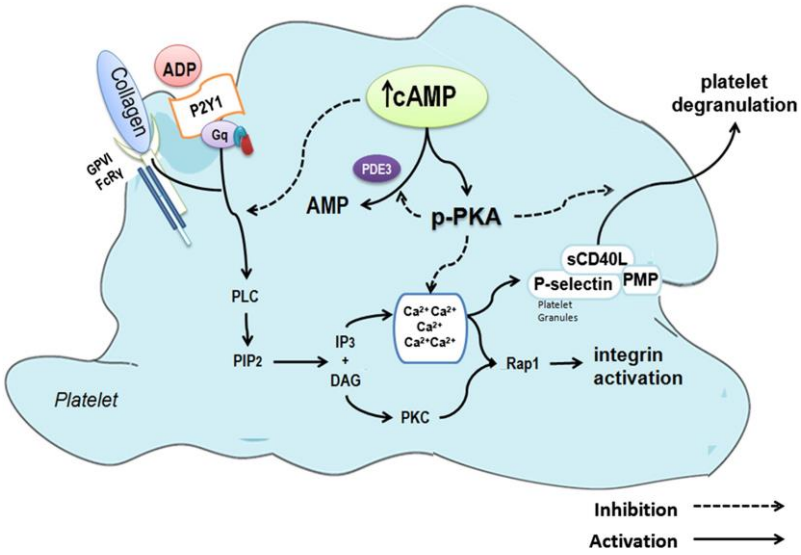

Figure1.5. Mechanism of antiplatelet action via cAMP levels.

1.5 Hemorrhagic complications in MDS

Hemorrhagic complications are among the major causes of death in MDS patients, particularly in those who progress to AML. In the MDACC chart review, 968 patients with MDS died without progression to AML. Of these, 460 patients had a coded cause of death: hemorrhage was a contributory cause death in 90 patients (20%) and was listed as

the only cause of death in 48 patients (10%)[29]. Other studies reported that hemorrhagic complications account for about 14%--45% of deaths in patients with MDS.

Both thrombocytopenia and platelet dysfunction contribute to hemorrhagic complications observed in MDS[30,31]. The estimated prevalence of thrombocytopenia in MDS ranged from 40% to 65%[32], but also patients without thrombocytopenia are at increased risk of bleeding because the risk of bleeding in MDS patients is typically attributed to both low platelet counts and abnormalities of platelet function[33].

Another evidence that the increased of risk of bleeding is not related to thrombocytopenia is that the frequency of fatal bleeding was evenly distributed among all four International Prognostic Scoring System (IPSS) risk groups[34]. The IPSS is used widely to predict survival and progression to AML and the platelet count is one of the four parameters evaluated[35].

To our knowledge the platelet count at which MDS patients with thrombocytopenia become at risk of hemorrhage has not been defined well. Some studies observed platelet hypo-reactivity in MDS patients, when stimulated with both weak and strong agonists. Merrelman 2000 studied 68 MDS patients and reported that MDS patients have reduced PA with EPI (75%), AA (54%), ADP (46%) , ristocetin (22%)[36]. Using impedance and lumiaggregometry, Manoharan et al found that, among 48 MDS patients, 35(73%) had at least 1 abnormal result indicating platelet hypoactivity; 7(15%) patients had mixed platelet hypoactivity and hyperactivity; and 4(9%) patients had platelet hyperactivity[37].

Bellucci S et al showed that one MDS patient had normal platelet expression of GPVI, normal binding of convulxin to platelets, but no platelet aggregation or secretion induced by convulxin[38].

Therefore, although there are some reports showing decreased platelet function in MDS patients, large studies exploring platelet function in MDS patients in depth are lacking

2. Aim of the study

We aimed at exploring several parameters of platelet function in MDS patients, including the regulation of platelet function by cAMP.

3. Subjects

The following groups of subjects were enrolled in the study:

- Healthy subjects
- MDS patients
- ITP patient

All subjects were eligible for enrolment in the study, based on the following inclusion criteria

- over 18 years of age
- free of drugs known to affect hemostasis (e.g. non-steroidal anti-inflammatory drugs, ticlopidine, dipyridamol, warfarin) in the two weeks before blood sampling
- no ischemic events or revascularization procedures not less than 3 months before
- no drug or alcohol abuse

Diagnoses of MDS and ITP were made based on standardized criteria.

4. Methods

4.1 Blood sampling

Blood samples were collected in the morning by venipuncture of the antecubital vein with a 21-gauge butterfly needle, the tourniquet released soon after needle insertion. The first 3 mL of blood was collected into K3EDTA (Sarstedt, Verona, Italy) for blood cell count MEDONIC CBC counter immediately following the blood draw. Subsequent 30mL blood was drawn with a polypropylene (PP) syringe (10 ml) and transferred immediately into three PP tubes containing sodium citrate anticoagulant solutions (109 mM, 1:9 v/v, final volume 10 ml), gently mixed, allowed 'to rest' at room temperature (RT) for 15 min, and centrifuged to obtain platelet-rich plasma (PRP)[1]. Blood samples for preparation of serum were collected into tubes without anticoagulant, and another 3mL of whole blood were collected into tubes of 3.2% citrate (Sarstedt, Verona, Italy) for flow cytometery. For the flowcytometry measurement of P-selectin, immediately after blood sampling, 4uL of proteinase inhibitors was added to 400uL of fresh whole blood, mixed gently, then take 200uL of the mixture added into 400uL PAMFIX(V_{pamfix}: V_{blood}=2:1), store at 4℃ overnight.

4.2 Light transmition aggregometry

Platelet aggregation was measured by light transmission aggregometry (LTA) using Chrono-Log 560 (Havertown, PA, USA). Platelet rich plasma (PRP) was obtained by centrifugation of citrate whole blood samples at 200 x g for 10 min at room temperature. Autologous platelet-poor plasma (PPP) was obtained by centrifugation of blood samples at 1.400 x g, at room temperature for 15 min. Autologous PPP was used to set the instruments' 100% light transmission, while un-stimulated PRP was used to set 0% light transmission. The individual platelet count of the PRPs was not adjusted to a pre-determined range, because this procedure may induce artefacts. All aggregation tests were performed within 3 hours after blood collection.

Samples of PRP (225 µL) were pre-warmed at 37°C for 3 minutes, then incubated with 25 µL luciferine/luciferase reagent at 37°C for 30 seconds and stirred at 1000 rpm in a lumi-aggregometer. After incubation, 5 uL of an aggregating agent was added, including ADP (2 or 4 or 20µM), epinephrine (5 or 10 µM), thrombin receptor active peptide (TRAP) (10 or 20 µM), synthetic liquid reactive peptide (SRP) (4 or 8ng/mL), collagen reactive peptide (CRP) (0.1 or 1 or 10µg/mL), Horm collagen (2, 4 or 10 µg/mL) and arachidonic acid (AA) (1 mM). Changes in light transmission (caused by the formation of platelet aggregates) were recorded for 3min. At the same time, ATP secretion tracings were recorded.

In addition, Prostaglandin E1 (PGE1) (0.1µM) response was tested in collagen induced platelet aggregation. PGE1 was incubated with PRP for 30 seconds before the addition of luciferase, after another 30 seconds incubation with luciferase, collagen was added to stimulate platelets aggregation. Maximal aggregation response to each agonist was measured and expressed as percent increase in light transmission.

Standard linear curve for ATP release measurement was made using the following concentrations of ATP: 2nM, 1 nM, 0.5 nM, 0.25 nM, 0.125 nM.

4.3 Delta granules contents measurement of ATP and ADP

1mL fresh PRP was mixed with 50µL 100mM EDTA and 950µL absolute Ethanol, then centrifuged at 16000g for 45mins at 4°C; the supernatant was stored at -80°C until analysis.

Nucleotide measurement adopted the firefly luciferin-luciferase system (Roche), used lumi-aggregometer Chrono-Log 560. Practically, add 30µL luciferase was added into 267.5µL buffer (4.5mM potassium acetate, 3.4mM EDTA, 0.09M TRIS-acetate), after incubation for 30 seconds, curves were made by 2.5µL of extracted samples, followed by 25pmol ATP. Light output by samples was proportional to the concentration of ATP. Subsequently, ADP was converted to ATP by the addition of pyruvate kinase/phosphoenolpyruvate (PK/PEP). ADP and ATP contents were expressed as pmoles

per 10^8 platelets.

Analysis of results:

The output is a unit of light emission. Then the results from ATP standard are used to determine the amount of ATP and ADP using the following formula:

$$STD\ (mm) : STD\ (mol) = R\ (mm) : R\ (mol) \quad \textbf{(4.1)}$$

Where: STD = ATP standard; R= Results that we are looking for.

- Using an ATP standard of 5 µL of 5 µM solution, 25 pmol are added to the solution
- The sample added has a dilution of ½ during preparation (see methods). (We add 2.5 µL in the reaction system)

$$R(mol) = [R(mm)*STD(mol)] / STD(mm) = I_1 \ / \ S_1 * 25\ pmol \quad \textbf{(4.2)}$$

$$R(concentration) = 25pmoli / 2.5\mu L * I_1 / S_1 * 2 = 20 * I_1 / S_1 \quad \textbf{(4.3)}$$

This is the formula that can convert the result obtained by instrument in concentration of our interest, where 5 is a conversion factor.

- If 2.5 µL of diluted sample at 50% was added, the conversion factor is 40.

4.4 Flow Cytometry Measurements

The presence of platelet specific antigens was analyzed in citrated whole blood of normal platelets and from patients diagnosed with platelet abnormalities, using a gating strategy based on ubiquitously expressed platelet membrane markers. We were able to detect the platelet antigens GPVI, CD41, CD42a, CD42b and CD61 in one single tube.

CD62P PhycoErythrin(PE)

The AK-4 monoclonal antibody specifically binds to CD62P. CD62P is a 140 kDa type I transmembrane glycoprotein that is also known as P-Selectin, Platelet activation-dependent granule membrane protein (PADGEM), or GMP-140. P-Selectin is stored in the α-granules of platelets and the Weibel-Palade bodies of endothelial cells, and is

rapidly transported to the plasma membrane upon activation. P-Selectin is thought to mediate the initial adhesive interactions of neutrophils and monocytes with endothelium in inflammatory responses, and of activated platelets to neutrophils and monocytes in hemostasis.

CD41 Fluorescein isothiocyanate (FITC)

The CD41 antigen is the integrin αIIb chain, also called platelet gpIIb. The molecular weight of the recognized antigen is 135 kDa. CD41 is non-covalently associated with the integrin β3 chain, also called gpIIIa or CD61. The P2 monoclonal antibody (mAb) reacts with gpIIb in the intact complex with gpIIIa, but not with gpIIb or gpIIIa separately

CD42a Fluorescein isothiocyanate (FITC)

The CD42a antigen (GPIX) is a transmembrane glycoprotein of 22 kDa which forms a non-covalent complex with CD42bc (GPIb) and CD42d (GPV). CD42a expression is restricted to platelets and megakaryocytes. The SZ1 monoclonal antibody reacts with CD42a only within the intact CD42a-d complex

CD42b Allophycocyanin (APC)

The HIP1 monoclonal antibody specifically binds to CD42b. CD42b is also known as the Platelet glycoprotein Ib alpha chain that is encoded by the *GP1BA* gene. CD42b is disulfide bonded to CD42c to form a 170 kDa heterodimer, GPIb. GPIb forms a noncovalent complex with CD42a and CD42d (CD42 complex) that is expressed on platelets and megakaryocytes. The CD42 complex serves as the von Willebrand Factor(vWF) surface receptor involved in the adhesion of platelets to the subendothelium of damaged vascular walls. HIP1 inhibits the ristocetin-dependent binding ofvWF to platelets and partially inhibits collagen-induced aggregation

CD61 phycoerythrin (PE)

The VI-PL2 monoclonal antibody specifically binds to CD61. CD61 is a 105 kDa

transmembrane glycoprotein that is also known as integrin β3 and platelet glycoprotein IIIa (GPIIIa or GP3A). It is expressed on platelets, megakaryocytes, osteoclasts and endothelia. Integrin β3 associates with gpIIa (CD41) to form the CD41/CD61 complex which mediates platelet adhesion and aggregation. CD61 also associates with CD51 to form the CD51/CD61 complex (vitronectin receptor). CD61 appears to bind to fibrinogen, fibronectin, vWF, vitronectin, and thrombospondin to mediate cell adhesion.

GPVI phycoerythrin (PE)

This CLONE 1G5 antibody recognizes the human Glycoprotein VI (GpVI) GpVI is a 62 kDa type 1 transmembrane receptor belonging to the immunoglobulin superfamily, and noncovalently associated with the signal-transducing FcRγ chain. GpVI is a major collagen receptor that plays a crucial role in the collagen-induced activation and aggregation of platelets. As well as collagen, other ligands such as collagen-related peptide (CRP) and snake venom protein (convulxin) can bind to the extracellular region of GpVI causing ectodomain shedding. This process is tightly regulated by a metalloproteinase, most likely ADAM10, providing a mechanism for the modulation of platelet responsiveness. Most GpVI is maintained in a monomeric form on resting platelets. GpVI dimerization is a cAMPcontrolled active process that primes platelet interaction with fibrillar collagen.

PAMFix (Platelet Solutions Ltd, Nottingham)

PAMFix was developed as a one-step procedure for stabilising platelets in blood for measurement of platelet activation markers (PAM) on the surface of platelets such as P-selectin (CD62P) from α-granules. It is also useful for measuring CD63 from dense bodies in platelets. PAMFix stabilises activation markers on the surface of platelets so that analysis can be performed up to 9 days following sample preparation. This enables remote testing of platelet function.

Figure 4.1. One-step procedure for stabilising platelets

Samples were analysed by using FACSverseTM BD FACS verse 6 color flow cytometry. Calibration beads automate the characterization of cytometer fluorescence detectors and the entire optical configuration by creating baselines with performance values, which have to be targeted prior to each measurement to ensure standardized performance.

BD FACSuite™ software controls the connection between the flow cytometer and instruments being the platform to perform calibration and acquisition.

All samples were acquired with a slow flow rate, and for each samples 5000 events were recorded.

4.4.1 Detector settings for rest platelets surface receptors

A set up experiment was made with a FS-log/SS-log plot and all fluorochrome combinations (FITC/PE, FITC/APC, PE/APC). FS and SS etectors where set to log-scale for detection of small particles. An unstained sample was used to adjust the FS/SS PMT voltages to a level that red blood cells and platelets could be separated and gated (Figure. 4.2).

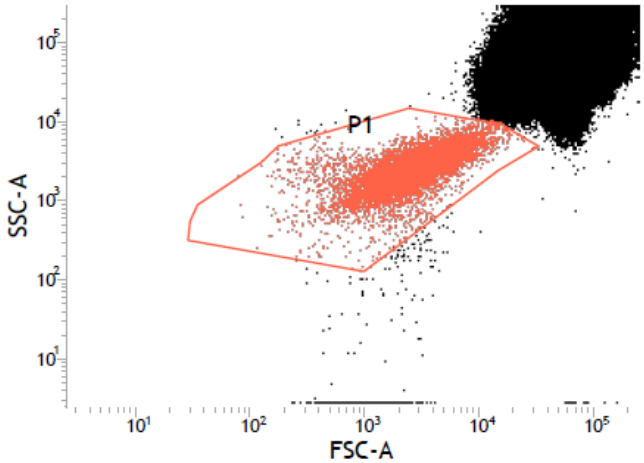

Figure 4.2. P1: FS-log/SS-log plot set for distinguish of platelet cloud

Gating platelets in P1, the double fluorochrome dot plots allow adjusting FL voltages to

a sensitivity level that all unstained platelets were negative. (Figure 4.2).

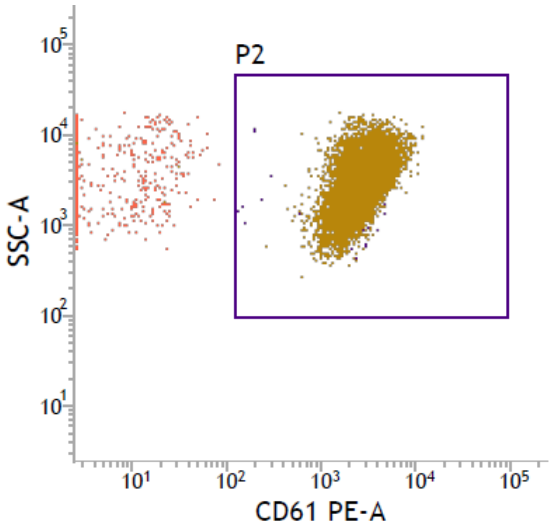

Figure. 4.3(a). Double fluorochrome dot plots to determine the purity of the platelet population. (CD61-PE)

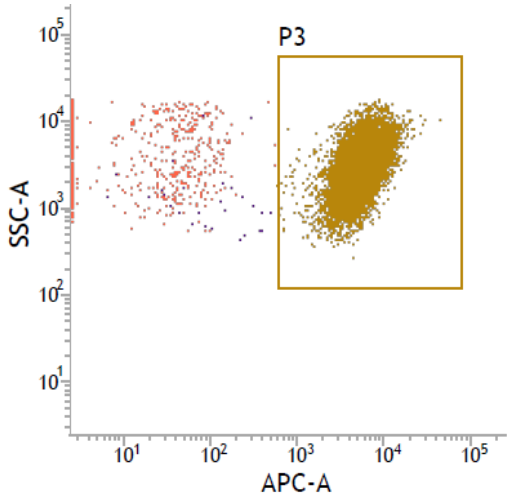

Figure. 4.3(b). Double fluorochrome dot plots to determine the purity of the platelet population.(CD42b -APC)

To detect the optimal antibody concentration, titration assays were performed with a fixed number of platelets (1*10^9). Settings were checked with proper fluorochrome and isotype matched controls.

The gating strategy determines the purity of the platelet population in whole blood. A variety of small particles can contaminate the platelet selection when the forward scatter, side scatter approach is used. These contaminations, do not express CD61 or CD42b. A proper platelet selection (>99%) can be performed by gating for platelet antigens that are ubiquitously present at platelet membrane such as CD61 and CD42b (Figure. 4.3).

4.4.2 Detector settings for rest platelets surface p-selectin expression

By using a dot plot CD42b-APC vs CD62P-PE, we plotted cells gated in "platelet" and we defined the positive cells basing on isotype control. We fixed the isotype control at a maximum of 4% of positive to CD62P cells. The analytic parameter used to evaluate the level of P-selectin expression was always the % of cells positive to the antibody CD62P.

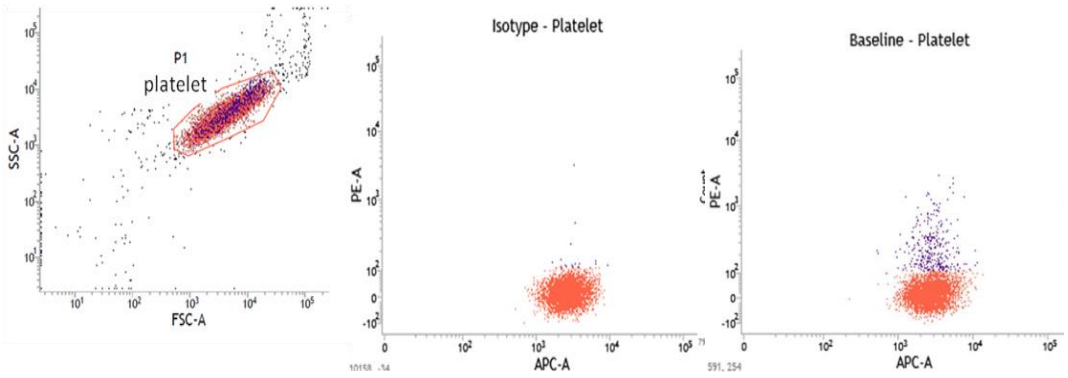

Figure 4.4. Detect of P-selectin by flow cytometer: isotype control and sample

The monoclonal antibody CD62P PE was used to detect P-selectin. 400µl Fresh cirate anticoagulated whole blood was treated with protease inhibitors (sigma), then added 2 times volumes of PAMFIX and mixed gently, stored at 4℃ degree overnight. 5µl of fixed samples mixed with 45µl PBS and antibodies then determined by flow cytometry.

4.5 TXB2 levels

Serum was prepared from non-anticoagulated venous blood that was allowed to clot at 37°C for 1 hour, centrifuged at 1,400 x g at RT for 15 min, and stored at -80°C until assay. TxA_2 levels are typically monitored by measurement of its stable metabolite TxB_2, which we measured by using a selective, competitive enzyme immunoassay (Thromboxane B_2 EIA kit, Cayman Chemical Company, Ann Arbor,MI, USA). Frozen samples were thawed at 37°C and diluted between 1:2 and 1:3000 with buffer and tested in duplicate. The plate was read at 405 nm wavelength in a standard 96-well plate reader (Ensight). Samples were assayed in parallel with known TxB_2 standards, prepared as outlined in the manufacturer's instructions, and a maximum binding control. The percent binding of known standards was calculated in reference to the maximum binding controls, plotted against the logarithm of concentration and analyzed by non-linear regression. Unknown samples were expressed in a similar fashion, interpolated from this standard curve and corrected for dilution. Samples with results outside the standard curve were re-assayed with appropriate dilution. The detection limit of the assay is 11 pg/mL. Data are expressed as pmoles/10^8 platelets.

4.6 cAMP levels

Platelet cAMP was measured by an ELISA assay, using a commercially available kit (Cyclic AMP Select ELISA Kit; Item No.501040, USA). Duplicate samples of 500μL citrated PRP containing 1 mM theophylline were incubated with Tyrode's buffer and PGE1 (1 μM), PGE1 (0.1 μM), or Tyrode's buffer alone in a control mixture. After incubation at 37°C (2 minutes), 500μL of 5% trichloroacetic acid added, samples were snap-frozen in liquid Nitrogen. Thawed them at ambient temperature, and rotated at 4°C for 45 minutes, followed by centrifugation at 4°C for 30 minutes, the supernatant was extracted with one volume of Trioctylamine as well as 2.5 volume of DCM, vortex for 2 min and then centrifuge at 3,000g for 15min, collect supernatant and store in -80 degree. When dosage by ELISA, Samples were thaw at room temperature and diluted with EIA buffer before assay. Samples of PPP with theophylline and Tyrode's buffer were also

collected, which should be subtracted for calculation to make sure all the cAMP from platelets.

4.7 Serotonin levels

Platelet serotonin was measured by Fluorescence spectrophotometry.

400µL of EDTA 0.1M was added into 1mL PRP, mix gently by inverting the Eppendorf several times, and then centrifuge at 12,000g for 3 minutes at room temperature, discard all the supernatant and make pellet dry for one hour, then froze at -80°C until dosage.

Thaw the pellet samples at room temperature, add 500uL of 10% TCA, homogenize and vortex, centrifuge for 2 minutes at 12000 rpm, transfer 400 L of supernatant to 10 mL in glass tubes with screw cap. Add 1.6 mL of OPT / HCl and vortex. Boil for 10 minutes, cool to RT, Add 5 mL of saturated dichloromethane (DCM) under the hood, vortex until separate well. Transfer 200µL of supernatant in a 96wells plate. Transfer 400µL standard of 4µM 5HT, 2µM, 1µM and 200 nM in 10mL glass tubes with screw cap, and treated the same as samples. Make a blank with 400 µL 10% TCA and treat it as the champions. Read by EnSight™ Multimode Plate Reader from PerkinElmer.

4.8 PF4 levels

20uL of Triton 20% was added into 400uL PRP, mix by inverting the Eppendorf several times, and froze the samples at -80°C until dosage. **PF4** was measured by Elisa (Human CXCL4/PF4 PicoKine ELISA Kit, BSR-EK0726, BOSTER, CANADA). Results were expressed as ng/10^6 platelets.

5.Results

5.1 Population characteristics

Thirty two MDS patients and 26 ITP patients, as well as 54 healthy subjects were enrolled in this study

Clinical data

MDS: Characteristics of MDS patients are presented in Table 5.1.

Table 5.1. Characteristics patients with MDS

Patient Characteristics		N(%)	
		ALL MDS patients	PLT/PRP>100,000 per µL, No anti-platelet drug
AGE	<55 yrs.	0	0
	55-64 yrs.	1	1
	65-74 yrs.	9	3
	>75 yrs.	22	10
SEX	Female	10	8
	Male	22	6
WHO	MDS-RA	6	3
	MDS-RARS	1	0
	MDS-LMMC	6	3
	MDS-RCMD	4	1
	MDS-RAEB-1	11	5
	MDS-RCMD-RS	0	0
	MDS-RAEB-2	1	0
	MDS-U	2	0
	MDS-5q	1	1
IPSS	Low	15	5
	Int1	11	6
	Int2	2	1
	High	1	1
	Unknown	3	0
R-IPSS	Very low	2	5
	Low	9	6
	Intermediate	7	1
	High	2	1
	Very high	0	0
	Unknown	11	5

Karyoty	Good	19	6
	Intermediate	8	1
	Poor	4	1
	Unknown	1	0
Karyotype	-7/del(7q) isolated or +1	1	1
	del(20q) isolated	1	1
	del(5q) isolated	1	1
	+8 isolated	1	1
	Complex	3	2
	Normal	17	4
	-Y	3	2
	OTHER	4	2
	Unknown	1	0
Blast %	< 5 %	6	3
	5–10	14	7
	11–20 %	3	0
	21–30 %	0	0
	Unknown	9	4
Hemoglobin (g/dl)	< 8.0	3	3
	8.0 – 9.99	7	7
	10.0 – 11.99	8	0
	≥12.0	14	0
	Unknown	0	4
Absolute Neutrophil Count (cells/µL)	< 500	0	3
	500 – 1,499	0	7
	1,500 – 9,999	28	0
	≥10,000	3	0
	Unknown	1	4
Platelets (per µL)	< 50,000	3	3
	50,000 – 149,000	15	7
	150,000 – 449,999	13	0
	≥ 450,000	1	0
	Unknown	0	4
Time from diagnosis to PLT funtion	Mean months	33.14±24.41	32.61±37.70

Table 5.2. Blood cell counts in three groups

	Healthy subjects	**MDS Patients**	**ITP Patients**	**p**	**p**	**p**
	(mean ± DS)	*(mean±DS)*	*(mean±DS)*	*Healthy vs MDS*	*Healthy vs ITP*	*MDS vs ITP*
WBC (10^3/µL)	6,953 ± 1,956	7,168±6,155	7,467±2,463	NS	NS	NS
RBC (10^6/µL)	4,744±0,545	3,927±1,211	4,581±0,603	< 0,0001	NS	0,006
Hgb(g/dL)	13,84±1,56	11,38±2,48	13,43±1,59	< 0,0001	NS	0,0035
Hct(%)	41,15±4,41	36,64±12,17	40,22±5,08	< 0,0001	NS	0,0038
MCV (fL)	87,02±6,63	88,18±20,46	88,09±5,77	0,0427	NS	NS
MCH (pg)	29,32±2,90	30,45±4,52	29,47±1,97	NS	NS	NS
MCHC (g/dL)	33,73±2,81	32,54±2,99	33,47±1,05	0,0213	NS	NS
PLT (10^3/µL)	229,1±42,48	152,9±111,9	76,52±48,30	< 0,0001	< 0,0001	0,0185
MPV (fL)	8,62±0,92	8,69±1,48	10,27±1,56	NS	0,0001	0,0011

5.2 Platelet aggregation and secretion

Because measurement of platelet aggregation by LTA may be inaccurate at platelet counts in PRP lower than 150*10^3/µL, we excluded from the analysis patients whose PRP platelet counts were lower than 150*10^3/µL

Patients on treatment with antiplatelet agents were also excluded from the analysis

Overall, data from 50 healthy subjects, 14 MDS patients and 11 ITP patients were available for statistical analysis (Dunn's multiple comparisons test).

5.2.1 ADP 2µM-induced platelet aggregation and secretion

The percentage of platelet aggregation after ADP 2uM stimulation showed significantly difference between healthy subjects and MDS patients, while ITP group showed no difference with any of the other two groups.

For ATP secretion, the method did not have limitation for platelet count, so for statistics, only data from patients who undergo antiplatelet therapy were excluded. Data from 50 healthy subjects, 21 MDS patients and 25 ITP patients were analyzed statistically, using Dunn's multiple comparisons test. Results showed both MDS and ITP groups were signigicantly lower than healthy subjects.

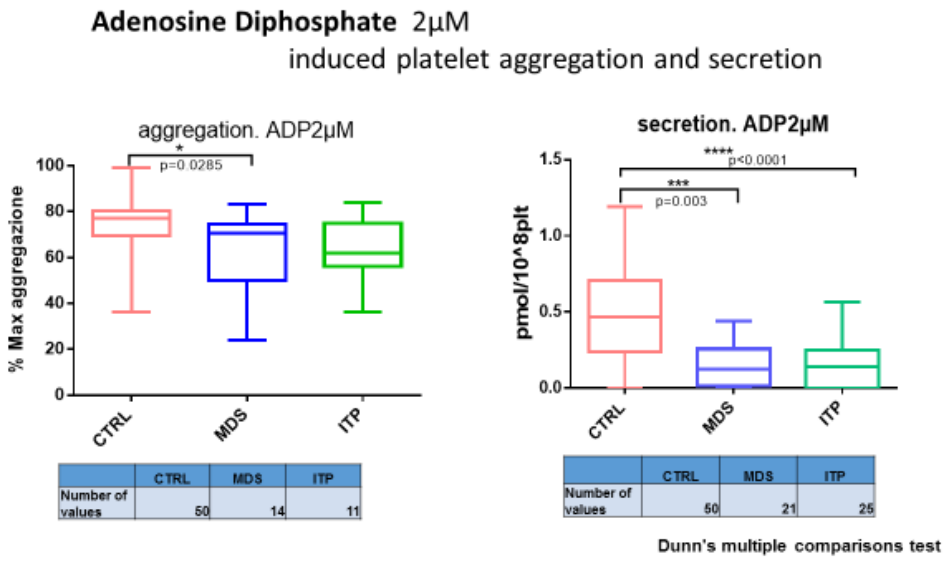

Figure 5.1. Adenosine Diphosphate 2µM induced platelet aggregation and secretion

5.2.2 Epinephrine 5µM-induced platelet aggregation and secretion

The same trend was observed when platelets were stimulated by Epinephrine at final concentration 5uM. The percentage of platelet aggregation was significantly lower in MDS patients than healthy subjects, while ITP group showed no difference with any of the other two groups.

ATP secretion was significantly lower in MDS and ITP groups than in healthy subjects. There was no statistically significant difference between MDS and ITP groups.

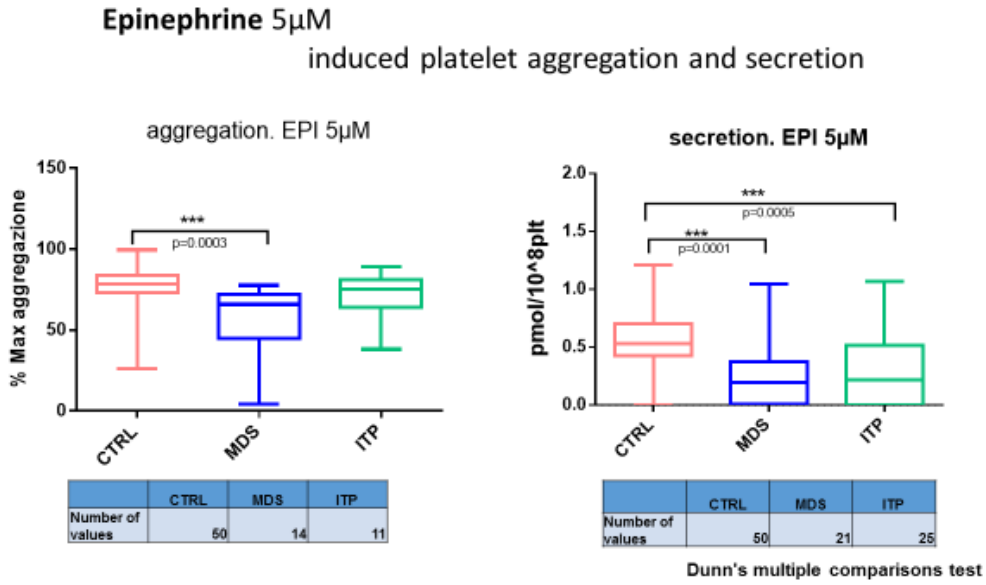

Figure 5.2. Epinephrine 5µM induced platelet aggregation and secretion

5.2.3 Thrombin receptor activator peptide (TRAP) 10µM induced platelet agg regation and secretion

The same trend was observed when platelets were stimulated by TRAP at final concentration 10uM. The percentage of platelet aggregation was significantly higher in healthy subjects compared to MDS patients.

ATP secretion was significantly lower in both MDS and ITP than in healthy subjects, There was no statistically significant difference between MDS and ITP groups.

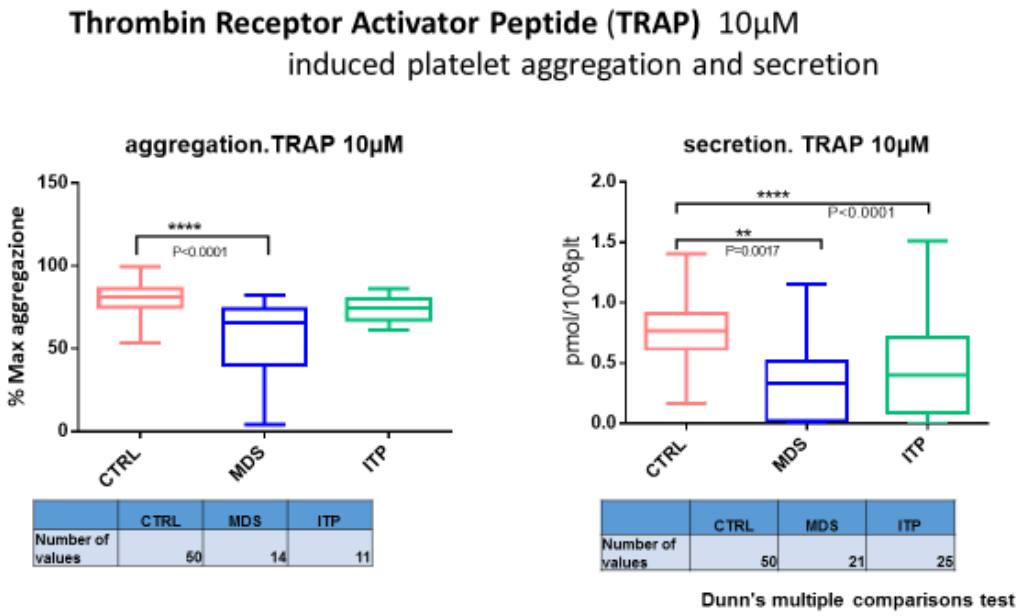

Figure 5.3. TRAP 10µM induced platelet aggregation and secretion

5.2.4 Collagen 2µg/mL-induced platelet aggregation and secretion

When platelets were stimulated by collagen at final concentration of 2µg/mL, the percentage of platelet aggregation was significantly lowers in MDS patients than in healthy subjects, while no statistically significant differences were observed between ITP and the other groups.

ATP secretion was lower in both MDS and ITP patients compared to healthy controls. There was no statistically significant difference between MDS and ITP patients.

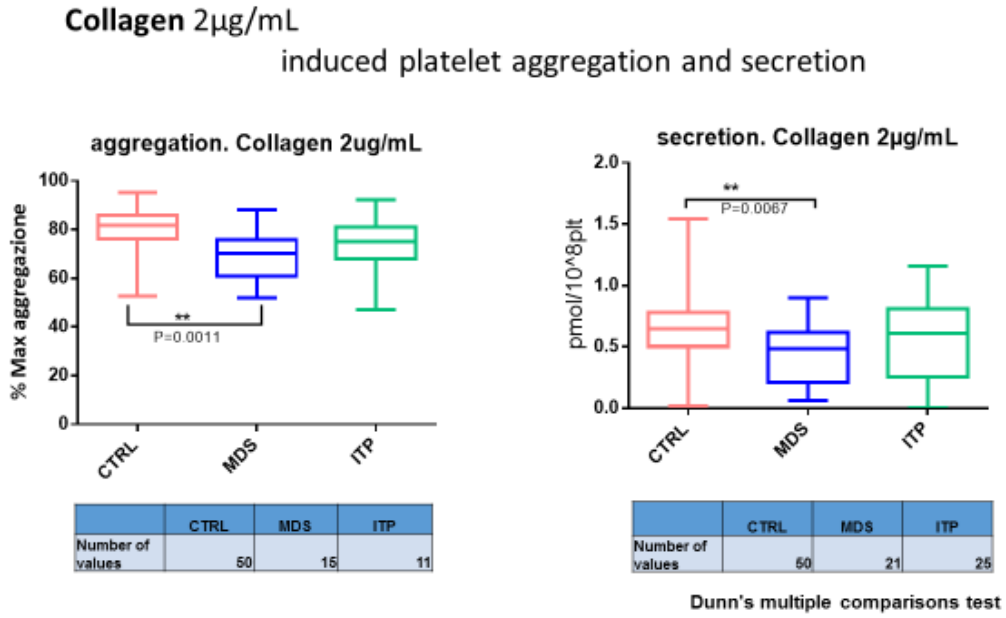

Figure 5.4. Collagen 2µg/mL induced platelet aggregation and secretion

5.2.5 SRP 4ng/mL and CRP 0.1µG/mL induced platelet aggregateion and secretion

We did LTA tests also for Synthetic Liquid Reactive Peptide SRP (final concentration 4ng/mL) and Collagen reactive peptide CRP (final concentration, 0,1µg/mL), they are two agonists specific for GPVI, one of the receptors for collagen.

After stimulating with SRP, platelet aggregation in MDS and ITP groups were both significantly lower than in healthy subjects. Platelet ATP secretion showed the same trend, because it was significantly lower in MDS and ITP patients than in healthy subjects.

CRP-induced platelet aggregation and ATP secretion in MDS and ITP patients were also significantly lower than in healthy subjects..

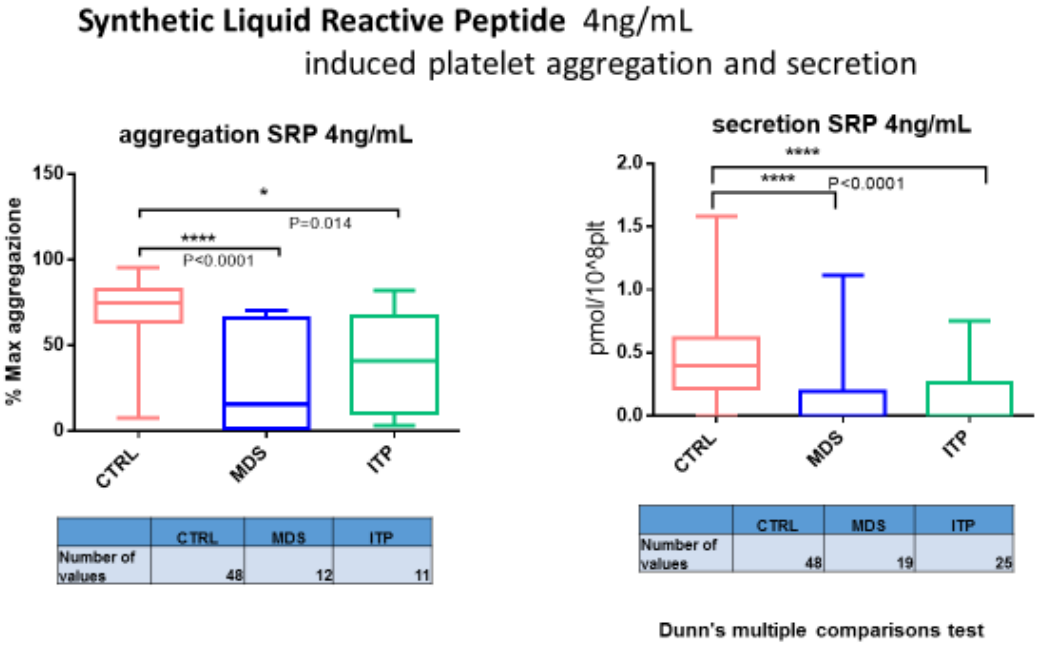

Figure 5.5. SRP 4ng/mL induced platelet aggregation and secretion

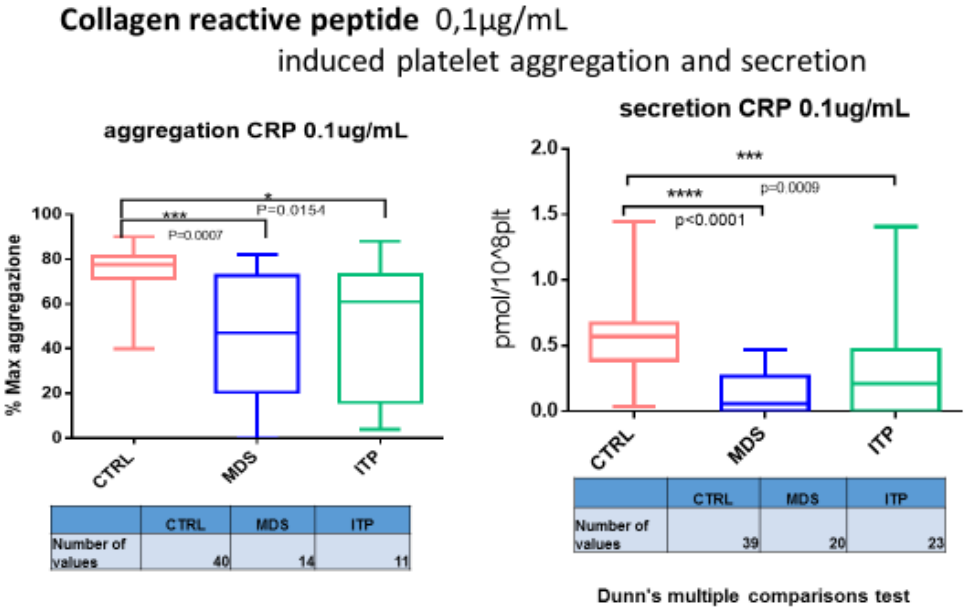

Figure 5.6. CRP 0,1ug/mL induced platelet aggregation and secretion

5.2.6 Arachidonic acid-1mM induced platelet aggregation and sec-retion

Arachidonic acid (1mM)-induced PA was significantly lower in MDS patients than in healthy subjects, while ATP secretion was lower in both MDS and ITP patients compared to healthy subjects. There was no statistical difference between MDS and ITP groups.

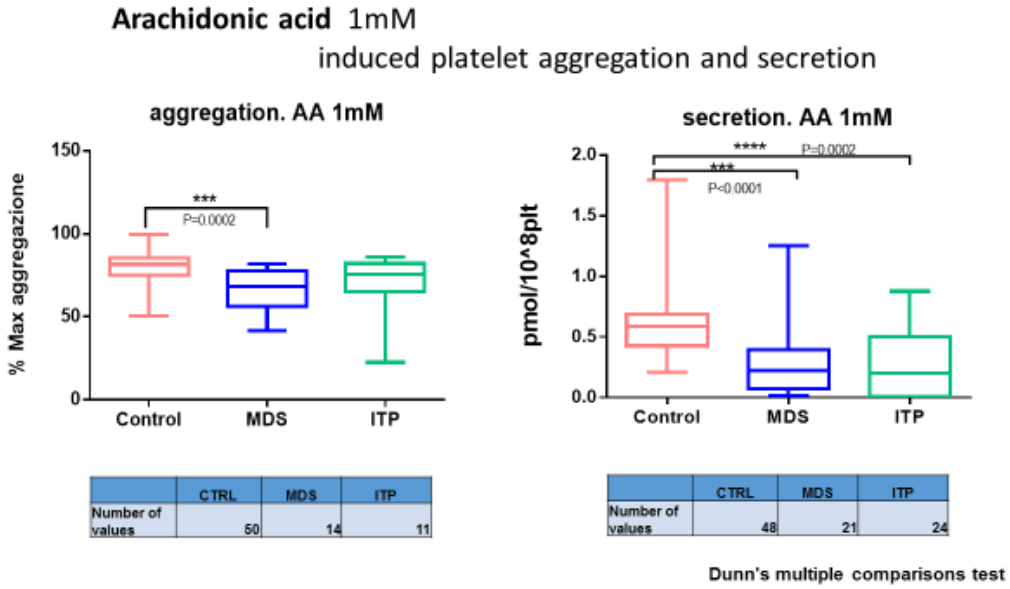

Figure 5.7. Arachidonic acid-1mM induced platelet aggregation and sec-retion

5.3 Inhibitory effect Prostaglandin E1 on collagen-induced platelet aggregation and secretion

Prostaglandin E1 inhibited collagen-induced platelet aggregation by 32.61%, 55.20%, 25.72% in healthy subjects, MDS patients and ITP patients respectively. Kruskal-Wallis test of Dunn's multiple comparisons test showed the decrease of platelet aggregation was statistically significant in MDS group compared with healthy subjects, while ITP patients showed no difference with the other two groups. In other words, platelets from MDS patients were more sensitive to Prostaglandin E1 than the other two groups. However, the decrease of ATP secretion were similar among the three groups.

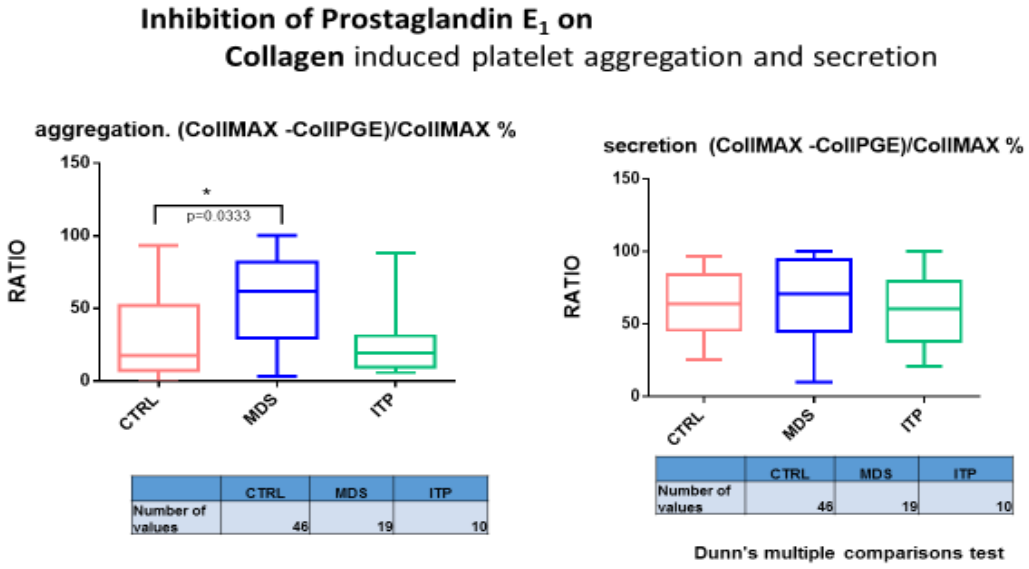

Figure 5.8. Inhibitory effect Prostaglandin E1 on collagen induced platelet aggregation and secretion

5.4 Platelet granules content

5.4.1. ATP and ADP

There was no statistically significant differences in the platelet ATP content among the 3 groups (Figure 5.9 a).

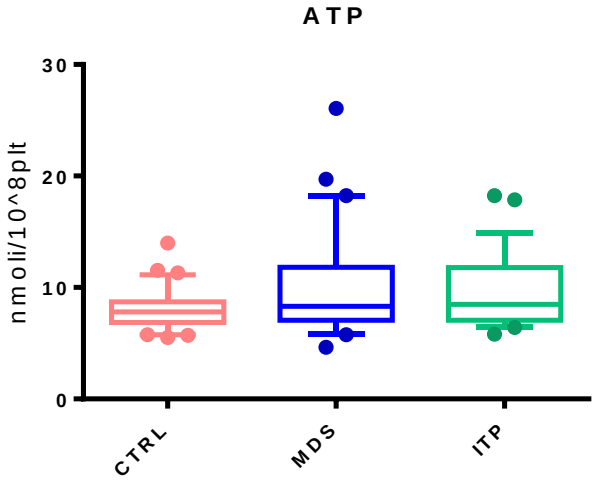

Figure 5.9 a. ATP contents in delta granules among three groups

In contrast, the platelet ADP content in MDS patients was significantly lower than in healthy subject(Figure 5.9 b). There was no statistically significant differences between ITP patients and any of the other two groups. However, the ratio between ATP and ADP was significantly higher in both MDS and ITP groups than in the healthy subjects group(Figure 5.10).

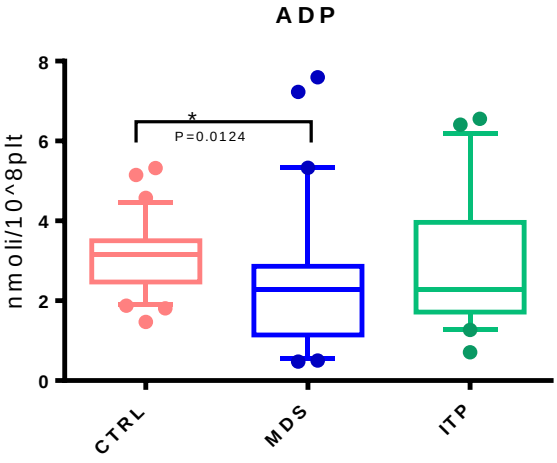

Figure 5.9 b. ADP contents in delta granules among three groups

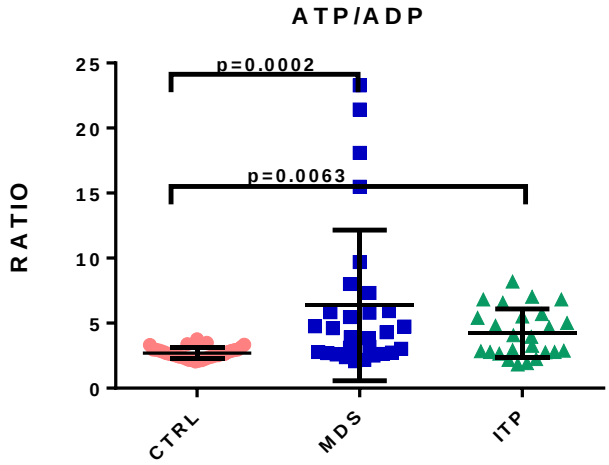

Figure 5.10. ATP/ADP RATIO among three groups

5.4.2 Platelet serotonin levels

Both MDS and ITP patients had lower levels of platelet serotonin than healthy subjects (Figure 5.11).

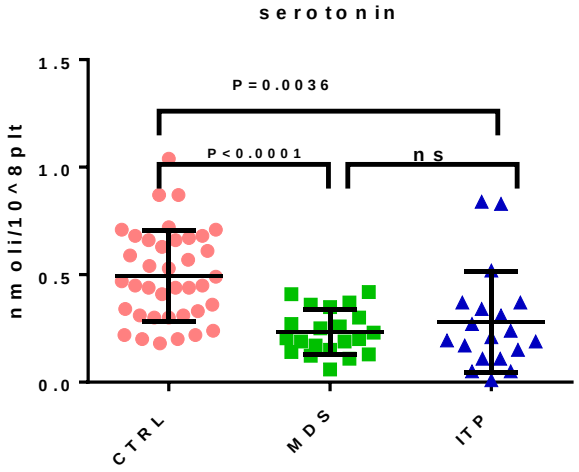

Figure 5.11. Serotonin level in delta granules

5.4.3. Platelet factor 4 levels

Both MDS and ITP patients had lower levels of PF4 than healthy subjects (Figure 5.12).

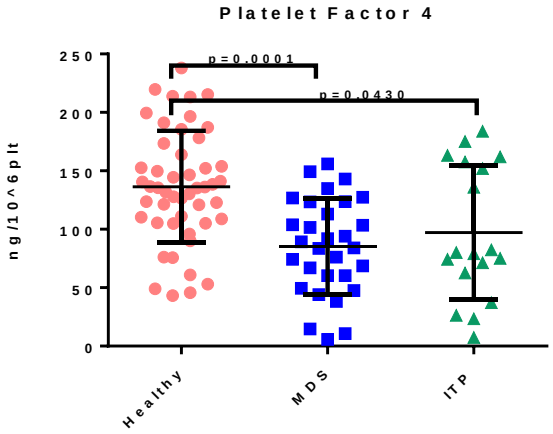

Figure 5.12. Platelet factor 4 level in alpha granules

5.5 Flow cytometry measurement of expression of surface receptors on resting platelets

Mean fluorescence intensity for CD41 and CD61 was similar in the three groups of subjects. However, CD42a expression was significantly higher in ITP patients than in the other two groups of subjects; CD42b and GPVI were higher in ITP than in MDS (Figure 5.13). P-selectin expression was similar among three groups (Figure 5.14).

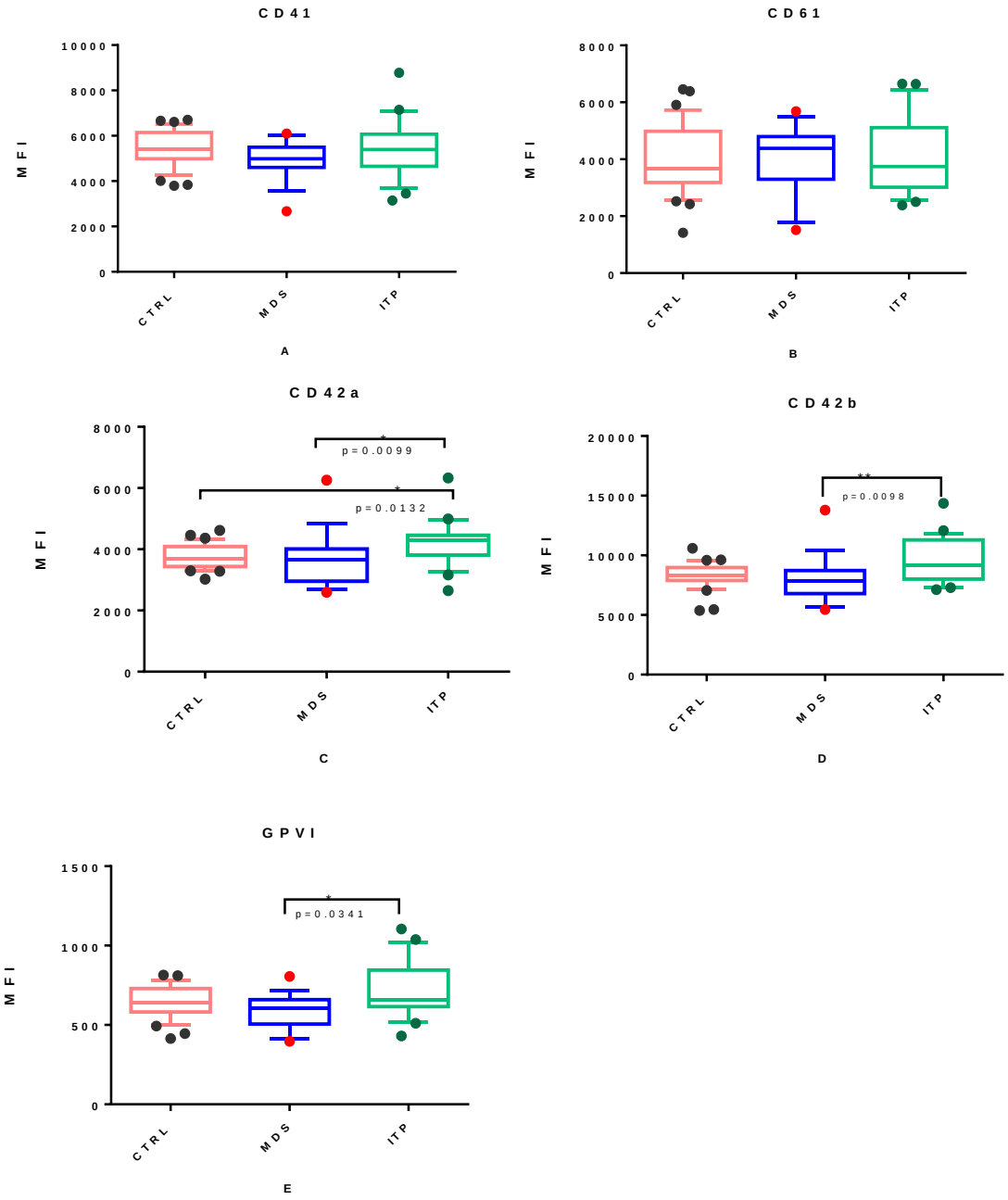

Figure 5.13. Comparison of platelet surface receptors expression

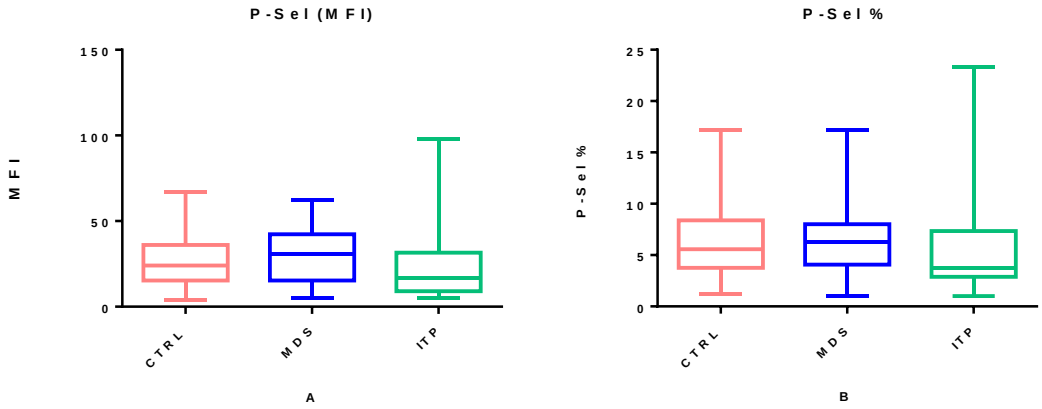

Figure 5.14. Comparison of rest platelet surface p-selectin expression

5.6 Serum TxB2 levels

Serum TxB2 levels in MDS patients were significantly lower than in healthy subjects, p=0.0153 (Figure 5.15), while there was no statistically significant differences between ITP patients and the other two groups of subjects.

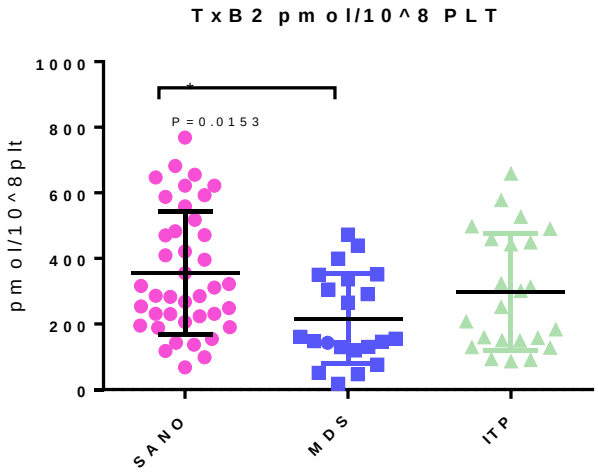

Figure 5.15. Serum TxB_2 level comparison in three groups.

5.7 Platelet cAMP levels

We measured cAMP levels from 54 healthy subjects, 32 MDS patients and 26 ITP patients, including cAMP levels in platelet poor plasma, platelet rich plasma, and also in PGE_1 stimulated platelet rich plasma. To calculate platelet cAMP level, we substract PPP cAMP from PRP cAMP, then divided by the count of platelet in PRP.

5.7.1 Baseline PPP levels of cAMP

There was no statistically significant difference of cAMP level in platelet poor plasma between among the study groups (Figure 5.16).

5.7.2 Baseline PRP levels of cAMP

There was no statistically significant difference of cAMP level in PRP between among the study groups (Figure 5.16).

5.7.3 PRP levels of cAMP after PGE1 stimulation

In the presence of Prostanglandin E1 (0.1 or 1.0 uM), cAMP levels in MDS and ITP patients were significantly lower than in healthy subjects; there was no difference between ITP and MDS patients (Figure 5.16).

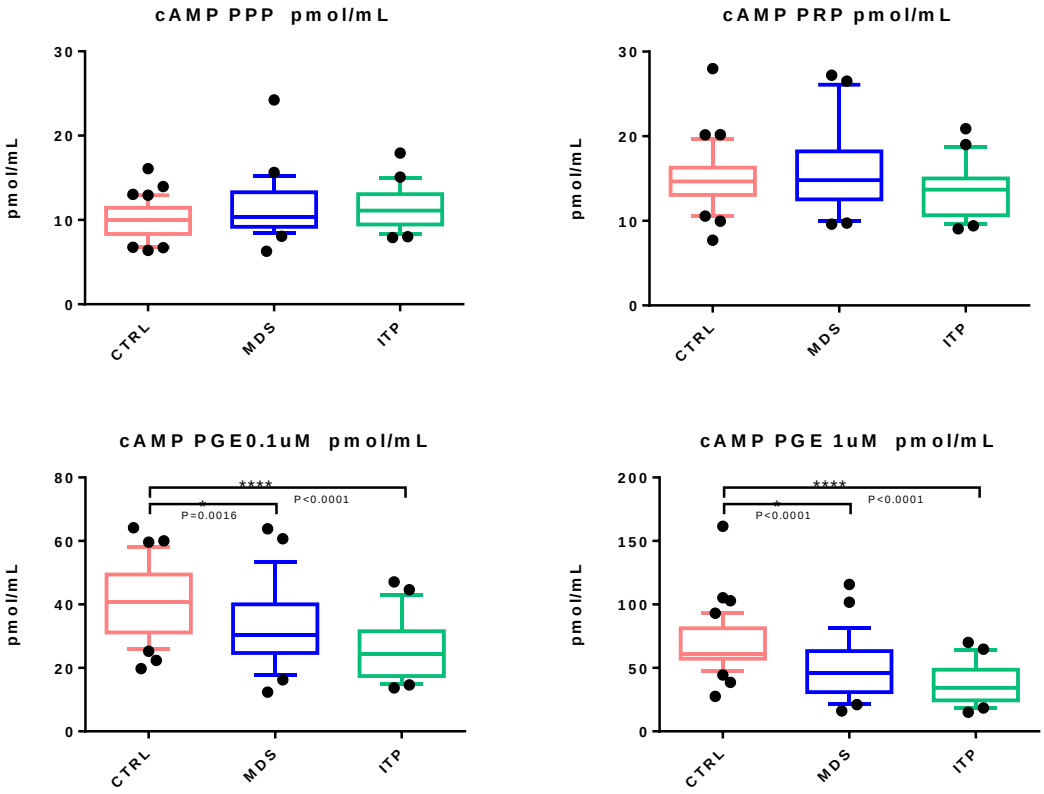

Figure 5.16. Total cAMP level (pmol/mL) comparisons in three groups

5.7.4 Platelet levels of cAMP pmol/10^9PLT

The intraplatelet levels of cAMP at baseline and in the presence of PGE1 0.1 or 1.0 uM were similar in the three groups of subjects.

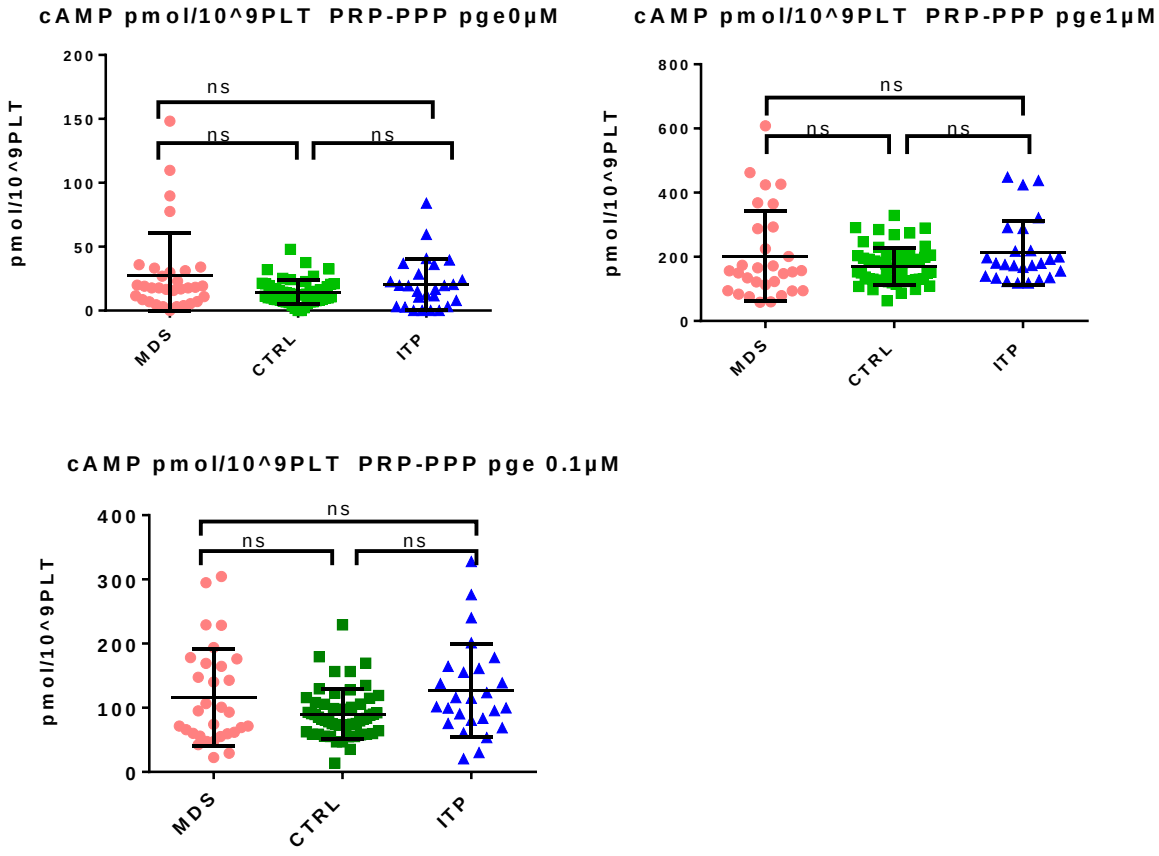

Figure 5.17. platelet cAMP level (pmol/10^9PLT) comparisons in three groups

5.7.5 Increase of cAMP concentration regard to platelet poor plasma level

The increase of cAMP was calculated as RATIO of the cAMP concentration in (PRP – PPP)/PPP.

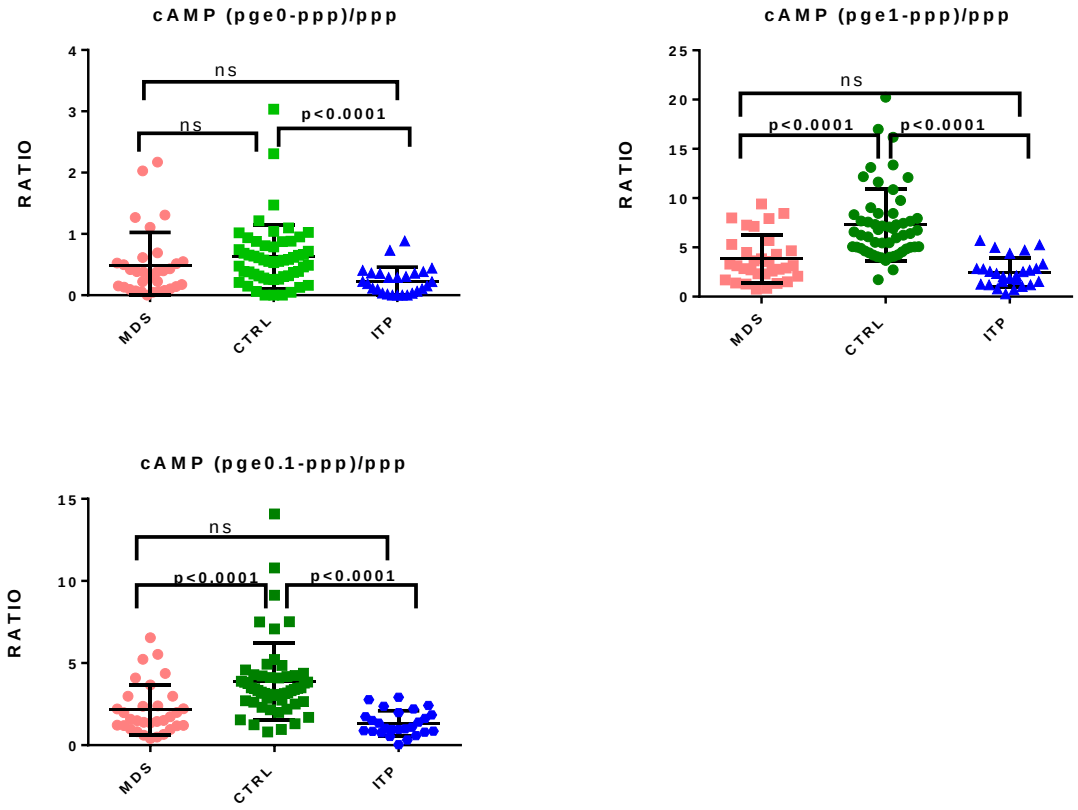

Figure 5.18. Increase of cAMP concentration regard to platelet poor plasma level

Three was no statistically significant difference in the baseline intraplatelet cAMP between MDS and healthy subjects, while lower levels were observed in ITP patients. In the presence of PGE1 0.1 or 1.0 uM, intraplatelet cAMPO levels were higher in healthy controls than in the other two groups of patients. (Figure 5.18).

6. Discussion and Conclutions

Documented evidence indicate that both thrombocytopenia and platelet dysfunction contribute to hemorrhagic complications observed in MDS, which account for 14-45% of deaths[2]. Bleeding in such patients is often attributed to the low platelet count, although in many patients platelet qualitative defects play an important role; a recent review of 838 MDS patients suggested qualitative rather than quantitative platelet abnormalities have prognostic significance[8]. A detailed study of the clinical features of 2900 patients with MDS reported that even among patients with platelet counts >50,000/uL, 19% had signs of bleeding at the time of diagnosis[39].

Several studies explored platelet aggregation by LTA or platelet activation by flow cytometry, platelet granules contents and the expression of surface receptors in MDS patients, reporting contradictory results[40]. In this study we aimed at exploring several parameters of platelet function in MDS patients, including the regulation of platelet function by cAMP, comparing it with normal controls and patients with ITP and comparable platelet counts as MDS patients.

The main results of our study can be summarized as follows: 1) platelet aggregation induced in PRP by all tested agonists was significantly lower in MDS patients than in both control groups, while there was no difference between ITP and healthy subjects. MDS and ITP patients had decreased ATP release measured during aggregation induced by all agonists compared to controls. 3) Platelet delta granules content of serotonin and ADP, alpha granules content of platelet factor 4 were lower than in healthy subjects, while serotonin only was lower than also in ITP patients. 3) Inhibition of platelet aggregation by exogenous PGE1 was significantly higher in MDS patients than in the other two groups. However, cAMP induced by PGE1, (0.1uM and 1 uM) was not higher in MDS patients, compared to ITP and healthy controls. 4) Platelet surface expression

of glycoprotein receptors in MDS group was normal while it tended to be higher in ITP platelets (CD42a, CD42b and GPVI).

The defect of platelet aggregation of MDS patients is not attributable to abnormalities of GPIIb/IIIa, the expression of which on the platelet membrane was normal. Reduced content of ADP in platelet delta granules, and, as a consequence, reduced ADP secretion during aggregation, could be responsible for the abnormal platelet aggregation in MDS patients. As a matter of fact, secreted ADP amplifies the platelet aggregation response to platelet agonists that induce platelet secretion. Reduced ADP secretion could also be responsible for the greater sensitivity of platelets to the inhibitory effect of external PGE1 on platelet aggregation, in the absence of increased platelet cAMP production, compared to other controls. As a matter of fact, it is known that ADP, by interacting with the platelet P2Y12 receptor, inhibits adenylate cyclase, the enzyme that is responsible for cAMP formation, which is stimulated by PGE1 (and other inhibitory prostaglandins) [41]. Lower than normal concentrations of secreted ADP will interact with P2Y12, resulting in lower inhibition of adenylate cyclase and lower cAMP production when platelets are stimulated by a secretion-inducing agonist, in the presence of PGE1. It is therefore likely that, although cAMP production by platelets exposed to PGE1 is normal, it would likely by reduced when PGE1 is added to platelets that are aggregating and secreting ADP as a consequence of stimulation of agonists. The finding of normal platelet content at baseline and after exposure to PGE1 in MDS patients contrasts with our previous observations in individual MDS patients at high risk, whose platelets contained high baseline levels of cAMP, which were greatly enhanced by exposure to PGE1, even at low concentrations (0.1 uM). Therefore, dysregulated cAMP production seems to be a rare finding in MDS patients, probably confined to patients at high risk, transitioning to acute myeloid leukemia.

Our observation of decreased content of both delta and alpha granules in MDS patients

suggest that generalized abnormality in platelet granules formation is characteristic of the disease. An alternative explanation of increased platelet activation in vivo, with platelet secretion of their granules contents in the circulation resulting in the presence of circulating “exhausted platelets”, is unlikely because our patients did not display any clinically relevant evidence of vascular disease, which could have caused platelet activation in vivo.

In conclusion, many MDS patients in the series that we studied displayed platelet dysfunction, associated with abnormalities of both delta and alpha granules, which could be categorized as acquired alpha-delta storage pool deficiency. Similar abnormalities have been described in other patient populations, such as myeloproliferative neoplasm and could contribute to the bleeding tendency of these patients.

Reference

1. James N George. Platelets. Lancet 2000;355:1531-9.
2. Seth J. Corey, Mark D. Minden, Dwayne L. Barber, Hagop Kantarjian1, Jean C. Y. Wang & Aaron D. Schimmer. Myelodysplastic syndromes: the complexity of stem-cell diseases. Nature Reviews Cancer 7, 118-129
3. Mufti GJ. Pathobiology, classification, and diagnosis of myelodysplastic syndrome. Best Pract Res Clin Haematol. 2004 Dec;17(4):543-57.
4. Vardiman JW. The World Health Organization (WHO) classification of tumors of the hematopoietic and lymphoid tissues: an overview with emphasis on the myeloid neoplasms. Chem Biol Interact. 2010 Mar 19;184(1-2):16-20
5. Thiele J1, Kvasnicka HM, Orazi A. Bone marrow histopathology in Myeloproliferative disorders--current diagnostic approach. Semin Hematol. 2005 Oct;42(4):184-95.
6. Schanz J1, Tüchler H, Solé F et al. New comprehensive cytogenetic scoring system for primary myelodysplastic syndromes (MDS) and oligoblastic acute myeloid leukemia after MDS derived from an international database merge. J Clin Oncol. 2012 Mar 10;30(8):820-9.
7. Itzykson R, Fenaux P. Optimal sequencing of treatments for patients with myelodysplastic syndromes. Curr Opin Hematol. 2009 Mar;16(2):77-83.
8. Rodeghiero F, Stasi R, Gernsheimer T. Standardization of terminology, definitions and outcome criteria in immune thrombocytopenic purpura of adults and children: report from an international working group. Blood. 2009 Mar 12;113(11):2386-93.
9. BornGV. Aggregation of blood platelets by adenosine diphosphate and its reversal.Nature1962;194:927-9.
10. PaiM, WangG, MoffatKA, LiuY, SeecharanJ, WebertK, et al. Diagnostic usefulness of a lumi-aggregometer adenosine triphosphate release assay for the assessment of platelet function disorders.AmJClinPathol2011;136:350-8.
11. Caen J, Sultan Y, Dreyfus B. Etude des fonctions plaquettaires dans 7 cas d'anémie réfractaire. Nouv Rev Fr Hematol. 1969 Jan-Feb;9(1):123–129.
12. Sultan Y, Caen JP. Platelet dysfunction in preleukemic states and in various types of leukemia. Ann N Y Acad Sci. 1972 Oct 27;201:300-6.
13. Rahman F, Brown CH. Qualitative platelet defects in preleukemia. Clin Res 1975;23:280A
14. Ressell NH, Keenan JP, Bellingham AJ. Thrombocytopathy in preleukaemia: association with a defect of thromxane A2 activity. Br J Haemetol 1979; 41:417-25.
15. Lintula R, Rasi V, Ikkala E, Borgstrom GH, Vuopio P. Platelet function in

preleukaemia. Scand J Haematol 1981;26:65-71
16. Tricot G, Criel A, Verwilghen RL. Thrombocytopenia as presenting symptom of preleukaemia in 3 patients. Scand J Haematol 1982;28:243-50
17. Stuart JJ, Lewis JC. Platelet aggregation and electron microscopic studies of platelets in preleukemia. Arch Pathol Lab Med 1982;106:458-61
18. Pamphilon DH, Aparicio SR, Roberts BE, Menys VC, Tate G, Davies JA. The myelodysplastic syndromes---A study of haemostatic function and platelet ultrastructure. Scand J Haematol. 1984;33:486-91
19. Aliza Zeidman, Nir Sokolover, Zinaida Fradin, Amos Cohen, Ophra Redlich and Moshe Mittelman, Platelet function and its clinical significance in the myelodysplastic syndromes. The Hematology Journal (2004) 5, 234–238
20. Blockmans D, Heynen MJ, Verhoef GE, Vermylen J. Platelet ultrastructural and functional studies in myelodysplasia. Haematologia (Budap). 1995;26(3):159-72.
21. Harrison P ,Cramer EM. Platelet alpha-granules. Blood Rev 1993;7(1):52-62.
22. CattaneoM. Inherited platelet-based bleeding disorders. J Thromb Haemost 2003;1:1628-36.
23. Michelson, A. D. (1996) Flow cytometry: a clinical test of platelet function. Blood 87, 4925–4936.
24. Michelson, A. D., Barnard, M. R., Krueger, L. A., Frelinger III, A. L., and Furman, M. I. (2002) Flow cytometry, in Platelets (Michelson, A. D., ed.), Academic Press, San Diego, CA pp. 297–315.
25. Jin J, Quinton TM, Zhang J, Rittenhouse SE, Kunapuli SP. Adenosine diphosphate (ADP)-induced thromboxane A(2) generation in human platelets requires coordinated signaling through integrin alpha(IIb)beta(3) and ADP receptors. Blood. Jan 1 2002;99(1):193-198.
26. Soderling SH ,Beavo JA. Regulation of Camp and cGMP signaling: new phosphodiesterases and new functions. Current Opin CellBiol2000;12:174-9.
27. Zaher Raslan, Khalid M. Naseem. The control of blood platelets by cAMP signaling. Biochem. Soc. Trans. (2014) 42, 289–294
28. Eduardo Fuentes, Iván Palomo. Relationship between Platelet PPARs, cAMP Levels, and P-Selectin Expression: Antiplatelet Activity of Natural Products. Evid Based Complement Alternat Med. 2013; 2013: 861786.
29. Kantarjian H1, Giles F, List A, Lyons R, Sekeres MA, Pierce S, Deuson R, Leveque J. The incidence and impact of thrombocytopenia in myelodysplastic syndromes. Cancer. 2007 May 1;109(9):1705-14
30. Maldonado JE, Pierre RV. The platelets in preleukemia and myelomonocytic leukemia. Ultrastructural cytochemistry and cytogenetics. Mayo Clin Proc. 1975;50:573–587.
31. Lintula R, Rasi V, Ikkala E, Borgstrom GH, Vuopio P. Plate-let function in preleukaemia. Scand J Haematol. 1981;26:65–71.
32. Hofmann WK, Ottmann OG, Ganser A, Hoelzer D. Myelo-dysplastic syndrome:

clinical features.Semin Hematol . 1996; 33:177–185.
33. Doll DC, List AF. Myelodysplastic syndromes. West J Med. 1989;151:161–167.
34. Judith Neukirchen, Sabine Blum, Andrea Kuendgen, Corinna Strupp, Manuel Aivado, Rainer Haas, Carlo Aul, Norbert Gattermann, Ulrich Germing. Platelet counts and haemorrhagic diathesis in patients with myelodysplastic syndromes. European Journal of Haematology 2009 83 (477–482)
35. Greenberg PL, Tuechler H, Schanz J, et al. Revised international prognostic scoring system for myelodysplastic syndromes. Blood 2012; 120: 2454–65
36. Mittelman M, Zeidman A. Platelet function in the myelodysplastic syndromes. Int J Hematol. 2000 Feb;71(2):95-8.
37. Manoharan A1, Brighton T, Gemmell R, Lopez K, Moran S, Kyle P. Platelet dysfunction in myelodysplastic syndromes: a clinicopathological study. Int J Hematol. 2002 Oct;76(3):272-8
38. Bellucci S1, Huisse MG, Boval B, Hainaud P, Robert A, Fauvel-Lafève F, Jandrot-Perrus M. Defective collagen-induced platelet activation in two patients with malignant haemopathies is related to a defect in the GPVI-coupled signalling pathway. Thromb Haemost. 2005 Jan;93(1):130-8.
39. Cattaneo M, Cerletti C, Harrison P, Hayward CPM, Kenny D, Nugent D, Nurden P, Rao AK, Schmaier AH, Watson SP, Lussana F, Pugliano MT, Michelson AD. Recommendations for the standardization of light transmission aggregometry: a consensus of the working party from the platelet physiology subcommittee of SSC/ISTH. J Thromb Haemost 2013;11:1183–1189

40. Bellucci S, Huisse MG, Boval B, Hainaud P, Robert A, Fauvel-Lafève F, Jandrot-PerrusM.Defective collagen-induced platelet activation in two patients with malignant haemopathies is related to a defect in the GPVI-coupled signalling pathway. Thromb Haemost. 2005 Jan;93(1):130-8.
43. CattaneoM. NewP2Y(12)inhibitors. Circulation2010;121:1719.