DETERMINATION OF PLATELET ACTIVATION AND ANTIBODY BINDING CAPACITIES OF PLATELET SURFACE RECEPTORS IN PATIENTS WITH MENSTRUAL CYCLE AND MALNUTRITION BASED IRON DEFICIENCY ANEMIA

by
Özgür Albayrak

Submitted to the Institute of Graduate Studies in

Science and Engineering in partial fulfillment of

The requirements for the degree of

Master of Science
In
Biotechnology

Yeditepe University
2013

DETERMINATION OF PLATELET ACTIVATION AND ANTIBODY BINDING CAPACITIES OF PLATELET SURFACE RECEPTORS IN PATIENTS WITH MENSTRUAL CYCLE AND MALNUTRITION BASED IRON DEFICIENCY ANEMIA

APPROVED BY

Assoc. Prof. Dr. Gülderen Yanıkkaya Demirel ....
(Supervisor)

Prof. Dr. Süleyman Sami Kartı ....

Assoc. Prof. Dr. Derya Özsavcı ....

DATE OF APPROVAL ..../..../....

To my mother, my
father, my cat ...

ACKNOWLEDGEMENTS

I would like to thank all healthy control volunteers who have helped with this study. I would like to thank Prof. Dr. Süleyman Sami Kartı for providing patient samples and for his moral support. I would also like to thank our laboratory specialist, my friend and my honorary big sister Fatma Tuba Akdeniz for her incredible support in the laboratory. I would like to thank Yeditepe University Hospital Biochemistry Laboratory for providing demographic results of patients and for ferritin measurement of healthy control samples. I would like to thank my honorary brothers Itri Atay and Kerem Özdönmez for their moral support. I would also like to thank all of our Laboratory team at Stem Cell Laboratory, Department of Hematology, Yeditepe University Hospital Sema Aktaş, Tuba Akdeniz, Zeynep Akbulut and Gökhan Terzioğlu for their support. I would like to thank my wonderful advisor Assoc. Prof. Gülderen Yanıkkaya Demirel for her incredible support. And last of all, I would like to thank my mother Melek Albayrak and my father Ahmet Erol Albayrak for their life-time support.

ABSTRACT

DETERMINATION OF PLATELET ACTIVATION AND ANTIBODY BINDING CAPACITIES OF PLATELET SURFACE RECEPTORS IN PATIENTS WITH MENSTRUAL CYCLE AND MALNUTRITION BASED IRON DEFICIENCY ANEMIA

IDA occurs when serum iron concentration decreases under a certain threshold and hemoglobin production is disrupted. Although there are several reasons for IDA to occur such as blood loss by disorders that cause chronic gastrointestinal bleedings, IDA has a high prevalence in women in developing countries due to heavy menstrual cycle and deficiency in iron uptake. In recent years, there are a few studies trying to determine the effects of IDA on platelets but there are conflicting reports whether IDA can affect the activation of resting platelets or not. In our study, platelet activations and ABCs of platelet surface receptors were examined with flow cytometry to determine the effects of IDA on platelet functions. 35 women patients with newly diagnosed IDA (16-50 years old) and 18 healthy women without IDA in same age group were included into study. We have found out that despite the increase in activated platelet population, ABC of CD41/CD61 fibrinogen binding complex was decreased in IDA patients. Also ABC of platelet activation markers was not changed in IDA patients in spite of platelet activation. This shows us that there may be conformational changes in fibrinogen binding to CD41/CD61 complex and surface activation molecules in IDA patients.

ÖZET

MENSTRUAL DÖNGÜ VE MALNÜTRİSYONA BAĞLI DEMİR EKSİKLİĞİ ANEMİSİ HASTALARINDA TROMBOSİTAKTİVASYONLARININ VE TROMBOSİT YÜZEY BELİRTEÇLERİNİN ANTİJEN BAĞLAMA KAPASİTELERİNİN BELİRLENMESİ

Demir eksikliği anemisi, vücuttaki demir oranı belirli bir eşik değerin altına düştüğünde ve buna bağlı olarak hemoglobin sentezinin sekteye uğradığı durumlarda ortaya çıkan bir rahatsızlıktır. Her ne kadar demir eksikliği anemisinin sebepleri arasında kronik gastrointestinal kanamalara yol açan rahatsızlıklar gibi etmenler gösterilse de, demir eksikliği anemisinde en yüksek prevalans, gelişmekte olan ülkelerdeki kadınlarda malnütrisyona ve ağır menstrual döngüye bağlı olarak görülmektedir. Son yıllarda demir eksikliği anemisinin trombosit sayıları ve aktivasyonları üzerindeki etkilerinin araştırılması amacıyla çeşitli çalışmalar yapılmış olsa da halen demir eksikliği anemisinin temel haldeki trombositlerin üzerinde bir etkisi olup olmadığı hakkında çelişkili raporlar bulunmaktadır. Bu çalışmada 35 yeni teşhis DEA (16-50 yaş) hastası ve 18 DEA bulunmayan aynı yaş grubundan sağlıklı kadınlarda trombosit aktivasyonları ve trombosit yüzey moleküllerinin antijen bağlama kapasiteleri (ABK) akan hücre ölçer metodu kullanılarak ölçülmüştür. Sonuç olarak DEA hastalarının trombositlerinde aktivasyon görülmesine rağmen, CD41/CD61 kompleksinin antijen bağlama kapasitesinde düşüş gözlenmiştir. Ayrıca aktivasyona rağmen, DEA hastalarının trombositlerinde aktivasyon belirteçlerinin ABKlerinde değişiklik gözlenmemiştir. Bu da bize DEAnin CD41/CD61 kompleksinde ve trombosit aktivasyon moleküllerinde konformasyonlara neden olabileceğini düşündürmektedir.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBLOS/ABBREVATIONS

c	Speed of Light
E	Energy
H	Planck's Constant
K	Potassium
S	Second
λ	Wavelength
ν	Frequency
ABC	Antigen Binding Capacity
ABCB10	ATP binding cassette super family B, member 10
ADP	Adenosine Diphosphate
bFGF	Basic Fibroblast Growth Factor
BD	Becton Dickenson
Ca	Calcium
CD	Cluster of Differentiation
CCL3	Chemokine C-C Motif Ligand 3
CCL5	Chemokine C-C Motif Ligand 5
COX	Cyclooxygenase
cMpL	Thrombopoietin Receptor
CTLA	Cytotoxic T-Lymphocyte Antigen
CXCL4	Chemokine (C-X-C motif. ligand 4
CXCR4	Chemokine C-X-C Motif Receptor 4
DcytB	Membrane-associated ferroreductase
dl	Deciliter
DMT1	Divalent Metal Transporter 1
DNA	Deoxyribonucleic Acid

EGF	Endothelial Growth Factor
EPO	Erythropoietin
FBC	Fibrinogen Binding Complex
FeS	Iron (II) Sulfur
FITC	Fluorescein isothyoCyanide
fL	Femtoliter
FL1	Fluorescence Channel 1
FL2	Fluorescence Channel 2
FS	Forward Scatter
GMP-140	Granule Membrane Protein 140
GP	Glycoprotein
HGB	Hemoglobin
HCT	Hematocrit
HLDA	Human Leukocyte Differentiation Antigens
HOX	Hemeoxygenase-1
IDA	Iron Deficiency Anemia
IGF	Insulin Like Growth Factor
IL	Interleukin
LAMP3	Lysosomal Membrane Associated Gllyco Protein 3
LPS	Lipopolysaccharide
LTA	Light Transmission Aggregometry
MALDI	Matrix Associated Laser Desorption and Ionization
MCH	Mean Corpuscular Hemoglobin
MCV	Mean Corpuscular Volume
MEA	Multiple Electrode Aggregometry
MFRN 1	Mitoferrin 1
Mg	Magnesium
MIP-1α	Macrophage Inflammatory Protein 1 Alpha
ml	Milliliter
mm	Millimeter
MoAB	Monoclonal Antibody

N-SAID	Non-Steroidal Anti-inflammatory Drug
P_2Y_1	Platelet ADP Receptor
P_2Y_{12}	Platelet ADP Receptor
PDGF	Platelet Derived Growth Factor
PE	Phycoerythrin
PGE-2	Prostaglandin E-2 Synthase
PF4	Platelet Factor 4
PMT	Photomultiplier Tube
PRP	Platelet Rich Plasma
RANTES	Chemokine (C-C motif. ligand 5
RBC	Red Blood Cell Counts
RNA	Ribonucleic Acid
SCF	Stem Cell Factor
SELP	Gene Expressing P-Selectin
SLC2537	Solute carrier family 25 member 37
SLE	Systemic Lupus Erythematosus
SPSS	Statistical Product and Service Solutions
SS	Side Scatter
TFR 1	Transferrin Receptor Protein 1
TGF	Transforming Growth Factor
TIBC	Total Iron Binding Capacity
TLR	Toll Like Receptor
TPO	Thrombopoietin
VEGF	Vascular Endothelial Growth Factor
VLA-3	Integrin α3-β1
VLA-6	Integrin-α6
vWF	von Willebrand Factor

1. INTRODUCTION

Iron Deficiency Anemia (IDA) is the most common type of anemia and is thought to be the cause of nearly 50% of total cases of anemia throughout the world [1]. Anemia can be caused by heavy blood loss by menstruation, parasite infections like hookworms, ascaris, and schistosomiasis, acute and chronic infections, including malaria, cancer, tuberculosis, and HIV and the presence of other micronutrient deficiencies, including vitamins A and B12, folate, riboflavin, and copper [1]. The term iron deficiency anemia means that anemia appears when the loss of body iron is faster than dietary iron absorption [2]. Iron deficiency anemia is characterized by pale pencil like small erythrocytes under light microscope. That is why IDA is also called hypochromic microcytic anemia [3]. Table 1.1 and Table 1.2 shows the global prevalence of IDA worldwide in data presented in worldwide prevalence of anemia according to 1993–2005 Global Databases on Anemia by World Health Organization [1]. Studies done by Erol Erduran [4] in 2010 reveals the IDA prevalence in Turkey according to the published material and the prevalence map is given in Table 1.3. IDA can be observed due to blood loss by disorders that cause chronic gastrointestinal bleedings such as Helicobacter Pylori infections and/or colorectal cancers. However IDA is mostly seen in women due to malnutrition, in the form of deficient iron uptake, or heavy menstrual cycle in developing countries and in those countries located in tropical climate the most common cause of IDA is infestation with hookworm. Although IDA is not a life threatening condition, chronic and untreated IDA can cause problems on metabolism due to the functions of iron as a structural component of certain proteins and enzymes. There have been studies reporting the effects of IDA on platelet aggregation and clotting time [5].

In our study, we analyzed the status of platelets in IDA by exploring platelet surface molecules for identification and activation of platelets by flow cytometry measurements. This report includes the general information about body iron and its functions, iron deficiency, iron deficiency anemia, general information about platelets and surface molecules used for this study; fluorescence emission and flow cytometry; previous studies about the effects of IDA on platelets along with explanations on why we have chosen this topic, methodology and results of our study and the interpretation of these results in light of the statistical analysis and suggestions for future studies that are needed to be done in order to fully understand the effects of IDA on platelets or vice versa.

Table 1.1. Global Prevalence of IDA in WHO Countries [1]

WHO Region/ Countries	Pre-School Age Children/Countries Age 0-4.99 Years	Pregnant Women /Countries	Non-Pregnant Women /Countries Age 15-49.9 Years	School Age Children/Countries Age 5-14.99 Years	Men/ Countries Age 15-59.9 Years	Elderly/ Countries Age ≥ 60 Years	All /Countries
Africa / 46	74.6% (26)	65.8% (22)	61.4% (23)	13.2% (8)	21.9 % (11)	0% (0)	40.7%
Americas / 35	76.7% (16)	53.8% (15)	56.2% (13)	47.1% (9)	34.3 % (2)	47.6 % (1)	58 %
South-East Asia / 11	85.1% (9)	85.6% (8)	85.4% (10)	13.6% (3)	4.1% (2)	5.2% (1)	14.9%

Table 1.1. Global Prevalence of IDA in WHO Countries [1] (Continue)

Europe / 52	26.5% (12)	8.3% (4)	28% (12)	9.3% (3)	14.1% (3)	8% (2)	22.9%
Eastern Mediterranean / 21	67.4% (11)	58.7% (7)	73.5% (11)	15.5% (6)	27.5% (6)	3.2% (3)	84.3%
Western Pacific / 27	90.4% (10)	90.2% (8)	96.9% (13)	83.1% (7)	96.2% (10)	93.3% (6)	13.8%
Global / 192	76.1% (84)	69% (64)	73.5% (82)	33% (36)	40.2% (34)	39.1% (13)	48.8%

Table 1.2: Global Population Effected by IDA [1]

Population Group	Prevalence of Anemia (%)	Effected Population x 10^6
Pre-School Age Children	47.4	293
School Age Children	25.4	305
Pregnant Women	41.8	56
Non-Pregnant Women	30.2	468
Men	12.7	260
Elderly	23.9	164
Total Population	24.8	1620

Table 1.3: Prevalence of IDA in Turkish Population According to Published Material [4]

Study Group	Population	Age (Years	IDA Positive (%)	Region
Children with Pica	107	1-6	76.6%	Urfa
School Age Children	2913	6-16	3.18%	Urfa
School Age Children	220	9-12	3.96%	Urfa
School Age Children in 2 different Elementary Schools	1633	6-18	1st School 4.2% 2nd School 13.8%	Malatya
Preschool Age	1004	0.5-6	6.5%	
Children Acquiring Iron Prophylaxis	90	1	2%	Ankara
School Age Children	848	7-11	24.7%	Manisa
School Age Children	724	7-16	2.9%	Diyarbakır
1st Grade Elementary School Children	680	7	%2.6	Konya

Table 1.3: Prevalence of IDA in Turkish Population According to Published Material [4] (Continue)

Adolescent School Age Children	1124	13-18	6.5%	Izmir
Adolescent School Age Children	1271	15-17	2.2%	Düzce
Adolescent School Age Children	329	12-18	5.5%	Sivas
Children and Adults	1223	0-≥14	Ages 0-2 18.3% Ages ≥14 16.3%	Adana
Random Patients Admitted to Hospital	343	0-14	18%	Manisa
Random Patients Admitted to Hospital	1656	2 Months-15	17.8%	Manisa

Table 1.3. Prevalence of IDA in Turkish Population According to Published Material [4] (Continue)

Random Patients Admitted to Hospital	742	0.5-14	18.48%	Ankara
Random Patients Admitted to Hospital	345	2-12	Ages 2-6 32% Ages 7-12 29.3%	Elazığ
Random Patients Admitted to Hospital	1362	0.25-16	Increased Frequency in children between 4-23 months	Kırıkkale
Healthy Volunteers	1491	2-69	Ages 2-5 34.5%	Kahraman Maraş

As will be seen in Table 1.3, the studies compiled above are mostly done on children so there is not enough and reliable data on IDA incidence in Turkish population and there is need for population based studies with large groups of patients from different ages.

2. PLATELETS

Platelets (or thrombocytes) are small enucleated cells that are two to three μm in size with irregular shapes and they are formed by fragmentation of special bone marrow cells called megakaryocytes which are the only polyploid hematopoietic cells [6].

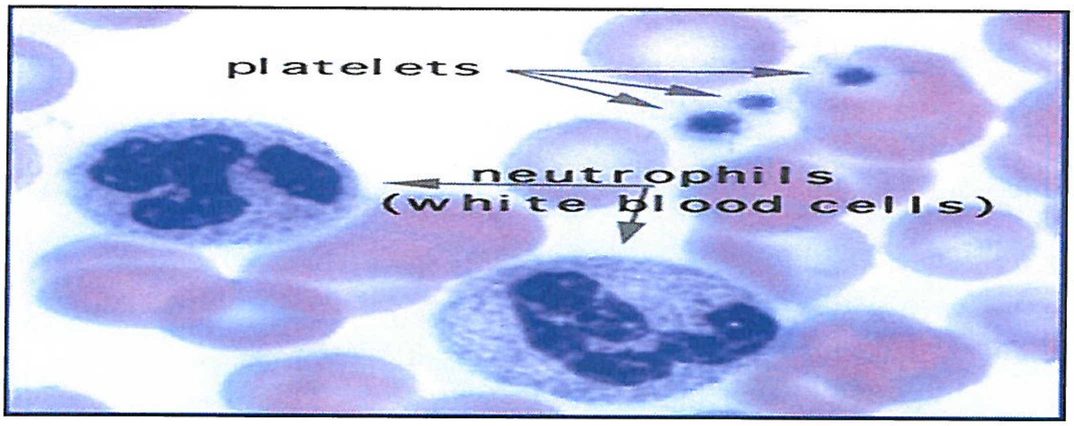


Figure 2.1. Platelets and blood cells under microscope [7]

Although platelets have no nucleus and genomic DNA, they contain small RNA molecules in their cytoplasm. Platelets have a lifespan between 24 to 48 hours in vivo and five to nine days when kept in anticoagulant agents in vitro. They are found in the peripheral blood of mammalians. Average human beings have 150 - 400 x 10^6 platelets per milliliter of blood.

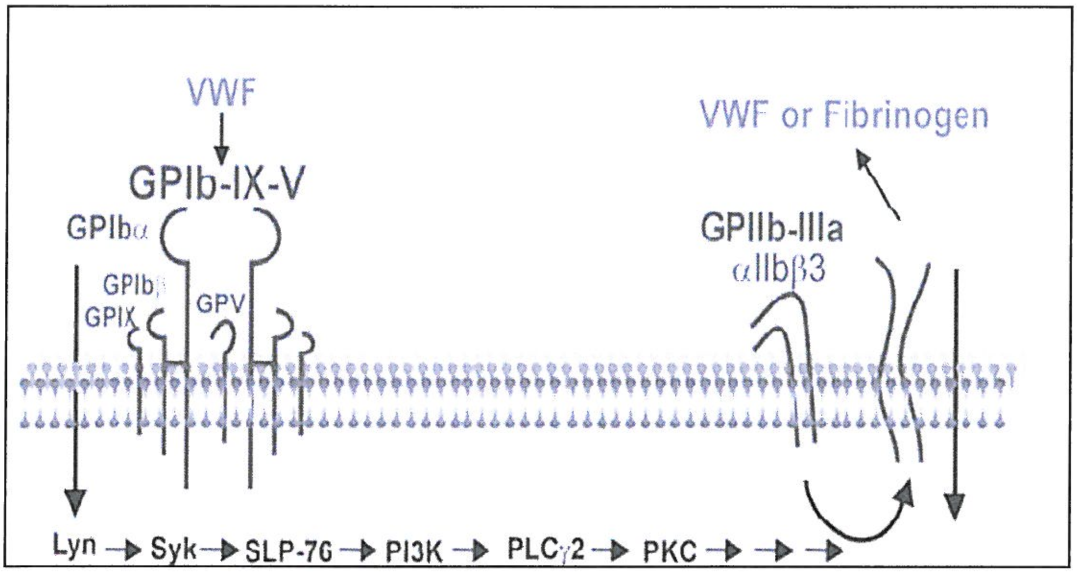


Figure 2.2. Platelet surface Receptors [6]

The main function of platelets is preserving hematopoiesis through blood clotting but in recent years there are increased number of studies that demonstrates other functions of platelets in wound healing and immune response [8]

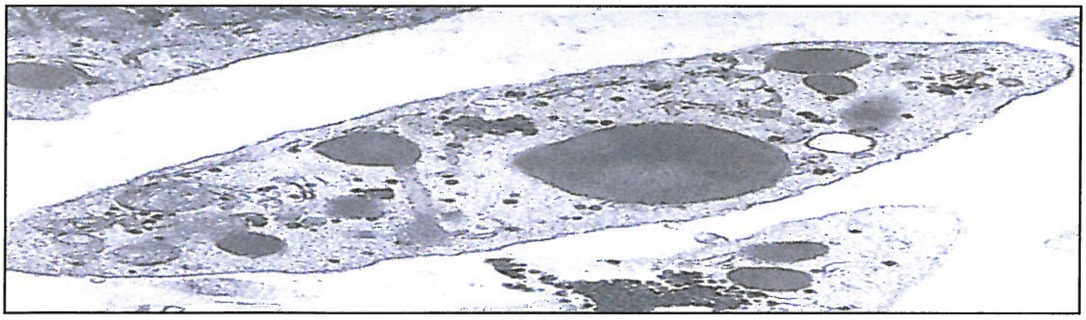


Figure 2.3. Normal discoid platelet containing a giant α-granule [6]

2.1. PLATELET FUNCTIONS

The main function of Platelets is preserving hematopoiesis through blood clotting by thrombus formation. However, studies in recent years demonstrated that platelets have several major functions in both immune responses, wound healing and signal transduction. A study done by Gunsilius et. al showed that platelets are the major source of Vascular endothelial growth factor (VEGF) in peripheral blood [9] and Weibrich demonstrated that platelets also synthesize platelet derived growth factor (PDGF), Insulin-like growth factor (IGF) and transforming growth factor β-1 (TGF- β1) that contributes to wound healing [10]. A study done by Faude also showed high amounts of PDGF, endothelial growth factor (EGF), basic fibroblast growth factor (bFGF) and IGF-1 are synthesized by platelets [11] and all of these studies shows that platelets are the natural source of growth factors in human body.

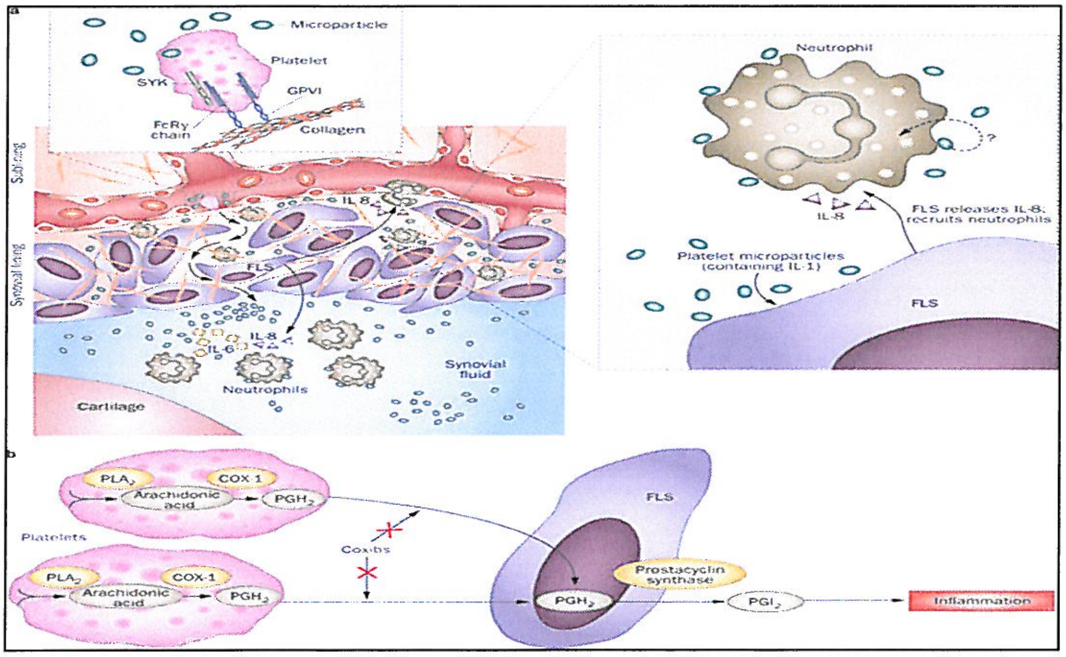


Figure 2.4. Enhancement of inflammation by platelets in arthritis by COX-1 mediated PGI_2 synthesis, IL-1 rich microparticle synthesis of platelets and how these platelet microparticles induce leukocyte chemoattraction mediators such as IL-6 and IL-8 [12]

Recent studies also showed that activated platelets regulate inflammation and leukocyte trafficking by secreting anti and pro-inflammatory cytokines such as interleukin-7 (IL-7), stem cell factor (SCF) and TGF-β, cMpL (Thrombopoietin Receptor) and Immunoglobulin E receptor subunits Fc epsilon RI alpha gamma mRNA expression as Soslau et. al showed [13], whereas Hartwig demonstrated expressions of interleukin-1β (IL-1β), interleukin-6, IFN-α cytokines in platelet concentrates [14]. Picker et. al also showed that platelets synthesize CCL3/MIP-1α, CXCL4/PF4 and CCL5/RANTES chemokines [15] to regulate immune responses. Activated platelets also mediate innate immune system via their surface expression of toll like receptors as Cognasse and Aslam showed that resting and activated platelets express surface Toll Like Receptors TLR-2, TLR-4 and TLR-9 [16, 17] whereas Andonegui demonstrated that LPS induced platelets express TLR-4 on their surfaces [18]. Platelets are also the main source of soluble CD40 ligand which activates neutrophils on binding. Evidence also demonstrates that platelets can act as phagocytes and can release reactive oxygen species on internalized bacteria as by Byrne et. al shows that von Willebrand Factor (vWF) bound Helicobacter pylori activates and aggregates platelets through surface Glycoprotein 1b/vWF receptor and is undergone phaghocytosis [19]. All the evidence above shows that platelets have a role in both innate and adaptive immune system responses.

In order to demonstrate their functions, platelets may need to be activated. This activation process can be in vivo and/or in vitro. Activation processes include fibrinogen release due to tissue damage, ADP binding to $P2Y_1$ and $P2Y_{12}$ receptors [20], hormonal activation by adrenaline (epinephrine in vitro), thrombin, serotonin, vasopressin [21]; inflammatory activation by enzymes Cyclooxygenase-1 and -2 (COX-1, COX-2), Prostaglandin E-2 Synthase (PGE-2) and Nitric Oxide; release of Indoleamine 2, 3-Dioxygenase release, high concentrations of Ca^{+2} and Mg^{+2} ions spontaneous activation occurring only in in vitro studies. Use of non-steroidal anti-inflammatory drugs (N-SAIDs), anti-inflammatory steroids like cortizol, anti-inflammatory cytokines like interleukin-10 (IL-10) and Cytotoxic T-Lymphocyte antigen 4 (CTLA-4) and platelet inhibitory drugs like Clopidogrel lead to platelet inhibition and loss of function.

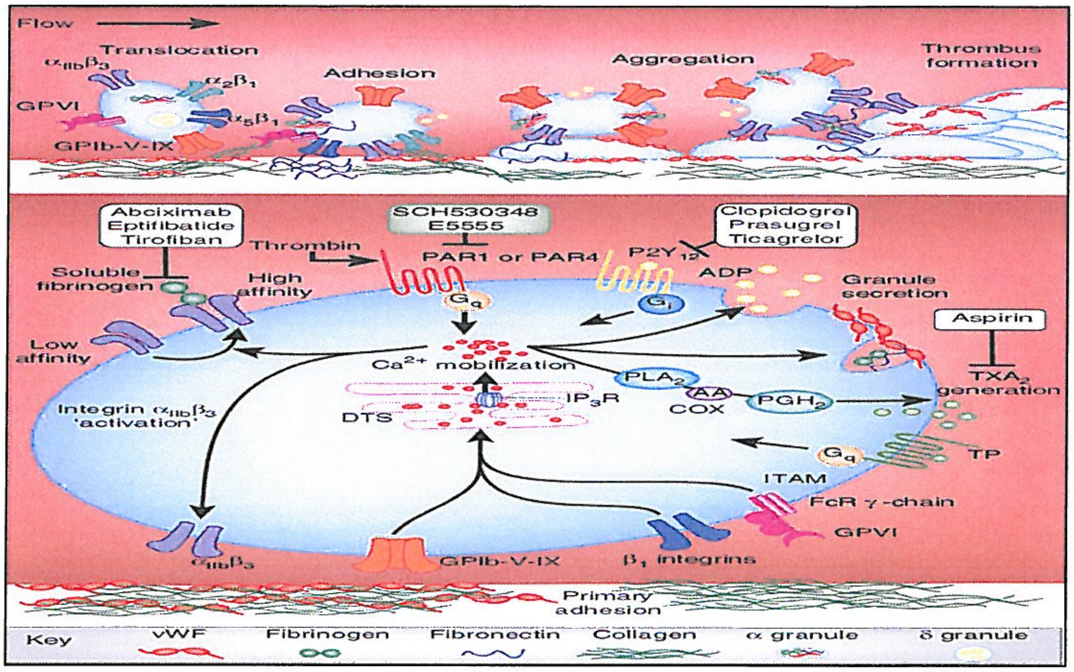


Figure 2.5. The activation and adhesion mechanisms of homeostatic and prothrombotic function of platelets by showing the locations of major platelet function mediating receptors and ligands such as free fibrinogen and vWF being on sites of vascular injury and how circulating platelets can be captured by these ligands, become activated and generate thrombus formation [22]

2.2. IN VITRO DETECTION OF PLATELETS

In order to analyze platelets and their functions, platelet populations are needed to be fully determined in heterogenic peripheral blood samples. This can be done in several ways such as peripheral smear method, fluorescence microscopy and flow cytometry. Also Light Transmission Aggregometry (LTA) and Multiple Electrode Aggregometry (MEA) are used for platelet function testing. In our study, flow cytometry method is used for both determining and analyzing platelets in peripheral blood samples. In flow cytometry, there are two platelet preparation methods to analyze platelets, Platelet Rich Plasma (PRP) and whole blood but because PRP preparation induces platelet activation through centrifugation, whole blood samples are used for platelet activation testing as recommended by Shattil et. al [23].

2.2.1. Flow Cytometry

Cytometry is the technique used for measuring the biological, physical and chemical properties of cells. Flow cytometry uses the fluid mechanics to put the cells in single lining as they pass through a capillary known as the flow channel. Main components of a flow cytometer are laser, fluidics, optics, and completing electronics. Currently used cytometry systems are capable of measuring multi-color experiments which provide more information on properties of intracellular media and surface of cells.

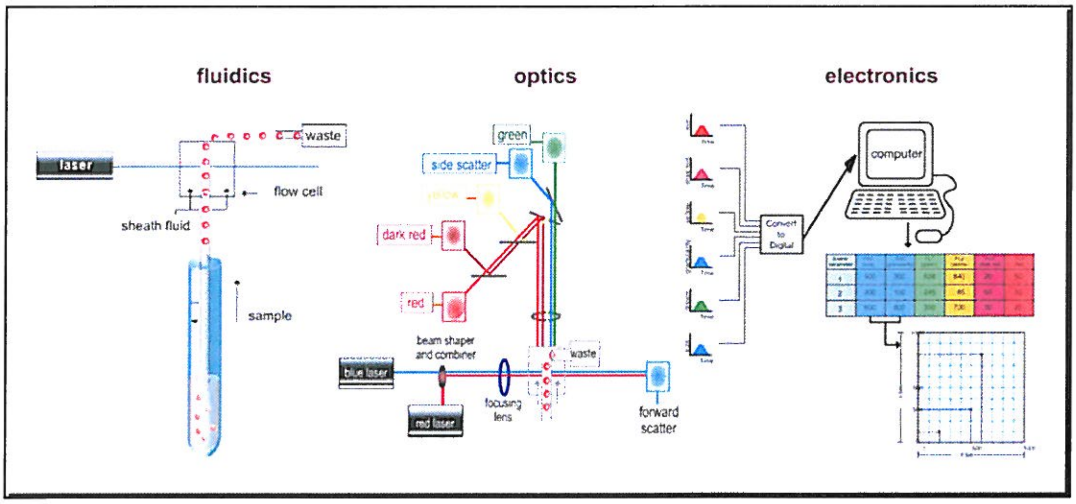


Figure 2.6. Basis of Flow Cytometric Detection

2.2.2. Light Scattering

Flow cytometry uses Laser technology to generate continuous ionizing laser beams for measurement. Several lasers with different wavelengths are used for detection such as 488nm Argon-Ion Laser, 632nm Helium-Neon Lasers and Ultraviolet Lasers. When single lined cells go through the flow channel, they are exposed to this continuous laser beam. When laser beam hits the cells, reflected beams scatter into two directions: Direct scattering (Forward Scattering, FS) and perpendicular scattering (Side Scattering, SS). In FS, light beam goes through the perimeter of the cell and gives an approximation of cell surface area and size of

5.2. PLATELET SURFACE IDENTIFICATION AND ACTIVATION MARKER RESULTS

Anti CD41-FITC and anti CD61-FITC MoABs were used on both patient and control peripheral blood samples in order to identify platelet population in whole blood and compare the functional differences of surface CD41-CD61 fibrinogen binding complex [GPIIb-IIIa] expressions between IDA patients and controls. Activation markers anti CD42b-PE, anti CD62P-PE and anti CD63-PE MoABs were used in order to find out the differences of platelet activation between control and IDA patient peripheral blood samples. Table 5.3 and Table 5.4 show surface identification and activation marker percentages and Table 5.5 and Table 5.6 show the ABCs of every molecule on platelets of IDA patients and healthy controls.

Table 5.3. Patient Platelet Surface Identification and Activation Marker Expressions

	CD41 %	CD61 %	CD42b %	CD62P %	CD63 %
Patient 1	84.30%	96.10%	44.20%	1%	0.80%
Patient 2	93.30%	97.60%	13.40%	0.20%	0.30%
Patient 3	94.80%	97.10%	3.10%	0.10%	0.10%
Patient 4	90.30%	93.30%	7.60%	1%	0.20%
Patient 5	95.70%	95.20%	97.40%	25.90%	17.20%
Patient 6	91.90%	93.10%	92.40%	46.30%	58.90%
Patient 7	87.10%	91.30%	93.00%	39.90%	31.30%
Patient 8	82.40%	93.30%	97.60%	22.50%	31.20%
Patient 9	90.30%	91.70%	95.10%	85.40%	86.70%
Patient 10	93.50%	94.20%	95.70%	23.20%	41.10%
Patient 11	87.50%	96.70%	97.90%	19.10%	16.00%
Patient 12	97.30%	98.60%	98.90%	19.60%	40.10%
Patient 13	97.40%	97.10%	90.50%	40%	63.60%
Patient 14	93.80%	88.60%	97.10%	55.30%	65.10%
Patient 15	95.60%	95.80%	97.00%	61.90%	74.76%

Table 5.3. Patient Platelet Surface Identification and
Activation Marker Expressions (Continue)

Patient 16	95.50%	95.00%	98.10%	63%	66.10%
Patient 17	95.70%	95.50%	99.60%	47.40%	71.20%
Patient 18	95.00%	95.80%	96.90%	46.54%	44.40%
Patient 19	95.80%	96.10%	98.20%	79.40%	91.90%
Patient 20	98.80%	99.00%	99.30%	23.50%	45.40%
Patient 21	96.50%	80.30%	94.60%	42%	51.30%
Patient 22	94.70%	95.30%	94.00%	27.40%	60.40%
Patient 23	98.20%	99.80%	99.40%	13.90%	45.40%
Patient 24	93.20%	95.00%	98.30%	35.20%	35.50%
Patient 25	90.60%	91.70%	99.50%	46.60%	65.30%
Patient 26	94.60%	97.50%	99.50%	46.30%	38.60%
Patient 27	96.10%	97.20%	99.70%	32.70%	36.10%
Patient 28	95.70%	93.90%	99.10%	6%	17.60%
Patient 29	97.30%	97.50%	99.70%	22.90%	26.00%
Patient 30	96.80%	96.80%	99.50%	33.90%	69.70%
Patient 31	97.10%	94.20%	99.40%	33.30%	46.00%
Patient 32	91.10%	86.40%	98.60%	34.20%	52.30%
Patient 33	93.10%	92.60%	97.60%	57.50%	44.20%
Patient 34	97.40%	97.00%	97.80%	32.80%	28.70%
Patient 35	95.70%	95.40%	98.00%	12.80%	40.30%

Table 5.4. Control Platelet Surface Identification and Activation Marker Expressions

	CD41 %	CD61 %	CD42b%	CD62P %	CD63%
Control 1	99.30%	99.00%	100.00%	8.80%	34.60%
Control 2	99.00%	98.90%	99.90%	18.60%	23.30%

Table 5.4. Control Platelet Surface Identification and Activation Marker Expressions (Continue)

Control 3	98.80%	99.30%	99.90%	7%	28.20%
Control 4	99.50%	99.30%	99.60%	10.90%	24.20%
Control 5	99.30%	99.30%	99.80%	11.30%	27.70%
Control 6	99.60%	99.50%	100.00%	18.55%	39.30%
Control 7	99.60%	99.40%	99.90%	9.70%	25.80%
Control 8	99.60%	99.40%	99.80%	2.70%	23.90%
Control 9	99.40%	99.10%	99.90%	7.80%	39.70%
Control 10	99.70%	99.30%	99.90%	5.80%	37.60%
Control 11	99.40%	98.90%	99.90%	9.20%	35.70%
Control 12	99.30%	99.00%	99.90%	9.60%	22.15%
Control 13	98.60%	98.00%	99.40%	18.80%	33.90%
Control 14	98.00%	97.30%	99.70%	9.40%	46.00%
Control 15	97.30%	98.00%	99.30%	17.40%	22.10%
Control 16	98.90%	99.30%	99.60%	13.70%	35.70%
Control 17	99.30%	99.20%	99.70%	1.70%	27.20%
Control 18	99.10%	98.80%	99.90%	3.30%	42.00%

Table 5.5. Patient ABCs

	CD41	CD61	CD42b	CD62P	CD63
Patient 1	28074.18	31156.55	906.46	381.15	361.61
Patient 2	29786.02	22253.92	713.44	403.14	359.16
Patient 3	31664.54	23623.40	649.92	681.68	425.13
Patient 4	28416.55	25677.60	740.32	476.44	388.48
Patient 5	20884.45	15286.73	9615.43	908.91	522.87

Table 5.5. Patient ABCs (Continue)

Patient 6	12821.68	23794.58	23497.83	2152.55	823.39
Patient 7	24136.95	36405.75	19488.60	1414.67	725.66
Patient 8	15543.51	23965.76	12487.24	1414.67	564.40
Patient 9	28587.73	35559.11	16435.21	10384.67	4000.02
patient 10	21398.00	27903.00	18081.38	1087.27	635.26
Patient 11	21226.82	30641.94	14576.62	1099.48	645.03
Patient 12	29957.20	30987.22	12897.50	984.65	569.29
Patient 13	11417.97	25677.60	18559.31	1133.69	689.01
Patient 14	19343.79	32341.85	16249.35	1649.23	779.41
Patient 15	27560.63	43178.91	16780.37	3564.12	706.11
Patient 16	24650.50	38607.03	15904.18	3487.20	1480.64
Patient 17	30817.89	37421.73	13487.24	1761.62	845.38
Patient 18	13352.35	32172.52	18346.90	1221.65	613.27
Patient 19	19001.43	40808.31	18187.59	3641.04	1624.79
Patient 20	17974.32	32172.52	12512.88	806.29	527.75
Patient 21	11554.92	33188.50	17311.40	806.29	583.95
Patient 22	10219.69	24821.68	17417.60	1026.19	571.73
Patient 23	21398.00	33527.16	22170.27	691.45	525.31
Patient 24	28074.18	36405.75	27082.25	1172.78	784.30
Patient 25	32172.52	39623.00	26816.74	1988.85	906.46
Patient 26	26019.97	37083.07	20019.62	1558.82	747.65
Patient 27	18659.06	33696.49	25329.87	1160.57	620.60
Patient 28	40638.98	36913.74	20816.16	1040.85	552.19
Patient 29	28074.18	32511.18	19010.68	918.68	488.66
Patient 30	31156.55	32849.84	22462.34	1153.24	652.36
Patient 31	29614.84	34373.80	24666.09	884.47	547.30
Patient 32	20713.27	33019.17	24294.37	1050.62	635.26

Table 5.5. Patient ABCs (Continue)

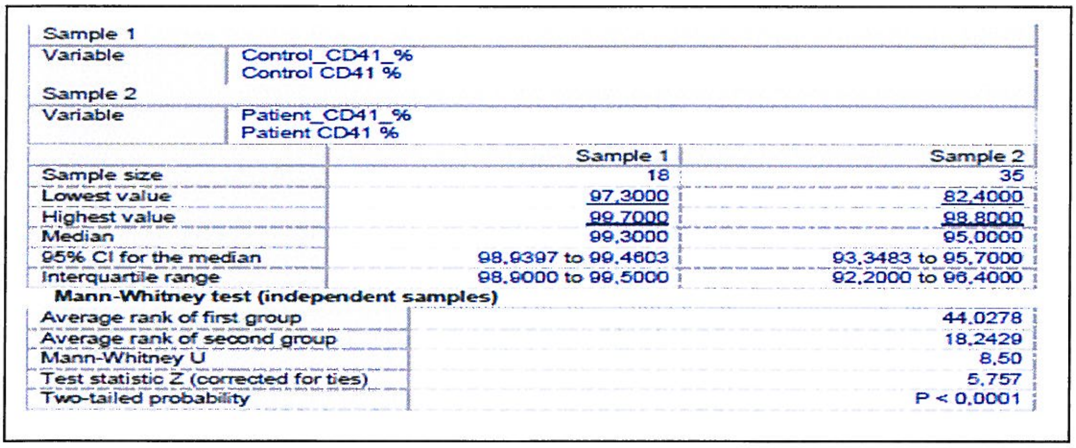
Patient 33	23965.76	39961.66	22541.99	2743.60	1126.36
Patient 34	9843.08	18830.24	20019.62	1116.59	608.38
Patient 35	29101.28	32511.18	26312.26	1145.91	740.32

Table 5.6. Control ABCs

	CD41	CD61	CD42b	CD62P	CD63
Control 1	38776.36	41993.61	24533.33	696.34	471.56
Control 2	36575.08	40469.65	23019.91	1177.67	725.66
Control 3	46904.15	46904.15	25728.14	701.23	471.56
Control 4	44533.55	34373.80	21878.21	1605.25	1859.35
Control 5	41146.96	36913.74	20444.44	1407.34	835.61
Control 6	51984.03	43856.23	22541.99	2245.39	801.40
Control 7	47581.47	36575.08	25914.00	952.89	552.19
Control 8	48428.12	40300.32	25276.77	662.13	544.86
Control 9	47412.14	38437.70	24453.68	894.25	603.49
Control 10	57741.21	45718.85	25329.87	933.34	691.45
Control 11	48766.77	41485.62	22515.44	864.93	588.84
Control 12	52830.67	46904.15	24055.41	950.44	581.51
Control 13	55201.28	48258.79	23073.01	1492.86	757.42
Control 14	38099.04	42162.94	19302.74	784.30	598.61
Control 15	32849.84	36236.42	18400.00	2208.74	857.60
Control 16	43348.24	37083.07	23975.76	1580.81	662.13
Control 17	52492.01	35897.76	25064.36	774.53	542.41
Control 18	57233.23	39453.67	24719.19	784.30	601.05

5.2.1. CD41 [GPIIb] Immunophenotyping

As a subunit of GPIIb-IIIa fibrinogen binding receptor complex, CD41 is both a major platelet surface identification and function marker in flow cytometry. Due to this importance, surface CD41 expressions were compared in both IDA patients and healthy controls. As it is seen in Figure 5.7 the mean percentage values of CD41 in IDA patients [n=35] were 93.83 % ± 0.65 whereas mean percentage of CD41 in healthy controls were 99.09 % ± 0.14 [n=18]. According to the statistical analysis, significant results were found between IDA Patient and healthy control surface CD41 expressions [P<0,0001].



Sample 1

Variable	Control_CD41_% Control CD41 %

Sample 2

Variable	Patient_CD41_% Patient CD41 %

	Sample 1	Sample 2
Sample size	18	35
Lowest value	97,3000	82,4000
Highest value	99,7000	98,8000
Median	99,3000	95,0000
95% CI for the median	98,9397 to 99,4603	93,3483 to 95,7000
Interquartile range	98,9000 to 99,5000	92,2000 to 96,4000

Mann-Whitney test (independent samples)

Average rank of first group	44,0278
Average rank of second group	18,2429
Mann-Whitney U	8,50
Test statistic Z (corrected for ties)	5,757
Two-tailed probability	P < 0,0001

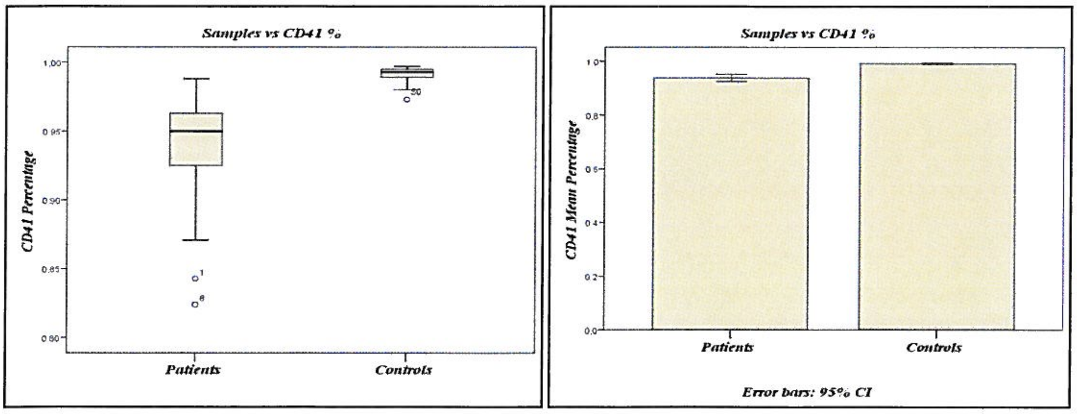


Figure 5.7. Statistical Analysis between Patients and Controls Platelet surface CD41 Expressions

the cell while in SS light beam refracts from the granules of cell and gives an approximation about granularity. As seen in Figure 2.7, there are two scales for cell measurement with light scattering: Linear scale and Logarithmic scale. In linear scale, FS x SS graphic plot is divided into segments corresponding to integers, giving us a zoomed out view of cells that are bigger than 8 µms like leukocytes, somatic cells and cancer cells. In logarithmic scale, the FS x SS graphic plot is divided into exponential numbers such as $10^0 - 10^1 - 10^2$ etc. giving us a zoomed in view of cells or microparticles smaller than 8 µms such as platelet populations, microbeads and nanoparticles.

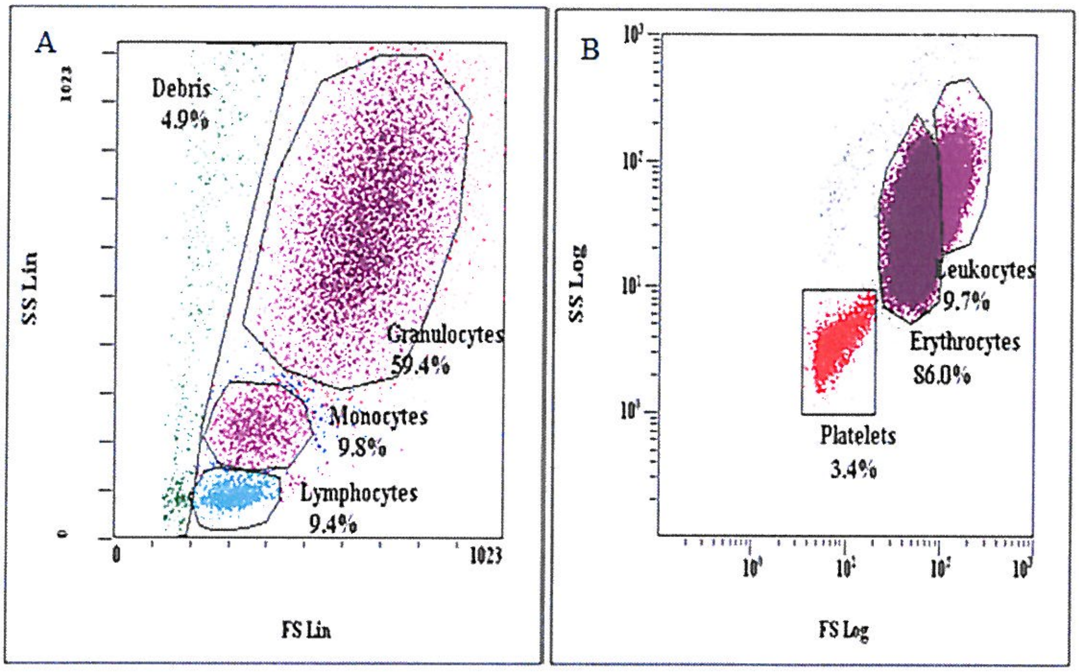


Figure 2.7. A - Forward Scatter vs. Side Scatter Graphic of leukocytes.
B - Forward Scatter vs. Side Scatter Graphic of platelets

2.2.3. Fluorescence Emission

Measurement by flow cytometry depends on the principal of fluorescence emission. First postulated by Albert Einstein [24], light has two forms interfering with each other known as the wave-particle duality in which millions of massless energy particles of light known as the photons move in the form of sinusoidal function in space which form the light wave. When this light wave interacts with matter, the resting valence electrons of that matter absorbs these photons and become excited electrons as their energy now increased and thus they move up to the upper orbitals with higher energy levels as shown in molecular orbital diagram, but excited electrons now become unstable and release the absorbed energy as a new photon to return to the resting state. However some of the photonic energy is lost to the internal quantum energy functions such as vibration and spinning. As Max Planck's energy equation [25], energy of a particle (E) equals to Planck constant (h, 6,626 x 10^{-27} erg.s) multiplied by the frequency (ν) which is the speed of light (c, 3x10^{8} m/s) divided by wavelength of the light (λ, nm)

$$E = h \times \frac{c}{\lambda} \quad (2.1)$$

As shown in the Equation 2.1 energy of a particle is inversely proportional to the wavelength of that particle thus the newly released photon becomes low energy particle with higher wavelength. This phenomenon is called the photoelectric effect. If the released photon has a wavelength in between the Ultraviolet-Visible Light spectrum then the phenomenon is called Fluorescence Emission. In Flow Cytometers, emitted light goes through a path consisting of reflective mirrors and dichroic filters that discriminates emitted light depending on the wavelength. After the light wave passes through a filter with appropriate wavelength interval, light is captured by photomultiplier tubes (PMT) that converts photonic energy to electrical energy and multiplies it 1x10^{8} times. Amplified electrical energy is then sent to an adjacent computer to be analyzed.

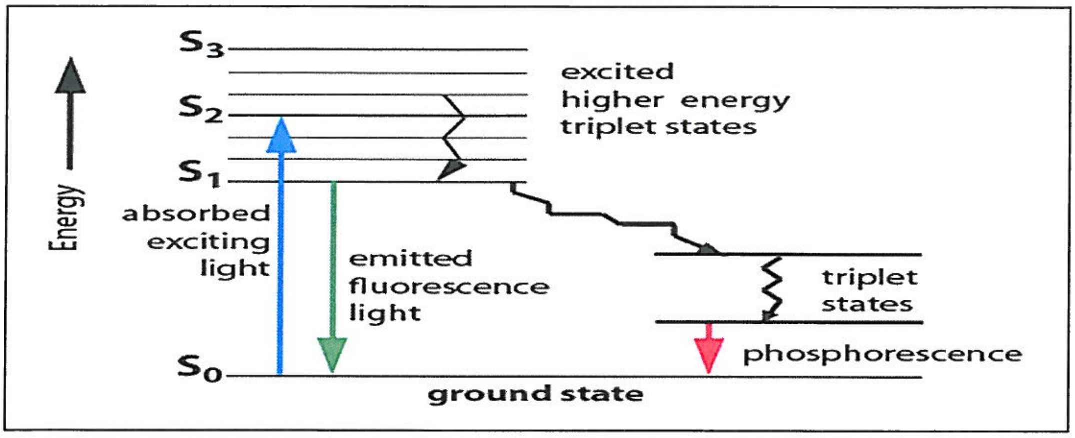


Figure 2.8. The Jablonski Diagram of Fluorescence Emission [26]

2.3. MONOCLONAL ANTIBODIES AND SURFACE IMMUNOPHENOTYPING

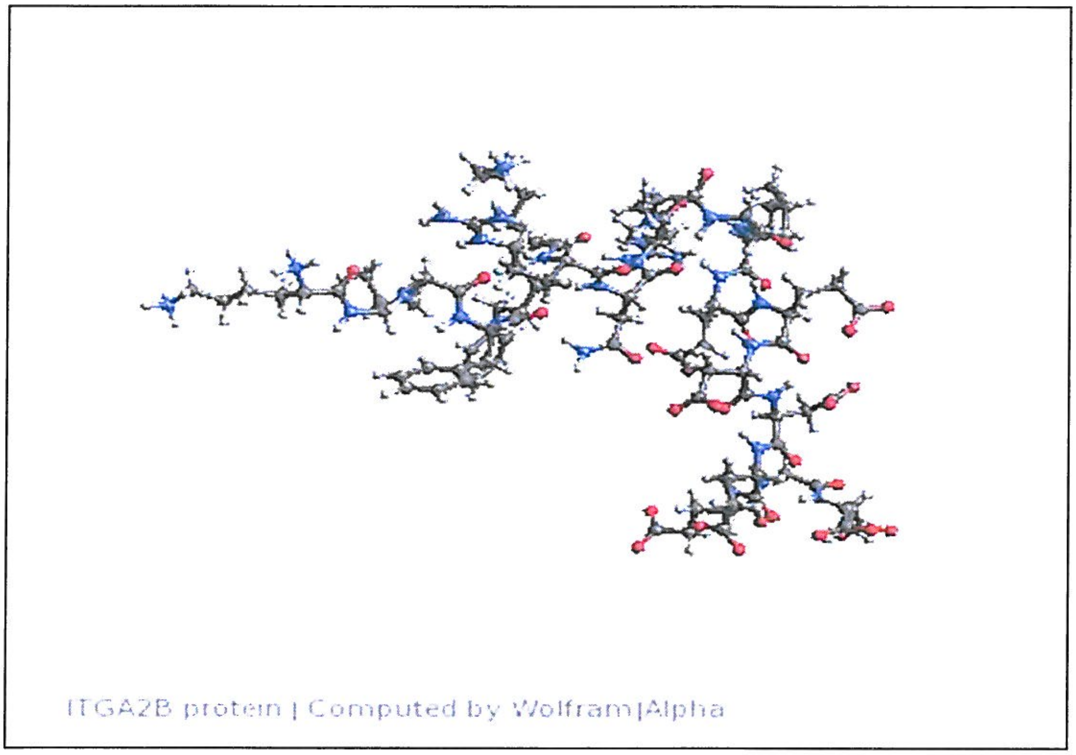
In order to measure the physical and biological properties of the cells, monoclonal antibodies are used for cell detection, characterization and functional analysis. As known, cells have certain molecules on their surfaces for different biological processes. These molecules can be transmembrane proteins, glycoproteins, carbohydrates and protein complexes etc. and they take part in functions such as adhesion, motility, migration, chemotacticity, cytotoxicity. These molecules have different names for their functions but in common nomenclature accepted in 1st International Workshop and Conference on Human Leukocyte Differentiation Antigens (HLDA) in Paris 1982, these molecules are now called Cluster of Differentiation (CD) molecules. Besides their functions, these CD molecules are also used as cellular markers for cell characterization in heterogeneous populations using monoclonal antibodies (MoAB) that can bind specifically to these CD molecules depending on the CD phenotype of specific cells. In Flow Cytometry, MoABs are conjugated with synthetic or naturally found fluorescence emitting polyaromathic organic molecules called “dyes”. These tagged MoABs that are specific to certain CDs can be detected with flow cytometers. This method is called Immunophenotyping. Besides cell characterization, immunophenotyping can be used for different applications due to the percentage of certain molecules expressed on cells such as in vitro diagnosis of hematological disorders like leukemias, lymphomas and myelodysplastic syndromes and activation state of cells.

2.3.1. Monoclonal Antibodies Used for Platelet Surface Immunophenotyping

In our study, we used five different MoABs for platelet detection and activation and two fluorescent bead kits for determining receptor-antibody binding capacity. The MoABs used were anti-CD41, anti-CD61, anti-CD42b, anti-CD62P and anti-CD63 antibodies.

2.3.1.1. cd41

CD41 antigen also known as Integrin Alpha-2b (ITA2b) and glycoprotein-IIb (GPIIb) is a transmembrane protein and the subunit of Glycoprotein IIb-IIIa (GPIIb-IIIa) complex expressed on Platelets. It has two chains linked by a disulfide bond known as GPIIb-α and GPIIb-β. CD41 non-covalently associates with CD61 Integrin-β3 which is the Glycoprotein-IIIa (GPIIIa) of the GPIIb-IIIa complex. GPIIb-IIIa complex is functional as fibrinogen receptor and in resting Platelets, binds to immobilized fibrinogen whereas in activated platelets this complex becomes specific to solubilized fibrinogen, fibronectin, vWF, vitronectin and thrombospondin. CD41 is also involved in platelet aggregation [27, 28]. CD41 is also expressed on megakaryocytes and a subset of $CD34^+$ hematopoietic stem cells. In healthy human beings, all platelets express CD41 on their surfaces; therefore anti-CD41 MoAB is used in Flow Cytometry to determine Platelets in heterogeneous populations. Mutations in the gene encoding CD41 can be the cause of Glanzmann Thrombasthenia [29].



ITGA2B protein | Computed by Wolfram|Alpha

Figure 2.9. GPIIb (CD41) Molecular Structure [30]

2.3.1.2. cd61

CD61 antigen, also known as GPIIIa and Integrin-β3 is the GPIIIa subunit of GPIIb-IIIa fibrinogen receptor complex expressed on the surface of platelets. By its own however, CD61 is also associates with Integrin-αV (CD51) to form vitronectin receptor. Platelets of healthy human beings all synthesize CD61 on their surfaces therefore anti-CD61 MoAB is also a surface marker for platelets to be used in flow cytometry for determining platelet populations. Mutations in the gene encoding CD61 can also be the cause of Glanzmann Thrombasthenia [29].

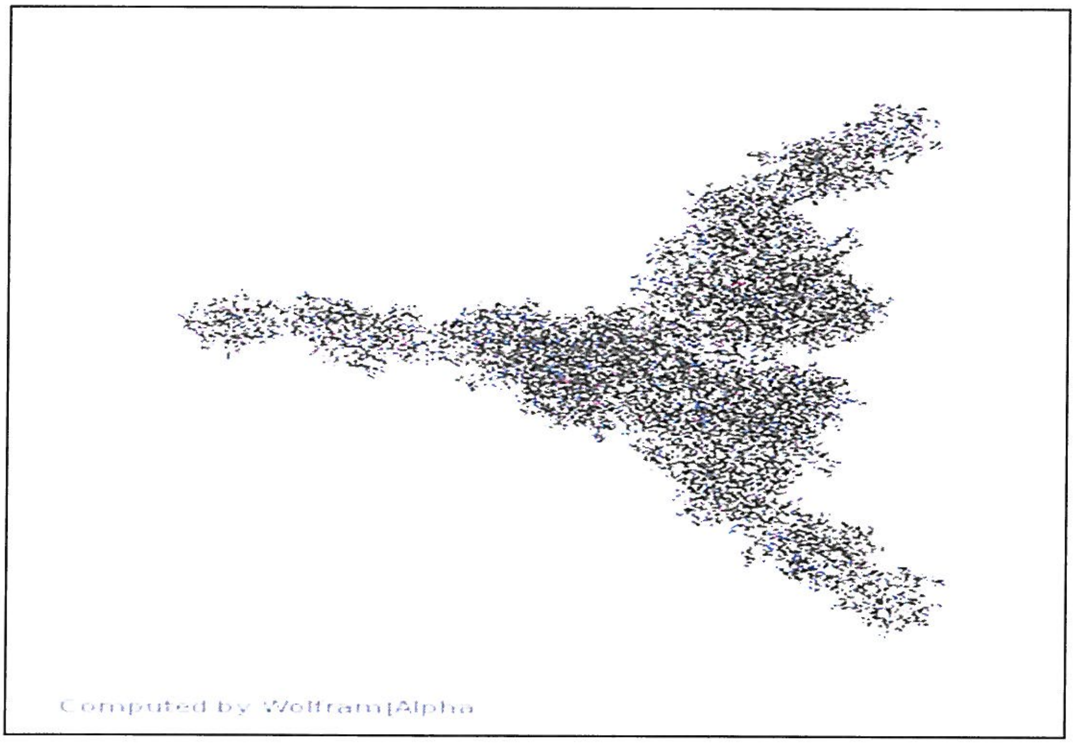


Figure 2.10. GPIIIa (CD61) Molecular Structure [31]

2.3.1.3. cd42b

CD42b, also known as glycoprotein-Ibα (GPIbα), is a glycoprotein expressed on Platelets, megakaryocytes and vascular and tosillar endothelial cells. CD42b with glycoprotein-Icα (GPIcα or CD42c) via disulphide bonds to form a functional Glycoprotein-Ib (GPIb) heterodimer. Functional CD42b forms a non-covalent complex with Glycoprotein-X (CD42a) and Glycoprotein-V (CD42d) to form CD42 membrane protein complex which becomes the receptor for vWF and Thrombin and mediates platelet adhesion to sub-endothelial upon tissue damage. Despite the complex formation, the active site for vWF binding is found on CD42b. Bernard-Soulier Thrombocytopenia occurs in the absence of CD42b on the surface of Platelets [32, 33]. Platelets of healthy individuals express CD42b on their surfaces; therefore in Flow Cytometry anti-CD42b MoAB is both a surface and an activation marker for platelets. In contrary to other activation markers, CD42b expression on platelets decreases by activation.

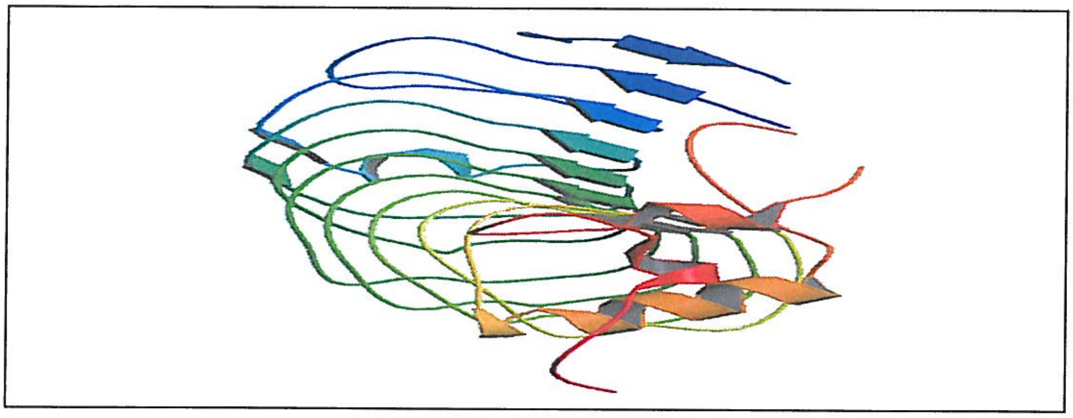


Figure 2.11. CD42b Molecular Structure [34]

2.3.1.4. cd62p

CD62P, also known as P-Selectin and Granule Membrane Protein-140 (GMP140) is an integral membrane protein expressed by platelets and endothelial cells, megakaryocytes and macrophages on the atherosclerotic regions [35] and expressed in humans by SELP gene [36]. CD62P is stored in the Weibel-Palade bodies of endothelial cells and α-granules of resting platelets. Upon activation of platelets and endothelial cells with agonists such as thrombin, CD62P is translocated to the membrane and expressed on the surface of activated endothelial cells and Platelets [37, 38]. In inflammation, when expressed on the surface of activated platelets and endothelial cells, CD62P becomes a receptor for circulating neutrophils and monocytes and recruits them to the inflammation site [39]. In Flow Cytometry, CD62P is a very effective activation marker for platelets thus anti-CD62P MoAB is used to differentiate resting and activated platelets in peripheral blood samples.

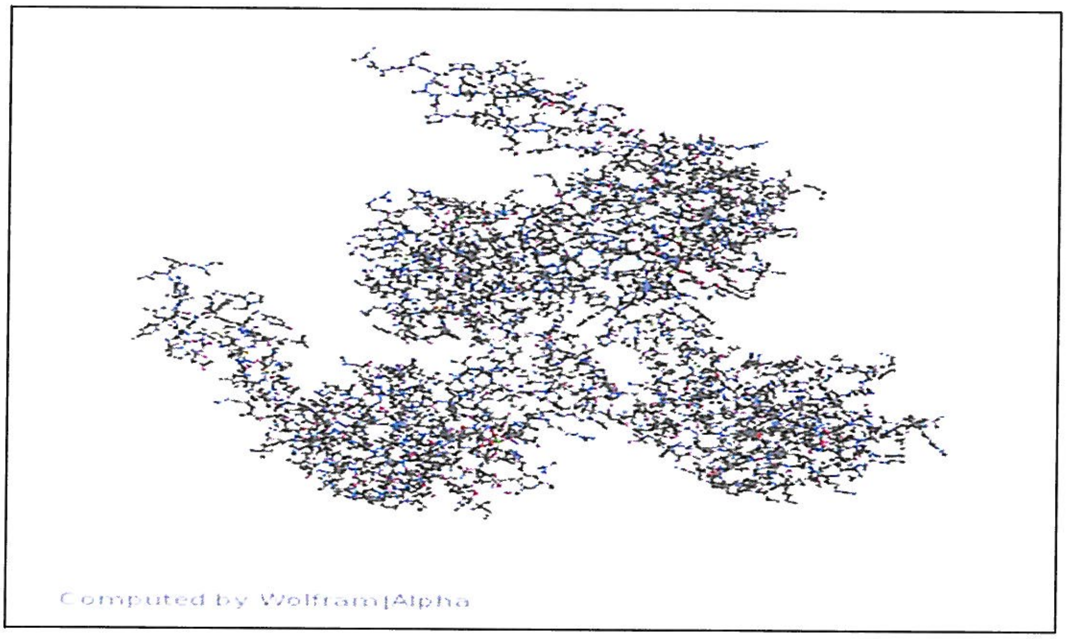


Figure 2.12. CD62P Molecular Structure [40]

2.3.1.5. cd63

CD63, also known as Lysosomal Membrane Associated Glico Protein 3 (LAMP3), is a member of Tetraspanins family [41]. CD63 and other tetraspanins CD9-CD81-CD82 form complexes with Integrin α3-β1 (VLA-3), phosphatidylinositol 4-kinase [42, 43], integrin-α6 (VLA-6) [44], CD11/CD18 and tyrosine kinase [45]. CD63 is first detected in the granules and surface of resting and activated platelets respectively [46]. Surface expression of CD63 is associated with Lysosomal secretion as resting neutrophils express CD63 in their azurophilic granules while activated neutrophils express CD63 on their surface [47]. Basophils also undergo the same expression patterns as neutrophils [48, 49]. In flow cytometry, CD63 is an effective activation marker for platelets. Therefore anti-CD63 MoAB is also used to discriminate resting and activated Platelets in peripheral blood samples.

3. IRON DEFICIENCY ANEMIA

3.1. BODY IRON

Iron is one of the most essential inorganic nutrients and it is required by every cell in human body. Iron plays crucial roles in multiple metabolic activities such as Ubiquinon redox reactions in electron transfer system, synthesis of metabolically active FeS proteins, enzymes that regulate oxygen metabolism (oxidases, peroxidases, catalases, and hydroxylases) but the most important role of body iron is the contribution to the synthesis of “Hemoglobin” protein found on erythrocytes that carries oxygen to cells [50].

3.1.1. Hemoglobin

Hemoglobin is the globular metalloprotein in RBCs responsible for oxygen transfer in all vertebrates. It is composed of four 3D myoglobin-like subunits coming together to form tetrameric Hemoglobin formation [51, 52] Hemoglobin takes up inhaled molecular oxygen from respitory tissues and organs and delivers it to somatic cells in order to be used in Electron Transfer System (ETS). In the last stage of ETS, molecular oxygen is reduced to CO_2 and H_2O and Hemoglobin then transports CO_2 back to the respitory tissues in order to be exhaled [53]. Figure 3.1 shows hemoglobin and myoglobin structures and mechanism of O_2 transport by hemoglobin

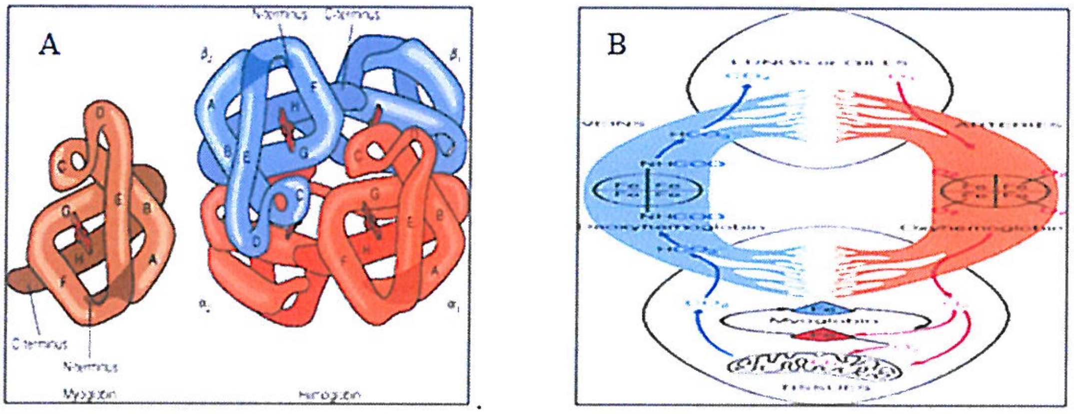


Figure 3.1. A - Hemoglobin and Myoglobin Structure
B - Oxygen Transport Mechanism by Hemoglobin [51]

In Hemoglobin protein, Iron is found in the form of Heme molecule which is the molecule composed of Fe (II) – Protoporphyrin IX complex. In this complex, Iron in Fe (II) form is chelated with the nitrogen atoms in the tetropyrrole ring system. Figure 3.2 shows the structure of Protoporphyrin IX and Heme molecule.

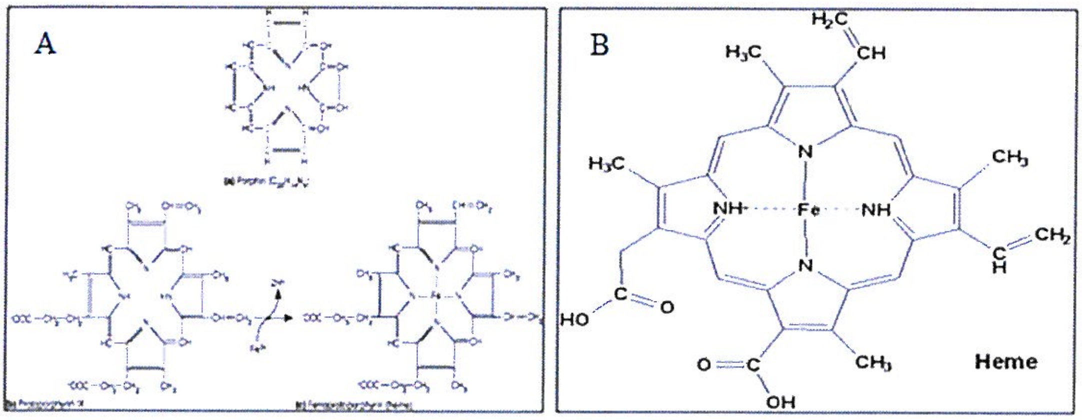


Figure 3.2. A - Protoporphyrin IX molecular structure. B - Heme Molecule [54]

Heme molecule is bound to a myoglobin-like subunit of hemoglobin in a non-covalent fashion by the hydrophobic space shown in Figure 3.3 where Fe (II) is coordinated octahedrally and thus has six binding sites. Four of these binding sites are nitrogens from the Protoporphyrin group and one of the remaining sites is bound to the nitrogen of a histidine aminoacid residing at the 93rd position of the F helix of the subunit. On the other side of Heme group, the remaining site is occupied with a water molecule. When hemoglobin takes up oxygen, it turns into oxyhemoglobin where O_2 makes a nucleophilic attack to the H_2O bound site and replaces water molecule via S_N1 mechanism. On the other side of oxygen lies another histidine molecule at the 64th position of the E helix of the subunit where the absolute positive charge of histidine increases the nucleophilic capability of oxygen, making it easier to bind to Heme group. After releasing oxygen to tissues, hemoglobin takes up CO_2 from cells with the same mechanism as oxygen [51, 55].

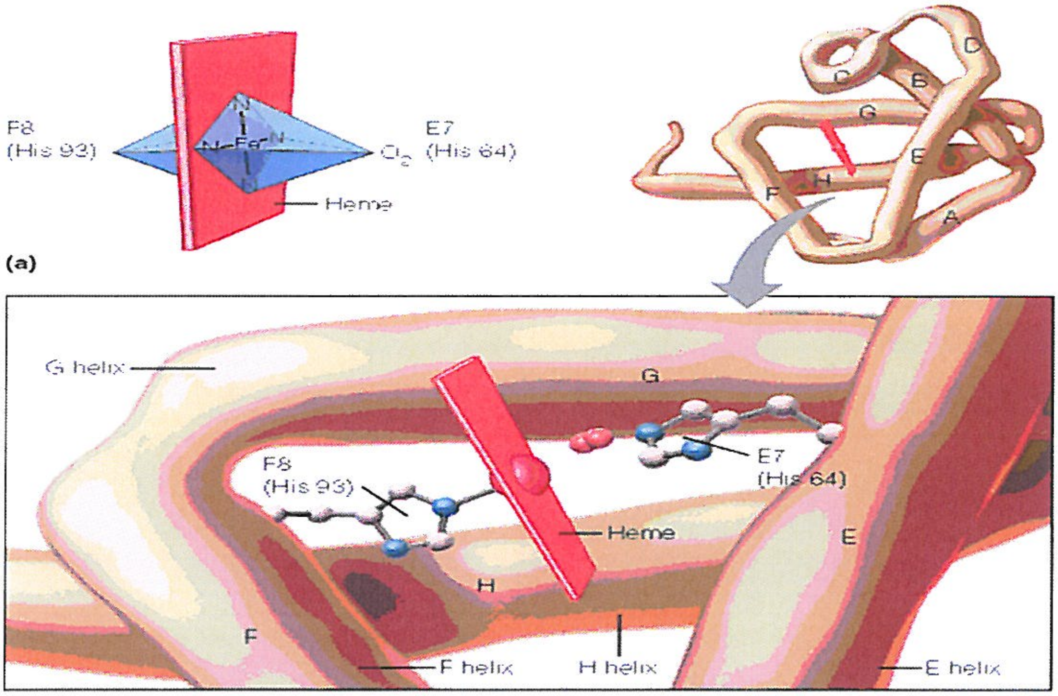


Figure 3.3. Coordination of Heme Group and Oxygen binding of Hemoglobin [51]

3.1.2. Iron Metabolism

Iron is absorbed into the body through duodenum and upper jejunum by Divalent Metal Transporter 1 (DMT1) protein [56, 28]. The average iron uptake in adults is 10 to 15 mgs per day and 1 to 2 mgs of total dietary iron is absorbed by duodenal erythrocytes. After absorption, ferric Fe^{+3} is reduced to ferrous Fe^{+2} by membrane-associated ferroreductase (DcytB) due to its oxidized state. Heme, on the other hand, is absorbed through unknown mechanisms [58] and Heme iron is released by inducible hemooxygenase 1 (HOX 1) [59]. Transportation of iron is conducted by basolateral iron exporter ferroportin [60, 61]. Major stores of iron in a mammalian body besides serum ferritin are liver, spleen, intestinal mucosa and bone marrow; kidneys, heart, skeletal muscle and brain are also minor sites of iron storage. Iron is stored in muscle, kidneys, brain liver and heart is used for myoglobin synthesis that would provide rapid oxygen for oxidative phosphorilation and cellular respiration and also for synthesis of FeS proteins. Iron stored in bone marrow is used for making new erythrocytes, while iron in intestinal mucosa is used for iron replacement and iron in spleen tissue is used for the survival of macrophages. Most of the iron is used for hemoglobin synthesis. Excess iron is stored in the form of ferritin [62] and transferrin [63]. Uptake of recycled iron into erythrocytes is regulated by TransFerrin Receptor protein 1 (TfR1). When acquired by erythrocytes, iron bypasses cytosole and is directly transported from endosomes to mitochondria via direct contact between these organelles. Iron then goes inside mitochondria via Mfrn1/SLC2537 (mitoferrin1//Solute carrier family 25 member 37) protein [64]. This process is accelerated with ABCB10 (ATP binding cassette super family B, member 10) protein [65]. When iron accumulation reaches limits, erythrocytes export this excess iron via ferroportin by expressing ferroportin Messenger RNA isoform b [66]. In a healthy person, daily need of iron is <10 % and is obtained by intestinal absorption. Rest of the iron is recycled internally by macrophages so that plasma iron undergoes turnover and is used repeatedly. When erythrocytes become aged or damaged, they are phagocytosed by macrophages and Heme is catabolysed by hemeoxygenase found in the phagosomal membranes of macrophages [67]. Figure 3.4 shows the altogether cellular iron metabolism [68].

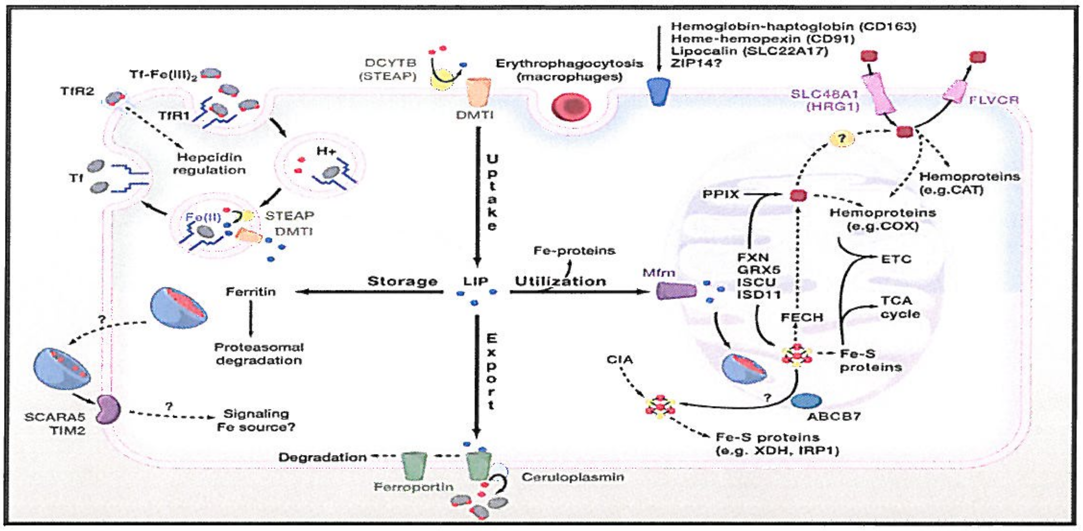


Figure 3.4. Cellular iron metabolism [68]

3.2. IRON DEFICIENCY

Iron Deficiency is the decrease of total body iron and occurs when the body iron supplies cannot meet the increasing requirements of iron. There are three stages of iron deficiency which are;

1) The decrease of iron storage without decrease in functional iron and is called Iron Depletion,

2) the lack of functional iron after the consumption of iron storage and limitations in iron based metabolically active compounds such as Hemoglobin and FeS proteins and the occurrence of Iron-Deficient Erythropoiesis,

3) Iron Deficiency Anemia due to further depletion of body iron [69]. There are several causes of iron deficiency such as chronic gastric and intestinal blood losses, intestinal parasites and worms, malnutrition and heavy menstrual cycle. Depletion of cellular iron due to one of these reasons initiates the release of stored iron to contribute to hemoglobin synthesis. When stored iron concentration decreases, iron absorption increases.

3.2.1. Iron Deficiency Anemia

When absorbed iron concentration cannot maintain the iron requirements and iron concentration decreases below minimum threshold, hemoglobin synthesis is reduced and erythrocyte functions become impaired. Erythrocytes become pencil like long and pale. Decreased hemoglobin concentration inhibits oxygen transportation to cells thus creating tissue damage. Continuous decrease of body iron concentration leads to iron deficiency anemia. If iron deficiency anemia becomes chronic, not only erythrocytes but also other metabolic activities are affected. Hair and nail thinning, bleeding in dental pulps are some of these symptoms. Iron dependent enzyme concentrations and immune system efficiency are also reduced. Especially infants with iron deficiency anemia may develop irreversible mental retardations [3].

Iron deficiency is very common, especially among women and in people who have a diet that is low in iron. The following groups of people are at highest risk for iron-deficiency anemia [2]:

- Increased iron loss (gastrointestinal)
    - Peptic ulcer (gastric, duodenal, Cameron's)
    - Cancer (gastric, esophageal, small bowel, colonic)
    - Vascular abnormalities (angiodysplasia, GAVE, HHT)
    - Inflammatory bowel disease
    - Colonic or gastric polyps
    - Gastritis, esophagitis
    - Parasitic infections (hookworm)
- Increased iron loss (nongastrointestinal)
    - Menorrhagia
    - Recurrent epistaxis
    - Urinary blood loss
    - Chronic intravascular hemolysis

  - Regular blood donation, phlebotomy
- Iron malabsorption
  - Coeliac disease
  - Previous gastrectomy
  - Achlorhydria and hypergastrinaemia
- Increased demand for iron
  - Adolescence
  - Pregnancy
  - Erythropoietin therapy
- Inadequate diet intake (vegetarians, vegans)

3.2.2. Diagnosis

In order to diagnose a patient with iron deficiency anemia (IDA), first a whole blood count is done to assess the hemoglobin concentration (HGB g/dl), Mean Corpuscular Volume (MCV) which is the mean volume of erythrocytes, Hematocrit (HCT) and mean hemoglobin mass (MCH). Second, serum ferritin levels and total iron binding capacity (TIBC) measurement is necessary to differentiate IDA from other anemias. Although low MCV, HCT and MCH levels are significant in IDA, low ferritin and TIBC assessment is necessary for full diagnosis. Also low reticulocyte counts after peripheral smear is discriminative [3].

3.2.3. Relationship Between Iron Deficiency Anemia and Platelets

The mechanism causing thrombocytosis in iron deficiency anemia is unknown. According to Akan et al thrombopoietic cytokines (thrombopoietin (Tpo), IL-6, IL-11) had no effect on reactive thrombocytosis seen in IDA [70]. In another study, it was reported that amino acid sequence homology of Tpo and erythropoietin (Epo) may explain the platelet elevations in IDA [71]. On the other hand, there are two reports suggesting negative participation of Epo and Tpo [72, 64].

Limited number of studies about effect of iron deficiency on platelet counts and functions has somehow conflicting results. In one series, the average platelet count was found to be twice the controls [74]. In some other studies it was reported that IDA may be associated with thrombocytopenia [75, 76]. In a recent study, performed by Kadikoylu et al, thrombocytosis and thrombocytopenia were detected in 27,9% and 2,3% of the patients respectively [77]. It has been reported that IDA may cause platelet aggregation dysfunction in a few studies [78, 41]. On the other hand there are reports claiming that reactive thrombocytosis secondary to IDA may cause stroke [80, 81].

In our study, we have analyzed the activation and functions of platelets and assessed the antibody binding capacities of platelet surface receptors in IDA patients and healthy volunteers using flow cytometry. Aggregation and activation by certain platelet activators like Adenosine Di-Phosphate (ADP) and Epinephrine were not used in our study to determine the effects of IDA on platelet activation due to the fact that native status of platelets in IDA was our primary aim.

In light of all of the above background information, we have aimed to explore the status of platelets in IDA patients and enlighten the thrombosis seen frequently in these patients in relation to platelet activation.

4. MATERIALS AND METHODS

4.1. PATIENT POPULATION

35 women diagnosed with IDA and 18 healthy women volunteers with same age groups without IDA at Yeditepe University Hospital Hematology Department Outpatient Clinics were selected for our study. For all of the patients, serological tests for diagnosis were performed by Biochemistry Laboratory of Yeditepe University Hospital in standard procedures. IDA diagnosis was made by determining Hemoglobin (HGB), Hematocrit (HCT), Mean Corpuscular Volume (MCV) and serum Ferritin levels. Platelets were counted to determine the presence of thrombocytosis or thrombocytopenia. Criteria for inclusion to study were to be in the age of fertility between 16-50, to have IDA caused by menstrual cycle and/or malnutrition, to be newly diagnosed with IDA and without usage of any drugs for IDA treatment. Criteria for healthy control selection was to be in fertile age between 16-50 years, not to be diagnosed with IDA, without any continuous usage of anti-inflammatory drugs or steroids, not having infection and/or inflammation during blood collection and not having chronic diseases such as diabetes mellitus or chronic hepatitis. Patients and healthy controls suitable to these conditions were included in the study and consent forms were signed by all participants prior to the study. Serological tests for HGB, HCT, MCV and Ferritin levels were also applied to the control group samples. This study was approved by the Yeditepe University Hospital Clinical Research Evaluation Committee. (Acceptance date 21st of November 2012 approval No: 249).

4.2. LABORATORY WORKFLOW CHART

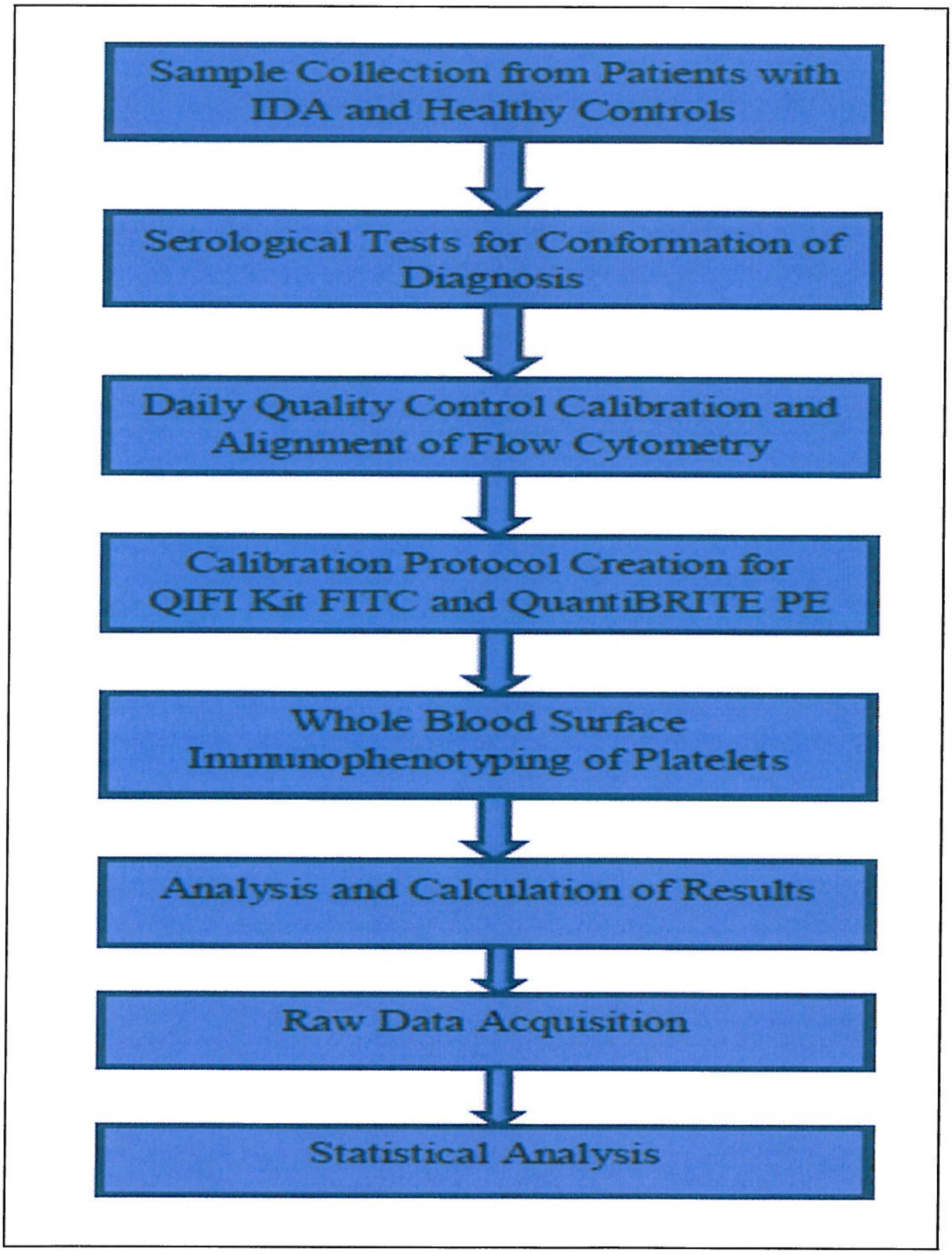


Figure 4.1. Workflow Chart for Laboratory Procedures

4.3. MATERIAL USED IN THE STUDY

Table 4.1. Every material used in the study

Material	Manufacturer	Catalog Number	Purpose of Use
Anti-Human CD41-FITC	e-Bioscience	11-0419-73	Platelet Marker
Anti-Human CD61-FITC	e-Bioscience	11-0619-73	Platelet Marker
Anti-Human CD42b-PE	e-Bioscience	12-0429-42	Platelet Marker and Activation Marker
Anti-Human CD62P-PE	e-Bioscience	12-0628-73	Platelet Activation Marker
Anti-Human CD63-PE	Beckman-Coulter	IM1914U	Platelet Activation Marker
QIFI Surface Receptor Quantification Kit FITC	DAKO	K007811	Surface Receptor Quantification for FITC Conjugated MoABs
QuantiBRITE PE Fluorescence Quantification Kit	Becton-Dickinson	340495	Surface Receptor Quantification for PE conjugated MoABs

Table 4.1. Every material used in the study (Continue)

FC-500 Cytomics Flow Cytometer	Beckman-Coulter	AJ31198	Experimentation and Analysis
12mm x 75mm Polystyrene Falcon Tubes	Becton-Dickinson	352052	Sample Preparation
Paraformaldehyde	Fluka	76240	Preparation of 1% Paraformaldehyde Solution
Isotonic Flow Sheath Fluid	Beckman-Coulter	8546859	Sample Preparation

4.4. WHOLE BLOOD SURFACE IMMUNOPHENOTYPING OF PLATELETS

Immunophenotyping of platelets were performed with whole blood samples. Depending on studies of White et. al [82] and latest studies by Oliveira et. al [83] and Okano et. al [84] that morphologies of platelets were unchanged and adhesion and spontaneous activation of platelets were inhibited, when whole blood samples were collected into Vacutainer tubes with Tri-Potassium Ethylene Diamine Tetra Acetate (K_3EDTA) as anticoagulant. In order to study the activation effects of IDA on platelets, Platelet Rich Plasma (PRP) separation was avoided because of the self activation of platelets by centrifugation and surface immunophenotyping of platelets were studied with whole blood samples. At blood collection phase, the first two mL of blood that contains activated platelets were taken into dry tubes for serum ferritin analyses thus activated platelet formation was eliminated upon blood collection. Vortexing, shaking and centrifugation was avoided in order to prevent spontaneous platelet activation. Samples were studied at 30 minutes after collection. Acquisition protocols for flow cytometric analysis were created using the voltage, gain, discriminator and color compensation parameters acquired from the calibration protocols of QIFI kit FITC (DAKO, Denmark) and QuantiBRITE PE (BD Biosciences, USA) respectively. And immunophenotyping experiments were conducted using these new protocols. Because platelets are two to three microns in size, logarithmic FS x SS graphics and decreased laser discriminator were used in order to expand the scale and observe platelet population without the interference of erythrocytes and leukocytes. Figure 4.1 shows the identification of platelets in whole blood sample. In order to solely asses the activation effects of IDA on platelets, platelet activating agents such as Adenosine Diphosphate (ADP), collagen and epinephrine were not used.

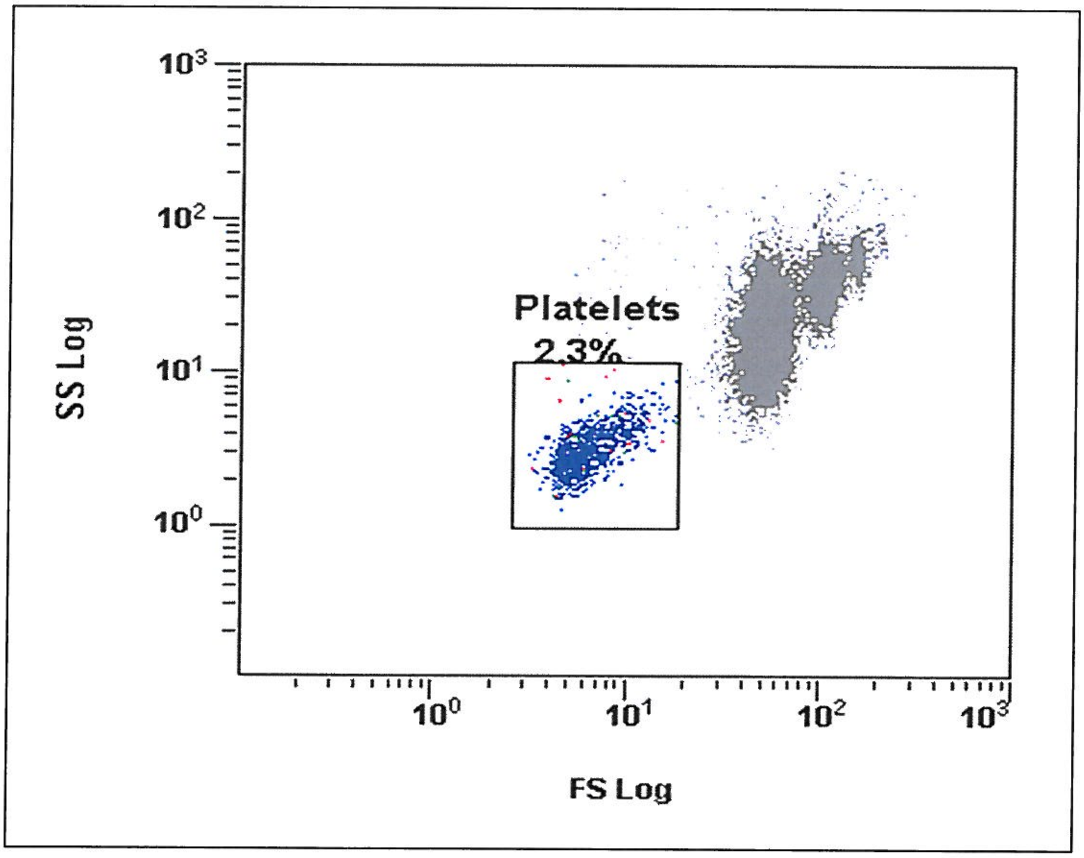


Figure 4.2. Identification of Platelets in Whole Blood

4.4.1. Antibodies Used For Surface Immunophenotyping of Platelets

Table 4.2. Antibodies used in surface immunophenotyping of platelets

Antigen	Function	MoAB	Role in Immunophenotyping
CD41	Glycoprotein – Subunit of the fibrinogen binding receptor complex GPIIb-IIIa	eBioscience Mouse - Anti Human CD41 FITC	Identification of Platelet populations in whole blood.
CD61	Integrin Protein – Subunit of the fibrinogen binding receptor complex GPIIb-IIIa	eBioscience Mouse - Anti Human CD61 FITC	Identification of Platelet populations in whole blood.
CD42b	Glycoprotein – Von Willebrand Factor binding receptor	eBiioscience Mouse – Anti Human CD42b PE	Identification and activation marker of platelets.
CD62P	Mediator of Platelet – Leukocyte interaction and platelet adhesion protein	eBioscience Mouse – Anti Human CD62P PE	Platelet activation marker
CD63	Member of the Tetraspanins Family	Beckman Coulter Mouse – Anti Human CD63 PE	Platelet activation marker

Antibodies used for immunophenotyping experiments were CD41-FITC (eBioscience USA), CD61-FITC (eBioscience USA), CD42b-PE (eBioscience USA), CD62P-PE (eBioscience USA) and CD63-PE (Beckman Coulter USA). Five µL of whole blood was used in order to minimize erythrocyte and leukocyte aggregation. All antibodies were titrated between five µL and ten µL to find the suitable staining concentration and mean channel values. Figure 4.2 shows antibody titration of anti-CD41-FITC staining with five and ten µL of MoAB. And according to titration results, five µl of antibodies were used for surface staining. QIFI kit FITC (Dako, Denmark) and QuantiBRITE PE (BD Biosciences, USA) quantification kits were used for determining antigen binding capacities of FITC and PE conjugated antibodies respectively. Antibodies were pipetted into the 15 mm x 75 mm falcon tubes for flow cytometer in the order of CD41 FITC – CD61 FITC for QIFI Kit protocol and CD42b PE – CD62P PE – CD63 PE for QuantiBRITE protocol. Five µl of whole blood was added to each tube with antibodies and mixed with gentle pipetting in order to avoid platelet activation and samples were incubated for 20 minutes at room temperature in dark. An unstained tube containing only blood sample for every protocol was used to eliminate autofluorescence. After incubation, one ml of cold 1% Paraformaldehyde was added onto all samples to avoid spontaneous platelet activation. Samples were run under FC-500 Cytomics Flow Cytometer (Beckman Coulter USA) with CXP Acquisition Software v2.2 (Beckman Coulter USA). 100000 events were acquired in each tube and platelets were gated in order to observe fluorescence signals specific to platelets. Results were analyzed using CXP Analysis Software v2.2 (Beckman Coulter USA).

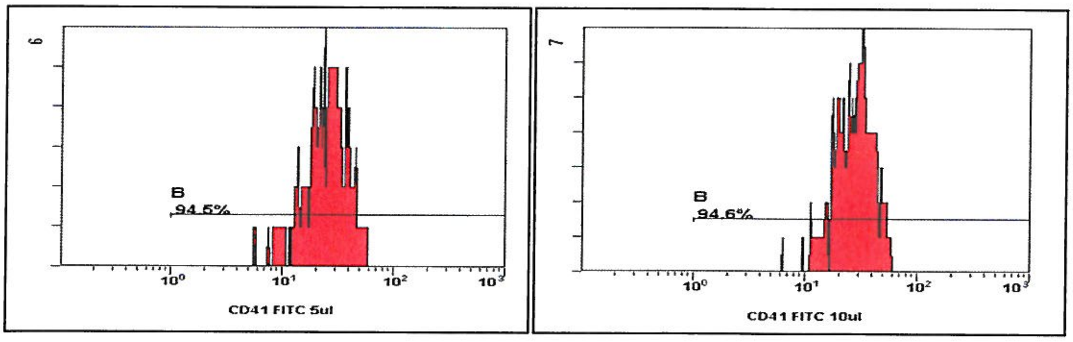


Figure 4.3. Antibody Titration for CD41 FITC

4.5. FLOW CYTOMETER CALIBRATION WITH QIFI KIT AND QUANTIBRITE FLUORESCENT BEADS

For evaluation of platelet surface and activation markers, flow cytometry system in Yeditepe University Hospital Stem Cell Laboratory was used. To ensure that our results were accurate, flow cytometry system was calibrated on daily basis with Flow Check (Beckman Coulter USA) and Flow Set (Beckman Coulter USA) fluorescent calibration beads. To assess the surface receptor numbers and antibody binding capacities of surface platelet receptors, QIFI kit and QuantiBRITE fluorescent quantification kits were used for FITC and PE conjugated MoABs respectively. In order to determine these parameters, FC-500 Cytomics Flow Cytometer was calibrated for accurately analyzing bead populations and calculating receptor numbers and binding capacities. Two separate calibration protocols were created for QIFI kit FITC and QuantiBRITE PE fluorescence beads prior to experimentation.

Figure 4.4. Beckman-Coulter FC-500 Cytomics Flow Cytometer

4.5.1. QIFI Kit Calibration

For calibrating FC-500 against QIFI Kit FITC, 100μl beads from setup vial with high number of bead concentration and 100 μl beads from calibration vial with different numbers of bead concentrations were put into two different 12mm x 75mm Falcon Tubes for Flow Cytometer (BD Biosciences USA). Three ml of isotonic flow sheath fluid (Beckman Coulter USA) were added to the tubes and after vortexing tubes were centrifuged at 300 x g for five minutes. Supernatants were discarded and approximately 50 μl of fluid was left at the bottom of each tube. FITC conjugate from vial 3 was diluted in isotonic sheath fluid at 1:50 concentration and 100μl of diluted FITC conjugate were added to each tube. Tubes were vortexed and incubated in dark at +4°C for 45 minutes. After incubation, beads were resuspended with 500 μl of isotonic sheath fluid and analyzed on FC-500 cytomics with acquisition software v2.2. Figure 4.4 shows the logarithmic histogram of FL1/FITC channel as x-mean values of histogram peaks refers to descending number of beads from bright to dim. X-Mean values of FITC conjugated MoABs on patient samples were compared against X-Mean values of the calibration beads for surface receptor quantification and antibody binding capacity.

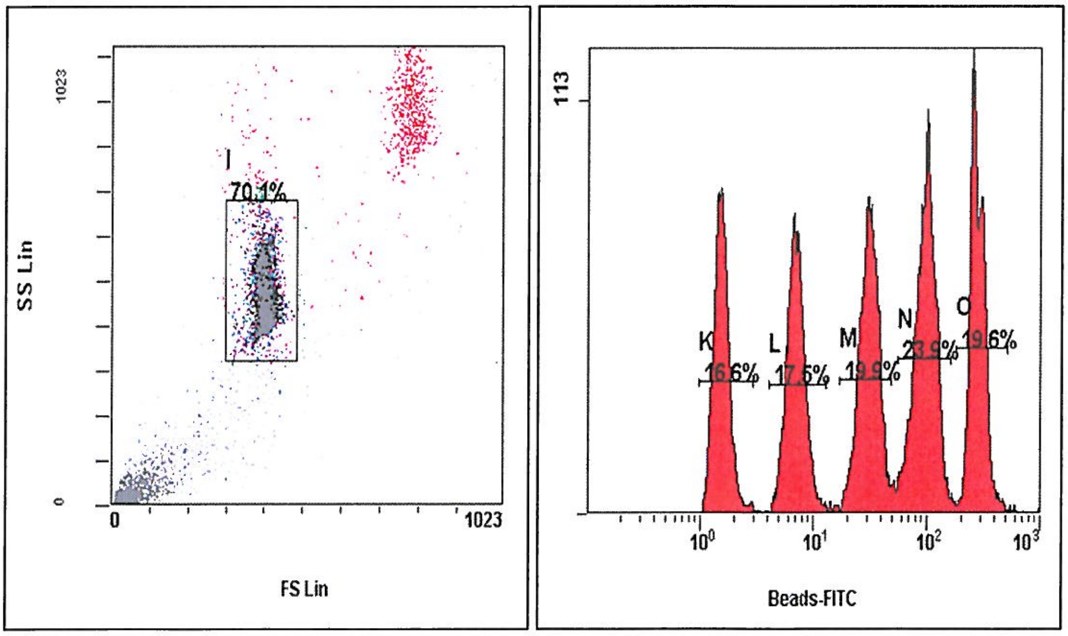


Figure 4.5. Calibration graphics of QIFI Kit FITC

4.5.2. QuantiBRITE Calibration

For calibrating FC-500 Cytomics against QuantiBRITE PE Fluorescent quantification kit, lyophilized beads in QuantiBRITE PE tube were reconstituted with 500µl of isotonic flow sheath fluid. Acquisition protocol was created for QuantiBRITE PE using FS x SS graphic to determine the beads and FL2/PE channel to determine the histogram peaks with declining bead concentrations from bright to dim. Voltage adjustments were made until four different peaks were observed on FL2 channel. Results were analyzed using CXP software v2.2. Figure 4.5 shows the logarithmic histogram of FL2/PE channel as x-mean values of histogram peaks refers to descending number of beads from bright to dim. X-Mean values of PE conjugated MoABs on patient samples were compared against X-Mean values of the calibration beads for surface receptor quantification and antibody binding capacity.

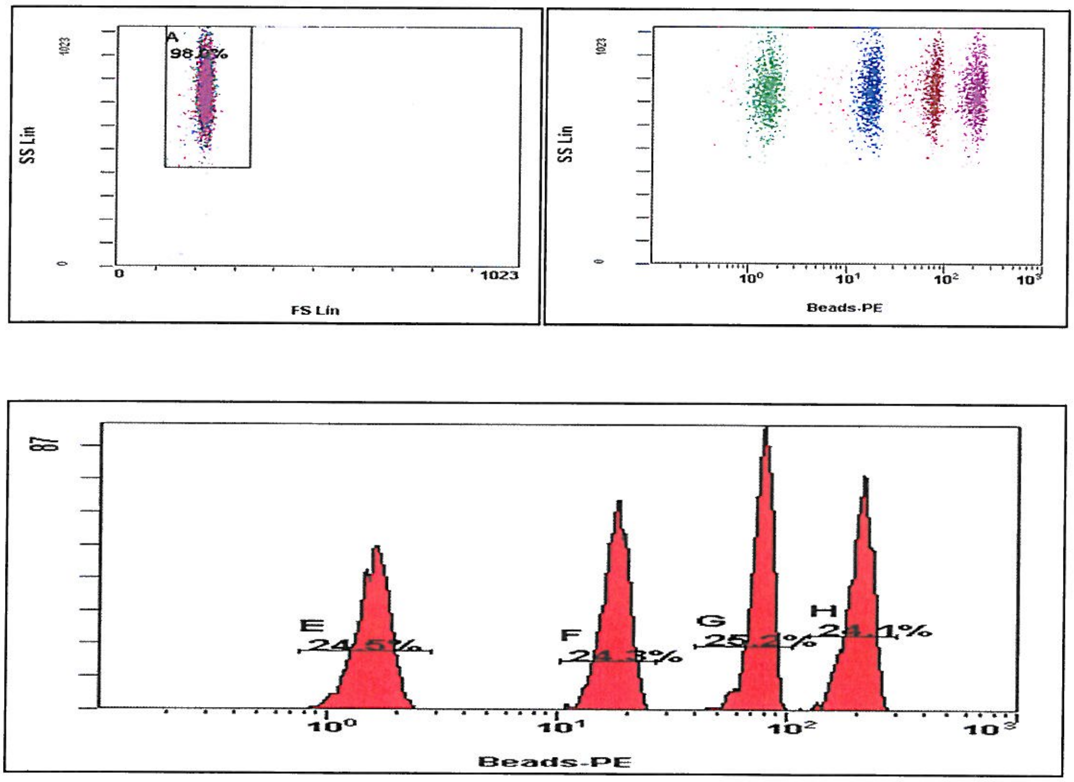


Figure 4.6. Calibration results of QuantiBRITE PE Fluorescence Quantification Kit

4.5.3. Calculation of Antibody Binding Capacities

Calculation of antibody binding capacities from QIFI Kit FITC and QuantiBRITE PE depends on the comparison of x-mean values of patient MoABs and bead concentrations of kits. In both QIFI Kit FITC and QuantiBRITE PE, x-mean values of different peaks from bright to dim corresponds to a certain number of descending number of beads. These bead concentrations were used as standards and in patient samples, x-mean results of MoABs were compared to the x-mean values of standards and depending on the x-mean result of patient MoAB, the neighboring standard was used as most similar and calculation was based on the ratio/proportion of patient and standard x-mean result and corresponding bead concentration depending on the formula:

$$\text{ABC} = \frac{\text{MoAB x-mean} \times \text{Corresponding Bead Concentration to Neighboring Standart x-mean}}{\text{Neighboring Standard x-mean}} \quad (4.1)$$

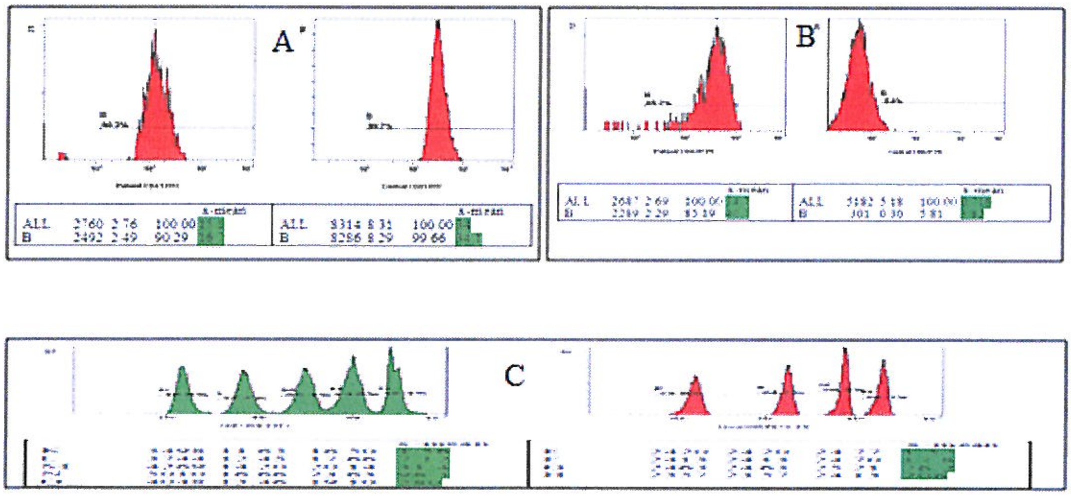


Figure 4.7 A - The x-mean values of CD41 FITC of a patient and a control sample

B - CD62P PE x-mean results of a patient and a control sample

C - x-mean values of QIFI Kit FITC and QuantiBRITE PE

4.6. STATISTICAL ANALYSIS

Statistical analysis was made by using Mann-Whitney U Rank Sum test for independent unpaired samples. Both SPSS Software version 20 (International Business Machines IBM, USA) and MedCalc Software version 12.2.1 (MedCalc Software, Belgium) were used to evaluate the same statistical data and results were compared between two softwares. Statistical analysis was applied to HGB, HCT, MCV and Ferritin values; Erythrocyte and platelet counts, and percentage and ABCs of CD41, CD61, CD42b, CD62P and CD63 molecules between patient and control samples. Confidence interval was 95% and $P < 0.05$ values were considered as significant.

5. RESULTS

5.1. PATIENT AND CONTROL DEMOGRAPHIC RESULTS

In order to fully diagnose female patients with IDA, patient peripheral blood samples were tested for RBC counts, HGB concentrations, HCT percentages, MCV values and Ferritin concentrations. Patients appropriately diagnosed with heavy menstrual cycle and malnutrition dependent IDA were selected for the study. Healthy controls without IDA were also tested for the same parameters. Because our study was conducted on platelets, platelet counts were also tested in both patient and control blood samples. Table 5.1. and Table 5.2. show the demographic results of IDA patient and Control blood samples respectively.

Table 5.1. Patient Demographic Results

	RBC 10^6 cells/µL	HGB g/dL	HCT %	MCV fL/cell	PLT 10^3 cells/µL	Ferritin ng/mL	Age
Patient 1	2.63	6.8	21.9	83.3	183	2.04	25
Patient 2	3.91	7.2	25.2	64.5	322	3.80	50
Patient 3	4.39	10.5	32.8	74.7	273	5.75	18
Patient 4	4.4	9.7	32.2	73.2	287	10.2	41
Patient 5	4.31	8.7	29.5	68.4	281	2.64	31
Patient 6	4.25	8.3	28.2	66.4	220	4.22	36
Patient 7	3.99	6.7	23.9	59.9	237	1.25	43
Patient 8	4.71	8.5	26.9	62.8	346	3.85	34
Patient 9	4.75	8.5	30.8	64.8	456	5.50	26
Patient 10	4.72	8.9	30.5	64.6	368	1.20	53
Patient 11	3.01	6.9	23.2	77.1	499	1.15	37
Patient 12	3.87	10.1	32.1	82.9	339	2.85	41

Table 5.1. Patient Demographic Results (Continue)

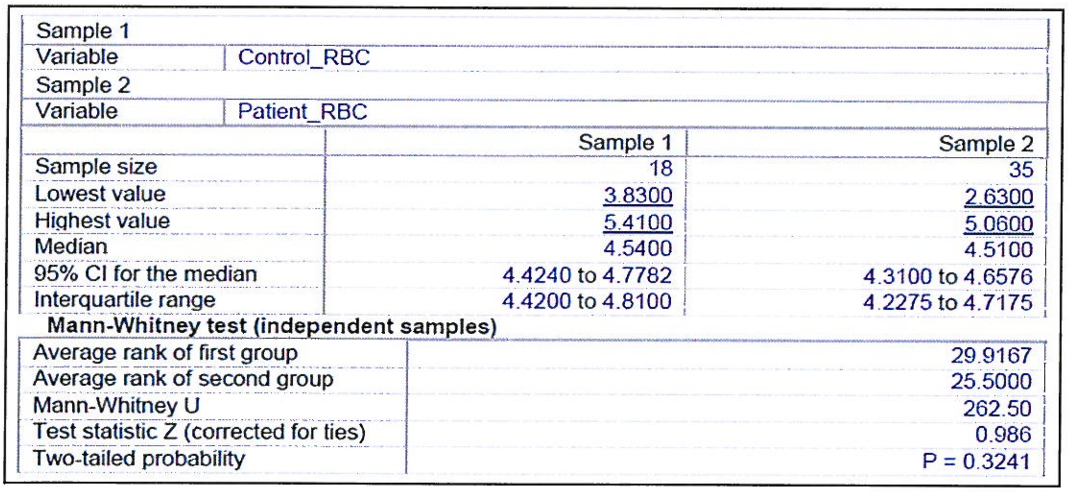
Patient 13	4.6	11.6	35.6	77.4	299	5.78	38
Patient 14	4.31	8.9	30.2	70.1	319	6.24	40
Patient 15	4.94	12.1	38.2	77.3	134	7.05	32
Patient 16	4.94	8.5	30.8	62.3	338	2.28	27
Patient 17	4.67	10.3	33.7	72.2	251	6.7	41
Patient 18	4.99	9.8	33.3	66.7	244	2.83	21
Patient 19	4.37	11.8	37	84.7	184	6.11	36
Patient 20	4.18	10.7	33.6	80.4	346	4.64	23
Patient 21	4.65	10.3	33.3	71.6	378	3.78	39
Patient 22	4.54	9.4	32.7	72	224	73.79	23
Patient 23	4.52	8.4	28.2	62.4	193	3.87	32
Patient 24	3.5	5.4	22	62.9	183	2.34	36
Patient 25	4.87	10	35.1	72.1	147	2.74	36
Patient 26	4.78	11	35.1	73.4	313	2.98	25
Patient 27	4.25	7.7	27	63.5	365	2.61	24
Patient 28	4.21	7.29	27.3	64.8	295	2.17	25
Patient 29	4.22	10.6	33.9	80.3	276	3.85	39
Patient 30	4.72	8	27.8	58.9	440	1.84	39
Patient 31	4.66	8	29.1	62.4	322	87.16	40
Patient 32	5.06	8.2	30.2	59.7	189	4.24	25
Patient 33	4.51	9.2	30.9	68.5	439	1.12	48
Patient 34	4.6	7.8	27.4	59.6	502	4.75	41
Patient 35	4.48	8.7	30.5	68.1	274	3.78	38

Table 5.2. Healthy Control Demographic Results

	RBC 10^6 cells/μL	HGB g/dL	HCT %	MCV fL/cell	PLT 10^3 cells/μL	Ferritin ng/mL	Age
Control 1	3,94	11,7	36,3	92,1	221	15,14	25
Control 2	4,42	11,7	35,9	81,2	294	30	35
Control 3	4,43	13,6	40	90,3	283	20,28	21
Control 4	4,84	13,4	41	84,7	446	15,78	23
Control 5	5,41	12,7	39,9	73,8	329	22,47	27
Control 6	4,55	13	41,3	90,8	252	23,5	32
Control 7	4,53	13,1	38,5	85	295	30,2	22
Control 8	4,52	13,3	40,2	88,9	322	51,64	22
Control 9	5,06	14,8	43,6	86,2	301	12,97	23
Control 10	3,83	12,6	37,4	97,7	265	20,81	19
Control 11	4,81	13,6	39,4	81,9	276	18,6	30
Control 12	3,91	11,8	35,6	91	199	19,05	23
Control 13	4,72	13,7	40,6	86	253	12,47	30
Control 14	4,48	12,7	38,7	86,4	290	22,43	28
Control 15	4,61	13,2	38,3	83,1	267	38,01	38
Control 16	4,73	13,7	41,3	87,3	301	10,12	28
Control 17	4,21	12,4	36,8	87,4	286	12,86	33
Control 18	5,09	14,4	44	96,4	197	20,52	28

5.1.1. Red Blood Cell Counts

RBC counts were compared between IDA patients and healthy controls blood samples. According to the statistical analysis, there were no significant differences between IDA patient [n=35] and healthy control [n=18] RBC counts in the form of 10^6 Cells/µl [P=0,3241]. As it was shown in Figure 5.1. the mean values of RBC counts of IDA patients and healthy controls were 4.386 x $10^6 \pm 0.09$ cells/µL and 4.56 x $10^6 \pm 0.098$ cells/µL respectively.



Sample 1	
Variable	Control_RBC
Sample 2	
Variable	Patient_RBC

	Sample 1	Sample 2
Sample size	18	35
Lowest value	3.8300	2.6300
Highest value	5.4100	5.0600
Median	4.5400	4.5100
95% CI for the median	4.4240 to 4.7782	4.3100 to 4.6576
Interquartile range	4.4200 to 4.8100	4.2275 to 4.7175

Mann-Whitney test (independent samples)

Average rank of first group	29.9167
Average rank of second group	25.5000
Mann-Whitney U	262.50
Test statistic Z (corrected for ties)	0.986
Two-tailed probability	P = 0.3241

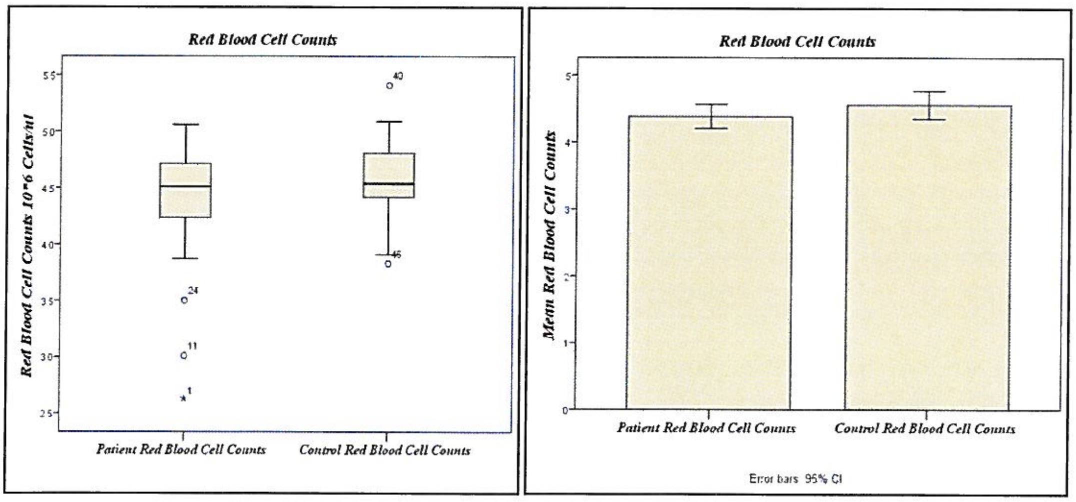
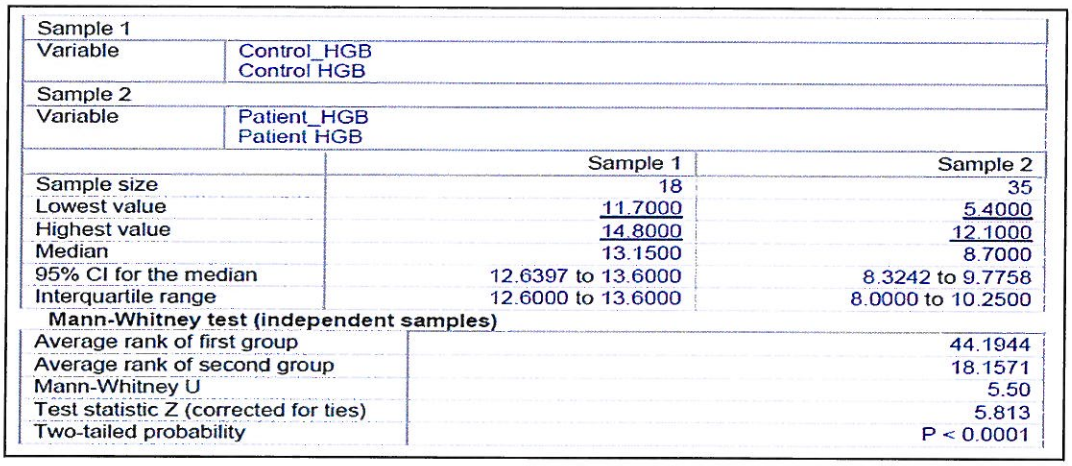


Figure 5.1. Statistical analysis for RBC counts in
IDA Patients and Healthy Controls

5.1.2. Hemoglobin Levels

Decreased hemoglobin level is one of the differential parameters to diagnose IDA. As it is seen in Figure 5.2., statistical analysis of Hemoglobin levels showed significant differences between IDA patients and healthy controls [P<0.0001] where mean levels of HGB in IDA patients and healthy controls were 8.98 ± 0.27 g/dL and 13.07 ± 0.2 g/dL respectively.



Sample 1	
Variable	Control_HGB Control HGB
Sample 2	
Variable	Patient_HGB Patient HGB

	Sample 1	Sample 2
Sample size	18	35
Lowest value	11.7000	5.4000
Highest value	14.8000	12.1000
Median	13.1500	8.7000
95% CI for the median	12.6397 to 13.6000	8.3242 to 9.7758
Interquartile range	12.6000 to 13.6000	8.0000 to 10.2500

Mann-Whitney test (independent samples)

Average rank of first group	44.1944
Average rank of second group	18.1571
Mann-Whitney U	5.50
Test statistic Z (corrected for ties)	5.813
Two-tailed probability	P < 0.0001

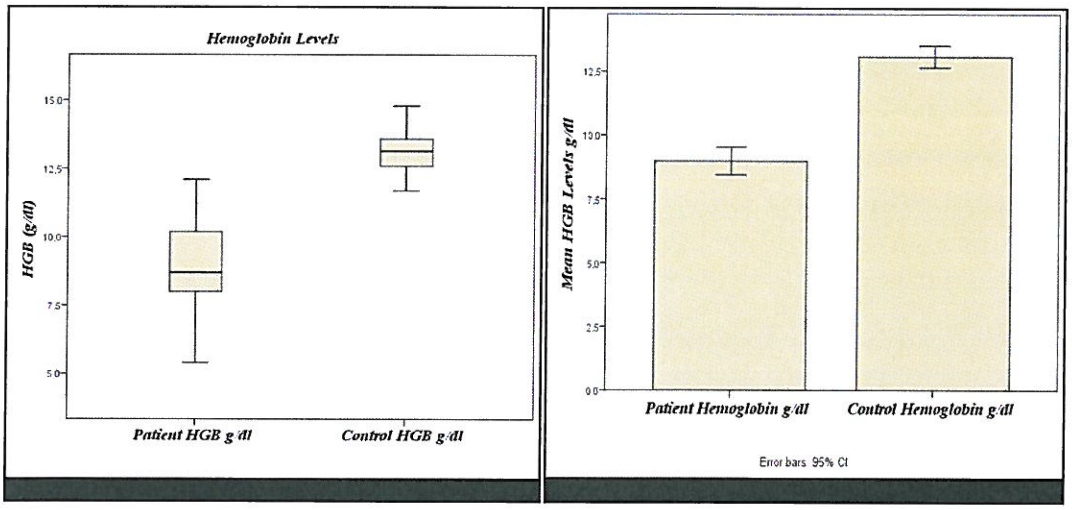
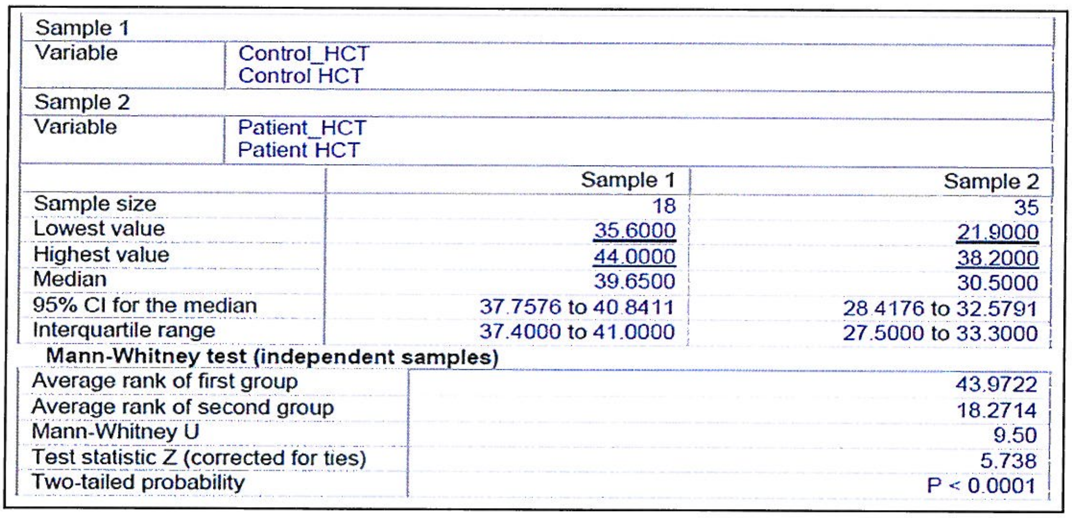


Figure 5.2. Statistical Analysis of HGB levels between IDA patients and healthy controls

5.1.3. Hematocrit Percentage

Hematocrit percentage was also analyzed as a diagnostic marker for IDA. Figure 5.3 shows that, IDA patients had a mean HCT percentage of 30.28 ± 0.69% whereas healthy controls had 39,37 ± 0.57% and statistical analysis showed significant differences between patient and control HCT percentages [P<0.0001].



Sample 1	
Variable	Control_HCT Control HCT

Sample 2	
Variable	Patient_HCT Patient HCT

	Sample 1	Sample 2
Sample size	18	35
Lowest value	35.6000	21.9000
Highest value	44.0000	38.2000
Median	39.6500	30.5000
95% CI for the median	37.7576 to 40.8411	28.4176 to 32.5791
Interquartile range	37.4000 to 41.0000	27.5000 to 33.3000

Mann-Whitney test (independent samples)

Average rank of first group	43.9722
Average rank of second group	18.2714
Mann-Whitney U	9.50
Test statistic Z (corrected for ties)	5.738
Two-tailed probability	P < 0.0001

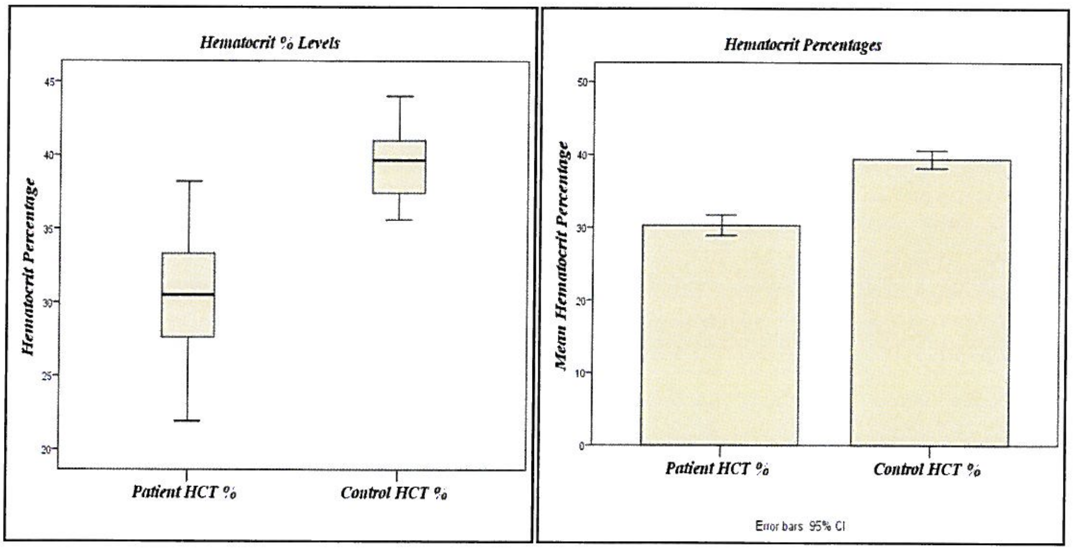
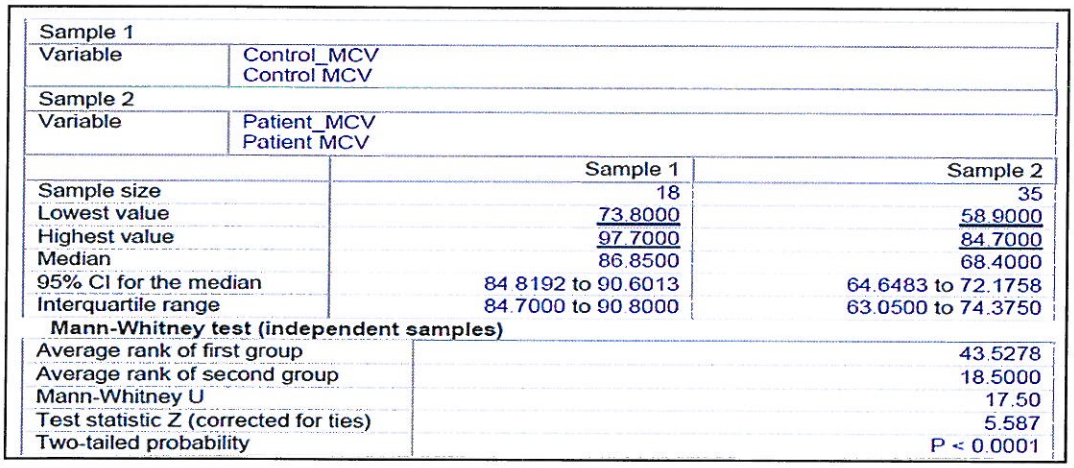


Figure 5.3. Statistical analysis between Patients and Controls HCT Percentages

5.1.4. Mean Corpuscular Volume [MCV] Results

MCV is used along with HGB, HCT, RBC and Ferritin results to determine whether a patient has IDA or not. Figure 5.4 shows that patients with IDA had significantly lower MCV values than healthy controls where IDA patients and healthy controls had mean MCV values of 69.54 ± 1.26 fL/cell and 87.2 ± 1.32 fL/cell respectively. Statistical analysis also showed a significant difference in MCV values in patients and controls [P<0.0001].



Sample 1	
Variable	Control_MCV Control MCV

Sample 2	
Variable	Patient_MCV Patient MCV

	Sample 1	Sample 2
Sample size	18	35
Lowest value	73.8000	58.9000
Highest value	97.7000	84.7000
Median	86.8500	68.4000
95% CI for the median	84.8192 to 90.6013	64.6483 to 72.1758
Interquartile range	84.7000 to 90.8000	63.0500 to 74.3750

Mann-Whitney test (independent samples)

Average rank of first group	43.5278
Average rank of second group	18.5000
Mann-Whitney U	17.50
Test statistic Z (corrected for ties)	5.587
Two-tailed probability	P < 0.0001

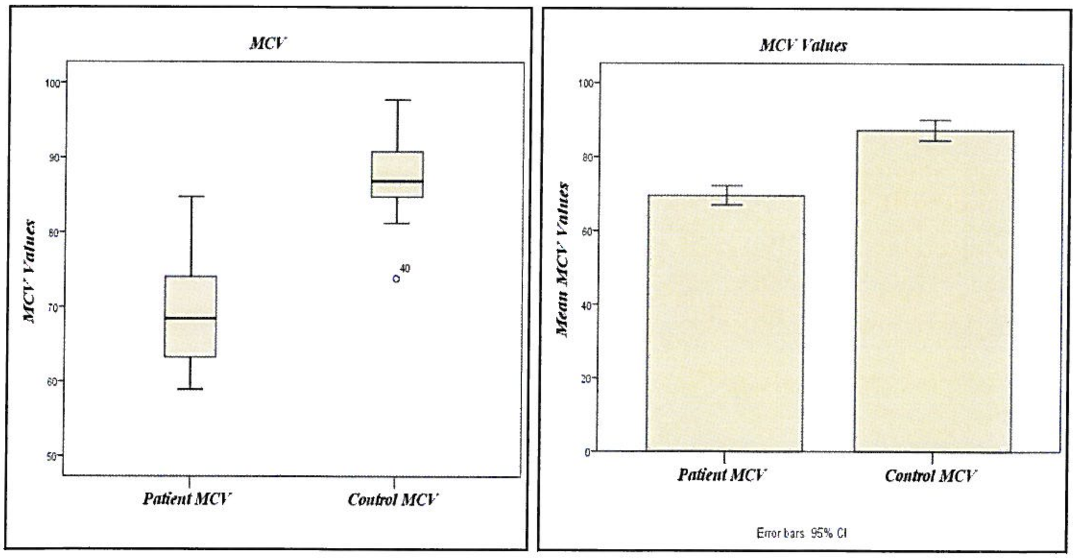
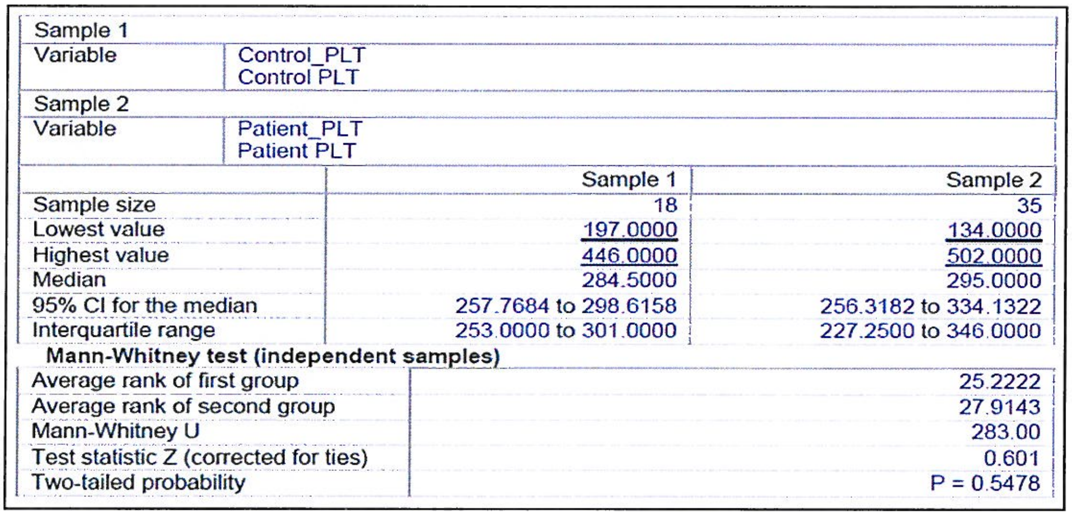


Figure 5.4. Statistical analysis of MCV values between IDA patients and Controls

5.1.5. Platelet Counts

As it is seen in Figure 5.5, IDA patients had slightly increased platelet counts [299.02 x 10^3 ± 16.06 Cells/μl, n=35] than healthy controls [288.05 x 10^3 ± 13.03 Cells/μl, n=18] but the difference was not statistically significant [p=0.5478]



Sample 1

Variable	Control_PLT Control PLT

Sample 2

Variable	Patient_PLT Patient PLT

	Sample 1	Sample 2
Sample size	18	35
Lowest value	197.0000	134.0000
Highest value	446.0000	502.0000
Median	284.5000	295.0000
95% CI for the median	257.7684 to 298.6158	256.3182 to 334.1322
Interquartile range	253.0000 to 301.0000	227.2500 to 346.0000

Mann-Whitney test (independent samples)

Average rank of first group	25.2222
Average rank of second group	27.9143
Mann-Whitney U	283.00
Test statistic Z (corrected for ties)	0.601
Two-tailed probability	P = 0.5478

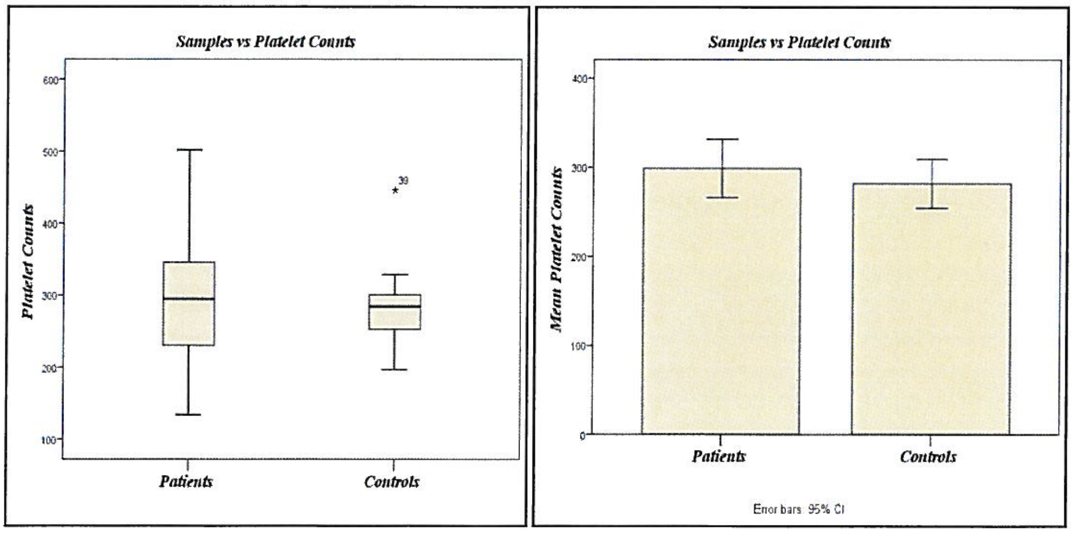
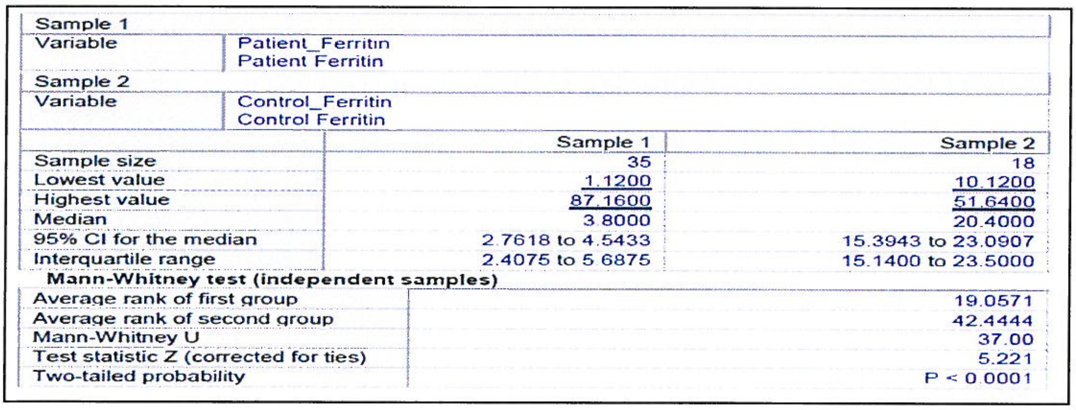


Figure 5.5. Statistical analysis of Platelet Counts values between IDA patients and Controls

5.1.6. Ferritin Results

In IDA diagnosis, lower serum Ferritin levels are most distinctive to properly diagnose IDA. In our study, ferritin levels in serums of IDA patients and healthy controls were analyzed. As predicted, mean ferritin levels of IDA patients were 8.202 ± 3.94 ng/ml n=35 while mean ferritin levels of healthy controls were 22.05 ± 4.30 ng/ml n=18. Statistical analysis also showed significant difference between healthy control and IDA patient serum ferritin levels (p<0.0001). Figure 5.6 shows the statistical analysis between IDA patients and healthy controls.



Sample 1	
Variable	Patient_Ferritin Patient Ferritin

Sample 2	
Variable	Control_Ferritin Control Ferritin

	Sample 1	Sample 2
Sample size	35	18
Lowest value	1.1200	10.1200
Highest value	87.1600	51.6400
Median	3.8000	20.4000
95% CI for the median	2.7618 to 4.5433	15.3943 to 23.0907
Interquartile range	2.4075 to 5.6875	15.1400 to 23.5000

Mann-Whitney test (independent samples)

Average rank of first group	19.0571
Average rank of second group	42.4444
Mann-Whitney U	37.00
Test statistic Z (corrected for ties)	5.221
Two-tailed probability	P < 0.0001

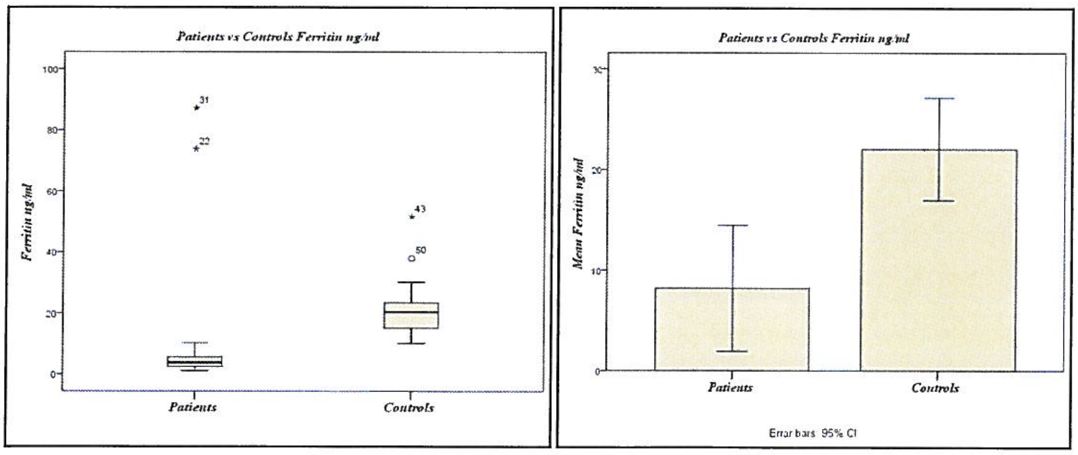
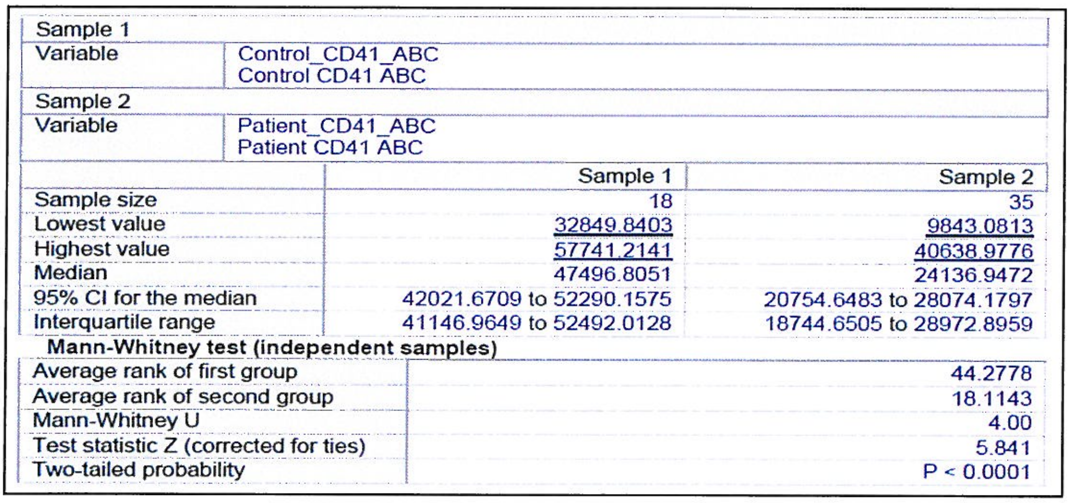


Figure 5.6. Statistical analysis of Serum Ferritin concentrations of IDA patients and Healthy controls

5.2.2. Platelet Surface CD41 Antigen Binding Capacity

Antigen Binding Capacities of platelet surface CD41 was compared between IDA patients and healthy controls. As seen in Figure 5.2.1.2, surface CD41 on healthy control platelets have increased antigen binding capacity [46772.45 ± 1705.94, n=18] than IDA patient platelets [23366.36 ± 1259.15, n=35]. Statistical analysis also shows significant differences of ABCs of surface CD41 between healthy control and IDA patient platelets [P<0,0001].



Sample 1	
Variable	Control_CD41_ABC Control CD41 ABC

Sample 2	
Variable	Patient_CD41_ABC Patient CD41 ABC

	Sample 1	Sample 2
Sample size	18	35
Lowest value	32849.8403	9843.0813
Highest value	57741.2141	40638.9776
Median	47496.8051	24136.9472
95% CI for the median	42021.6709 to 52290.1575	20754.6483 to 28074.1797
Interquartile range	41146.9649 to 52492.0128	18744.6505 to 28972.8959

Mann-Whitney test (independent samples)

Average rank of first group	44.2778
Average rank of second group	18.1143
Mann-Whitney U	4.00
Test statistic Z (corrected for ties)	5.841
Two-tailed probability	P < 0.0001

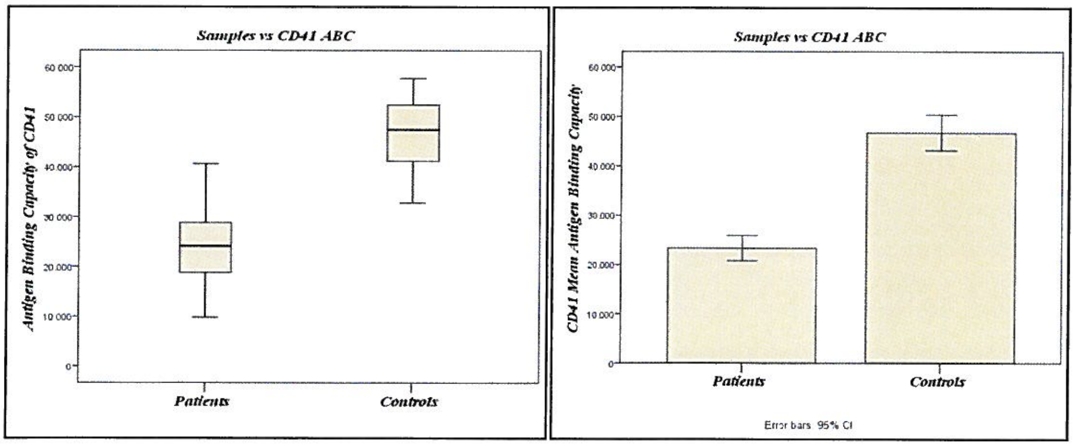
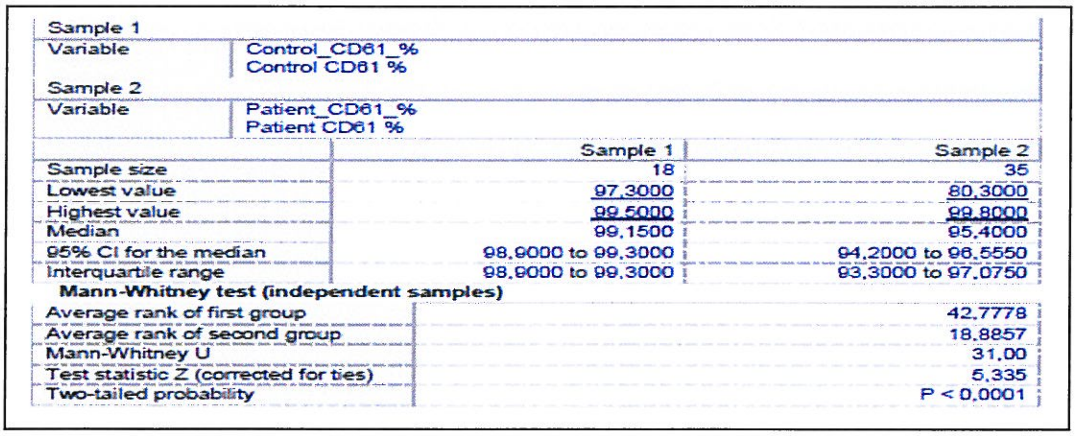


Figure 5.8. Statistical Analysis of surface CD41 ABCs of patients and controls platelets

5.2.3. CD61 [GPIIIa] Immunophenotyping

As the second subunit of GPIIb-IIIa fibrinogen binding receptor complex on platelets, CD61 is also a major platelet identification and function marker in Flow Cytometry. Therefore surface CD61 expressions were also compared between IDA patients and healthy controls. Statistical analysis in Figure 5.9 shows significant differences between patients and healthy controls [P<0,0001] as IDA patients have lower surface CD61 expressions [94.62 % ± 0.63 n=35] than healthy controls [98.94 % ± 0.14 n=18].



Sample 1	
Variable	Control_CD61_% Control CD61 %
Sample 2	
Variable	Patient_CD61_% Patient CD61 %

	Sample 1	Sample 2
Sample size	18	35
Lowest value	97,3000	80,3000
Highest value	99,5000	99,8000
Median	99,1500	95,4000
95% CI for the median	98,9000 to 99,3000	94,2000 to 96,5550
Interquartile range	98,9000 to 99,3000	93,3000 to 97,0750

Mann-Whitney test (independent samples)

Average rank of first group	42,7778
Average rank of second group	18,8857
Mann-Whitney U	31,00
Test statistic Z (corrected for ties)	5,335
Two-tailed probability	P < 0,0001

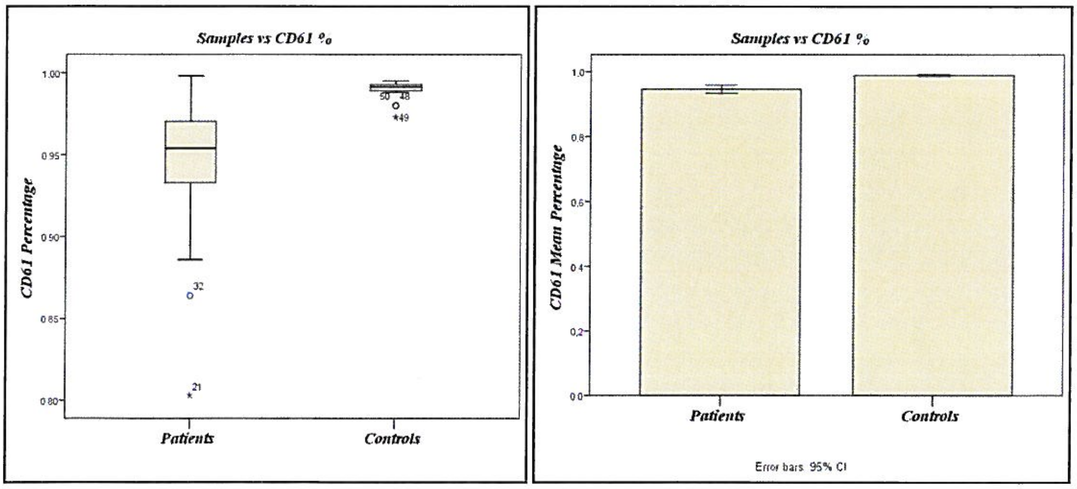
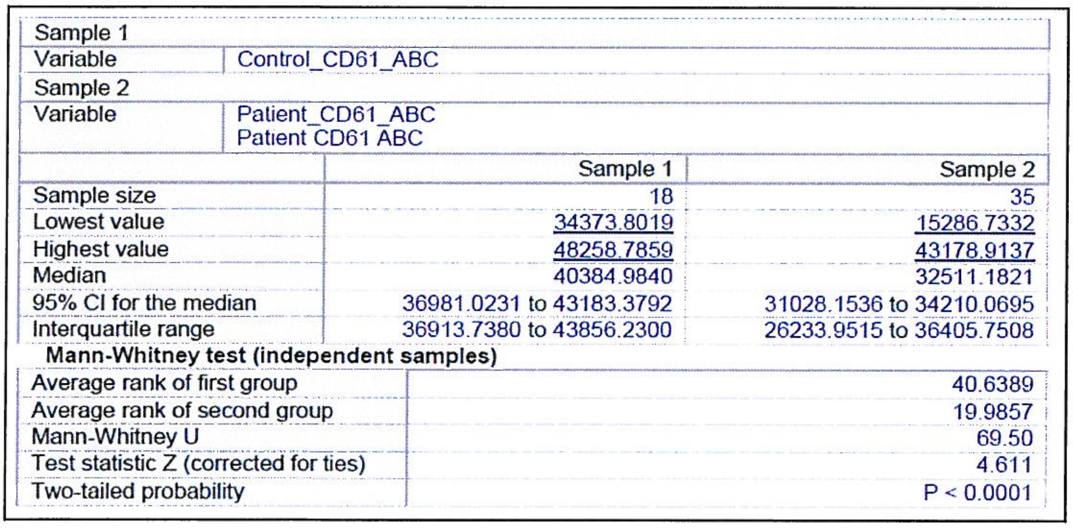


Figure 5.9. Statistical Analysis between Patient and Control platelet surface CD61 expressions

5.2.4. Platelet Surface CD61 Antigen Binding Capacity

ABCs of platelet surface CD61 was also compared between IDA patients and healthy controls. Figure 5.10 shows that surface CD61 on healthy control platelets have increased antigen binding capacity [40723.64 ± 1003.28, n=18] than IDA patient platelets [31684.35 ± 1089.08, n=35]. Statistical analysis also shows significant differences of ABCs of surface CD41 between healthy control and IDA patient platelets [P<0,0001].



Sample 1	
Variable	Control_CD61_ABC
Sample 2	
Variable	Patient_CD61_ABC Patient CD61 ABC

	Sample 1	Sample 2
Sample size	18	35
Lowest value	34373.8019	15286.7332
Highest value	48258.7859	43178.9137
Median	40384.9840	32511.1821
95% CI for the median	36981.0231 to 43183.3792	31028.1536 to 34210.0695
Interquartile range	36913.7380 to 43856.2300	26233.9515 to 36405.7508

Mann-Whitney test (independent samples)

Average rank of first group	40.6389
Average rank of second group	19.9857
Mann-Whitney U	69.50
Test statistic Z (corrected for ties)	4.611
Two-tailed probability	P < 0.0001

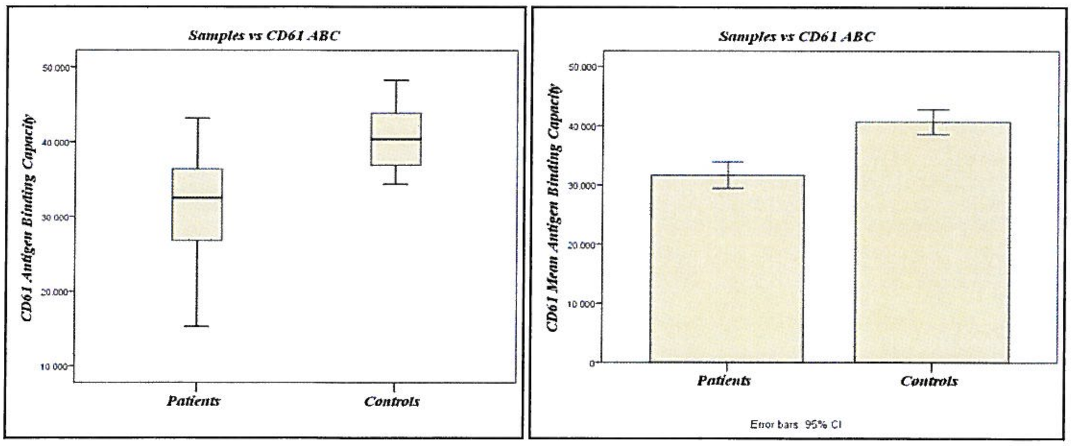
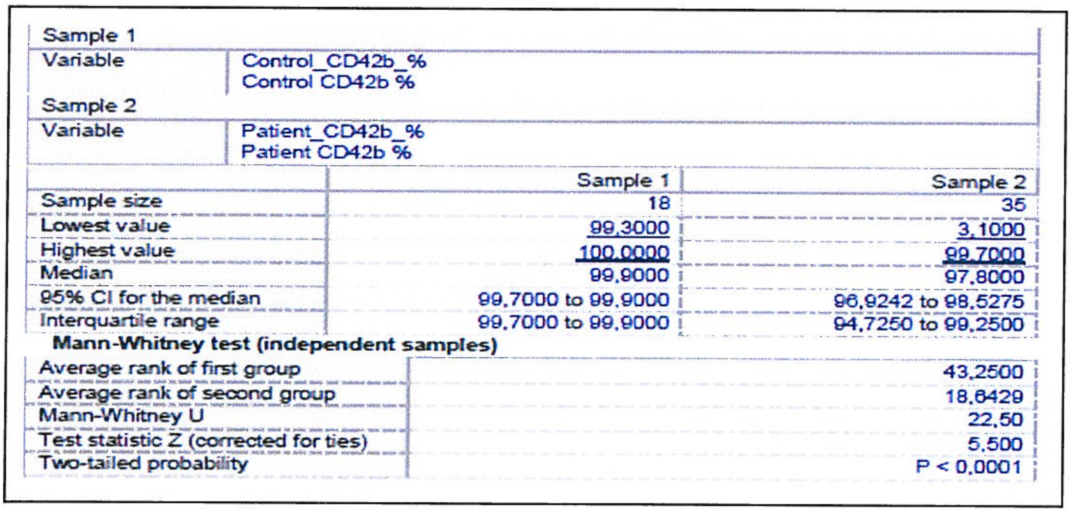


Figure 5.10. Statistical Analysis of surface CD61 ABCs of patients and controls platelets

5.2.5. CD42b [von Willebrand Factor Receptor] Immunophenotyping

As one of the most important functional platelet surface marker, CD42b was also analyzed in our study. Figure 5.11 shows that platelets of IDA patients had lower expression of CD42b [88.22 % ± 4.5 n=35] on their surface than healthy controls [99.78% ± 0.05]. Statistical analysis also showed significant differences between patient and control surface CD42b percentages [P<0.0001]



Sample 1		
Variable	Control_CD42b_% Control CD42b %	
Sample 2		
Variable	Patient_CD42b_% Patient CD42b %	
	Sample 1	Sample 2
Sample size	18	35
Lowest value	99,3000	3,1000
Highest value	100,0000	99,7000
Median	99,9000	97,8000
95% CI for the median	99,7000 to 99,9000	96,9242 to 98,5275
Interquartile range	99,7000 to 99,9000	94,7250 to 99,2500

Mann-Whitney test (independent samples)

Average rank of first group	43,2500
Average rank of second group	18,6429
Mann-Whitney U	22,50
Test statistic Z (corrected for ties)	5,500
Two-tailed probability	P < 0,0001

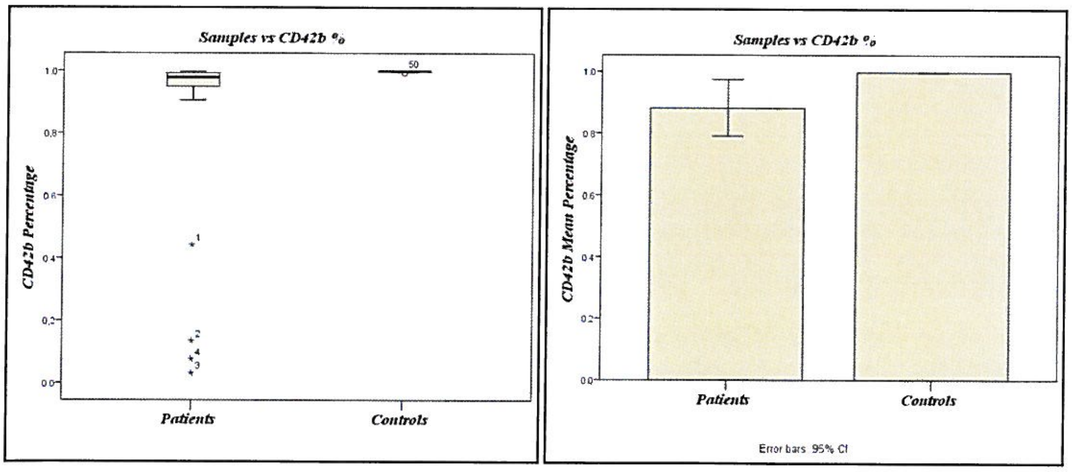
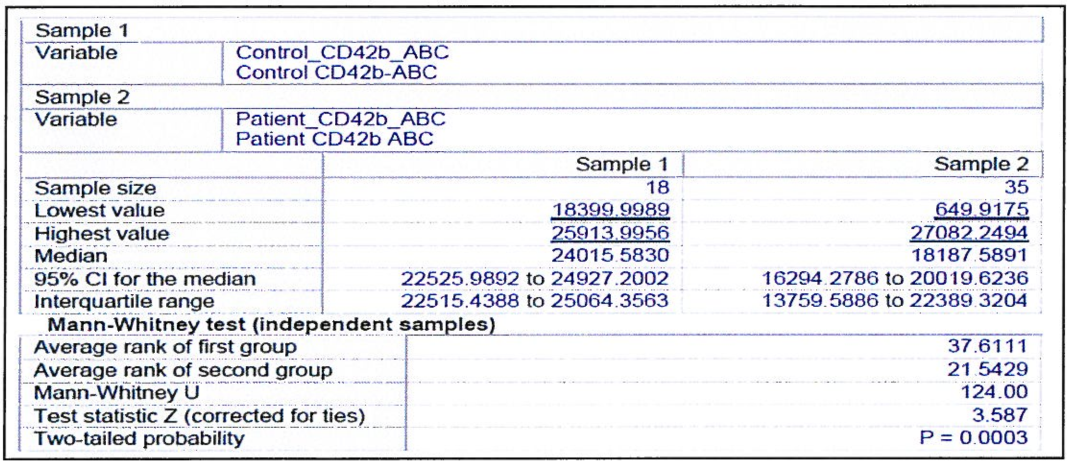


Figure 5.11. Statistical Analysis between Patients and Controls platelet surface CD42b expressions

5.2.6. Platelet Surface CD42b Antigen Binding Capacity

Platelet Surface CD42b ABCs were compared between IDA patients and healthy control samples. Figure 5.12 shows that platelets of IDA patients have decreased surface CD42b ABC [17039.68 ± 1241.4, n=35] than healthy control platelets [23345.90 ± 515.16, n=18]. Statistical analysis also showed significant differences of surface CD42b ABCs between IDA patient and control platelets [P=0,0003].



Sample 1		
Variable	Control_CD42b_ABC Control CD42b-ABC	
Sample 2		
Variable	Patient_CD42b_ABC Patient CD42b ABC	
	Sample 1	Sample 2
Sample size	18	35
Lowest value	18399.9989	649.9175
Highest value	25913.9956	27082.2494
Median	24015.5830	18187.5891
95% CI for the median	22525.9892 to 24927.2002	16294.2786 to 20019.6236
Interquartile range	22515.4388 to 25064.3563	13759.5886 to 22389.3204
Mann-Whitney test (independent samples)		
Average rank of first group		37.6111
Average rank of second group		21.5429
Mann-Whitney U		124.00
Test statistic Z (corrected for ties)		3.587
Two-tailed probability		P = 0.0003

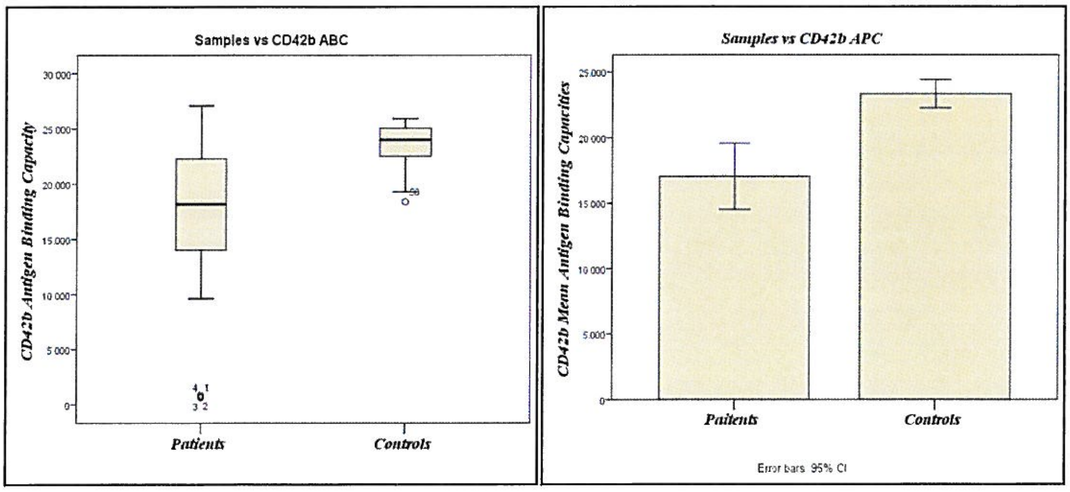
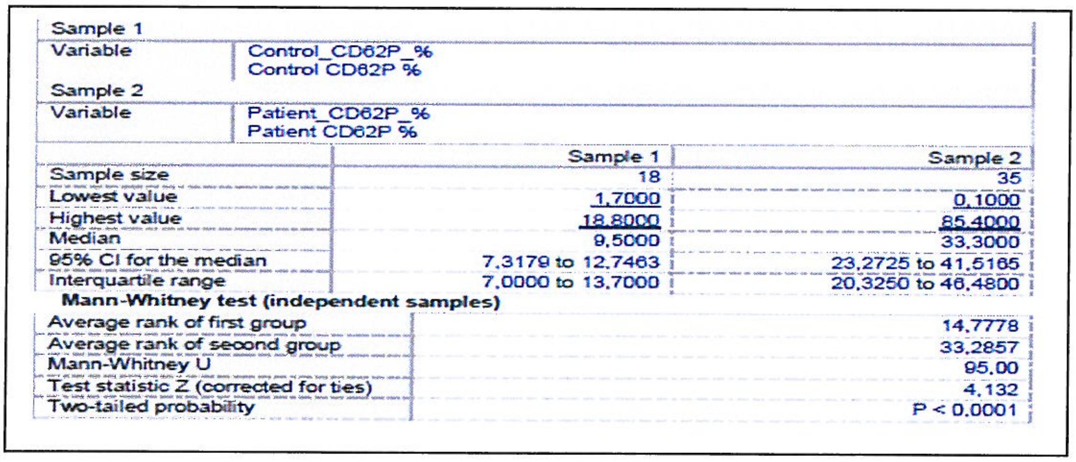


Figure 5.12. Statistical Analysis of surface CD42b ABCs of patients and controls platelets

5.2.7. CD62P [P-Selectin] Immunophenotyping

As one of the major platelet activation marker, surface CD62P expressions were also compared between IDA patients and healthy controls. Figure 5.13 shows that IDA patient platelets showed higher activation [34 % ± 3.6, n=35] than healthy control platelets [10.24 % ± 1.27]. Statistical analysis also shows significant differences between IDA patient and control platelet activations [P<0,0001]



Sample 1	
Variable	Control_CD62P_% Control CD62P %
Sample 2	
Variable	Patient_CD62P_% Patient CD62P %

	Sample 1	Sample 2
Sample size	18	35
Lowest value	1,7000	0,1000
Highest value	18,8000	85,4000
Median	9,5000	33,3000
95% CI for the median	7,3179 to 12,7463	23,2725 to 41,5165
Interquartile range	7,0000 to 13,7000	20,3250 to 46,4800

Mann-Whitney test (independent samples)

Average rank of first group	14,7778
Average rank of second group	33,2857
Mann-Whitney U	95,00
Test statistic Z (corrected for ties)	4,132
Two-tailed probability	P < 0,0001

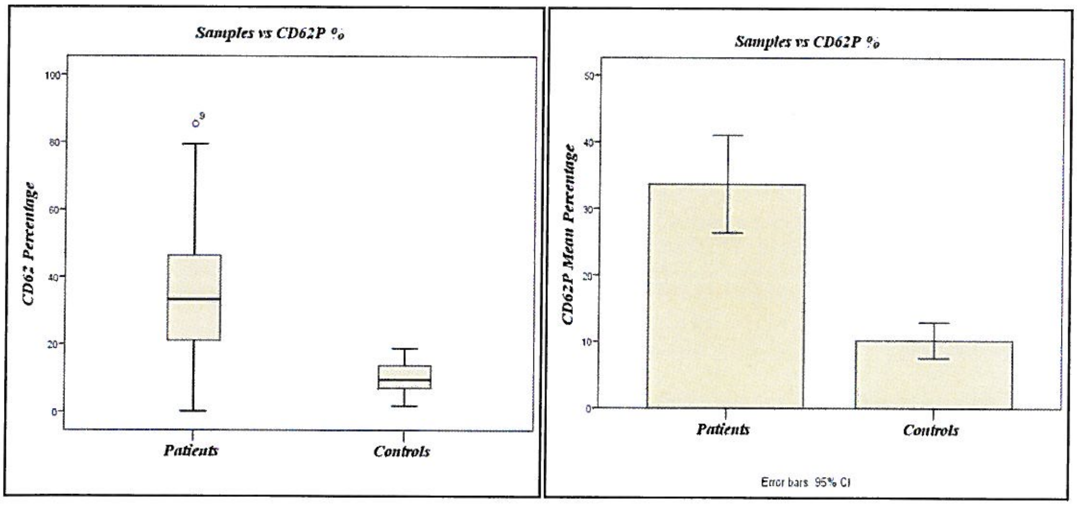
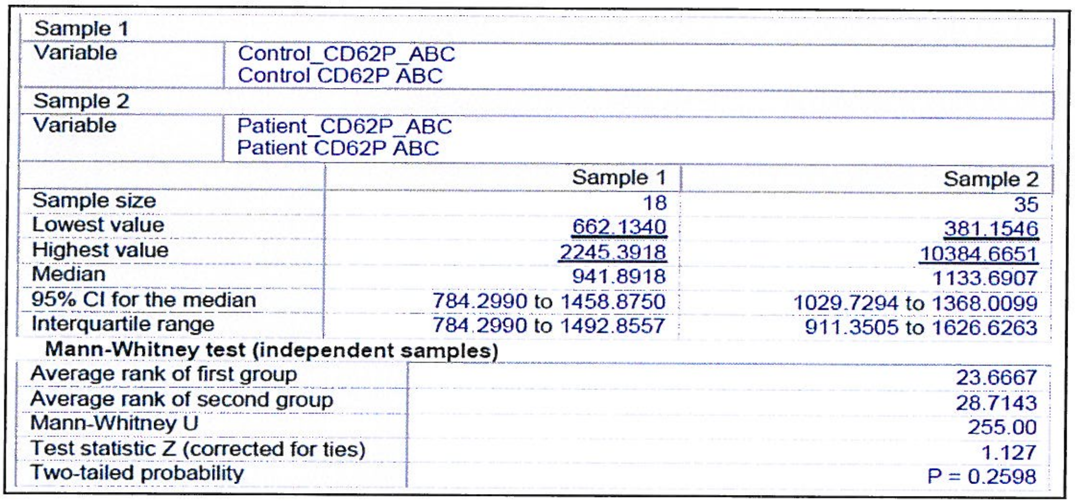


Figure 5.13. Statistical Analysis between Patients and Controls platelet surface CD62P expressions

5.2.8. Platelet Surface CD62P Antigen Binding Capacities

Platelet surface CD62P antigen binding capacities were compared between platelets of IDA patients and healthy control samples. As it was shown in Figure 5.14 platelets of both IDA patients and healthy controls have approximate values of surface CD62P ABC [1631.80 ± 293.28, n =35; 1150.92 ± 117.5, n=18]. Statistical analysis also did not show significant differences of platelet surface CD62P ABCs between IDA patients and healthy controls [P=0.2598].



Sample 1	
Variable	Control_CD62P_ABC Control CD62P ABC

Sample 2	
Variable	Patient_CD62P_ABC Patient CD62P ABC

	Sample 1	Sample 2
Sample size	18	35
Lowest value	662.1340	381.1546
Highest value	2245.3918	10384.6651
Median	941.8918	1133.6907
95% CI for the median	784.2990 to 1458.8750	1029.7294 to 1368.0099
Interquartile range	784.2990 to 1492.8557	911.3505 to 1626.6263

Mann-Whitney test (independent samples)

Average rank of first group	23.6667
Average rank of second group	28.7143
Mann-Whitney U	255.00
Test statistic Z (corrected for ties)	1.127
Two-tailed probability	P = 0.2598

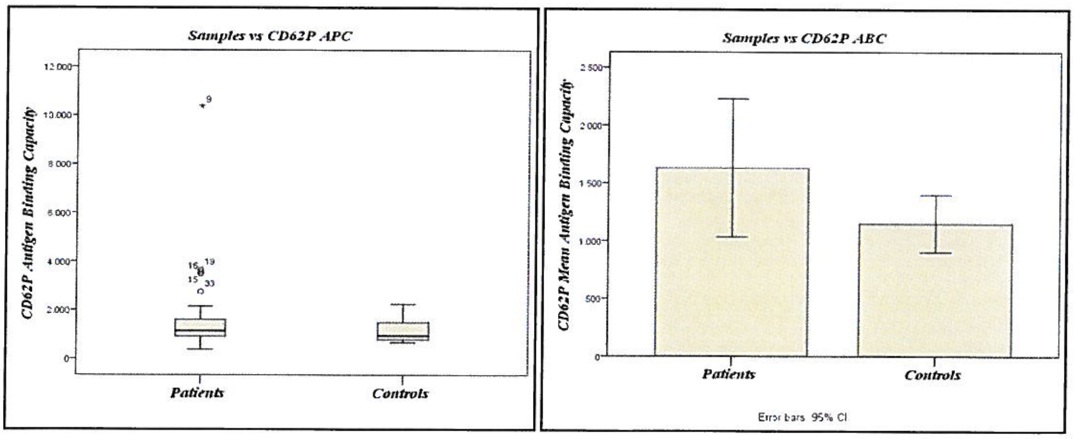
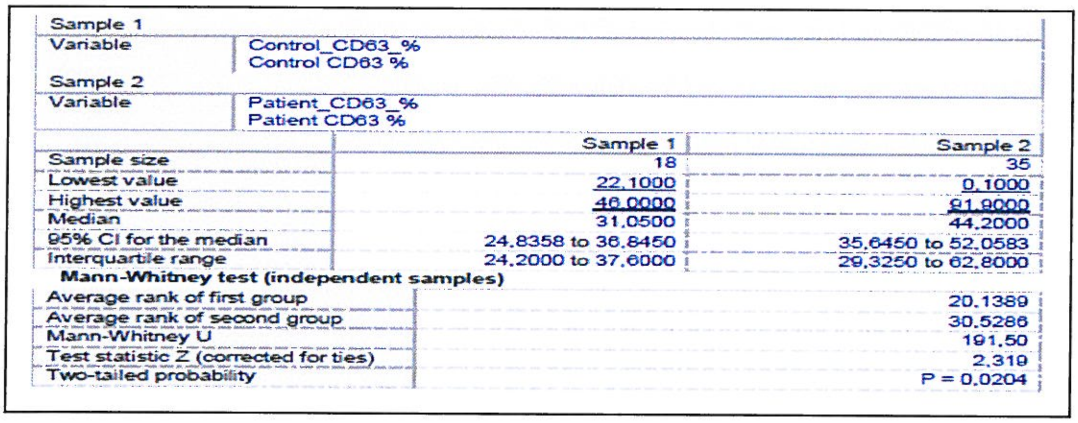


Figure 5.14. Statistical Analysis of surface CD62P ABCs of patients and controls platelets

5.2.9. CD63 Immunophenotyping

CD63 percentages were also analyzed and compared between patients and controls because of the nature of platelet surface CD63 as an activation molecule. As same as CD62P results, surface CD63 expression was higher in IDA patients [42.96 % ± 4.07, n=35] than healthy controls [31.61 % ± 1.78, n=18]. Statistical analysis also showed significant differences between IDA patient and control platelet surface CD63 expressions [P=0.0204] Figure 5.15 shows the statistical analysis of patient and control platelet surface CD63 expression levels.



Sample 1	
Variable	Control_CD63_% Control CD63 %
Sample 2	
Variable	Patient_CD63_% Patient CD63 %

	Sample 1	Sample 2
Sample size	18	35
Lowest value	22.1000	0.1000
Highest value	46.0000	91.8000
Median	31.0500	44.2000
95% CI for the median	24.8358 to 36.8450	35.6450 to 52.0583
Interquartile range	24.2000 to 37.6000	29.3250 to 62.8000

Mann-Whitney test (independent samples)

Average rank of first group	20.1389
Average rank of second group	30.5286
Mann-Whitney U	191.50
Test statistic Z (corrected for ties)	2.319
Two-tailed probability	P = 0.0204

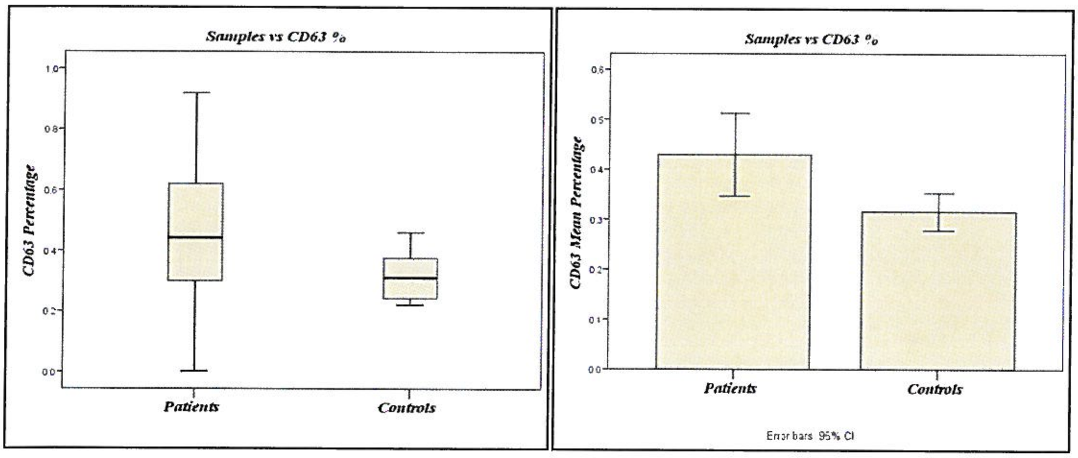
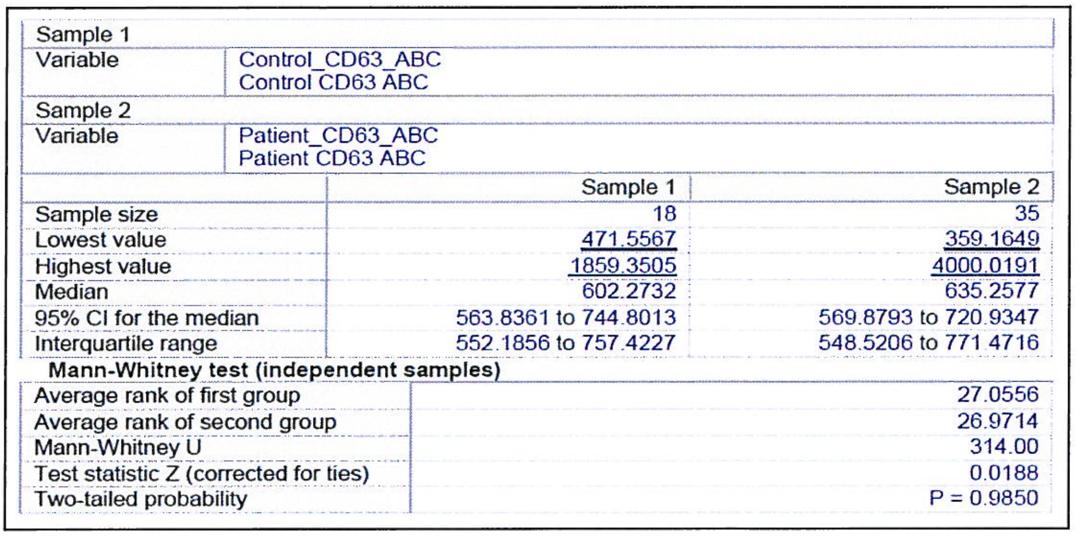


Figure 5.15. Statistical Analysis between Patients and Controls platelet surface CD63 expressions

5.2.10. Platelet Surface CD63 Antigen Binding Capacities

Platelet surface CD63 antigen binding capacities were also compared between platelets of IDA patients and healthy control samples. Figure 5.16 shows that platelets of both IDA patients and healthy controls have approximate values of surface CD63 ABC [782.21 ± 104.85, n=35; 708.15 ± 73.05, n=18]. Statistical analysis also showed no significant differences of platelet surface CD63 ABCs between IDA patients and healthy controls [P=0.9850].



Sample 1	
Variable	Control_CD63_ABC Control CD63 ABC

Sample 2	
Variable	Patient_CD63_ABC Patient CD63 ABC

	Sample 1	Sample 2
Sample size	18	35
Lowest value	471.5567	359.1649
Highest value	1859.3505	4000.0191
Median	602.2732	635.2577
95% CI for the median	563.8361 to 744.8013	569.8793 to 720.9347
Interquartile range	552.1856 to 757.4227	548.5206 to 771.4716

Mann-Whitney test (independent samples)

Average rank of first group	27.0556
Average rank of second group	26.9714
Mann-Whitney U	314.00
Test statistic Z (corrected for ties)	0.0188
Two-tailed probability	P = 0.9850

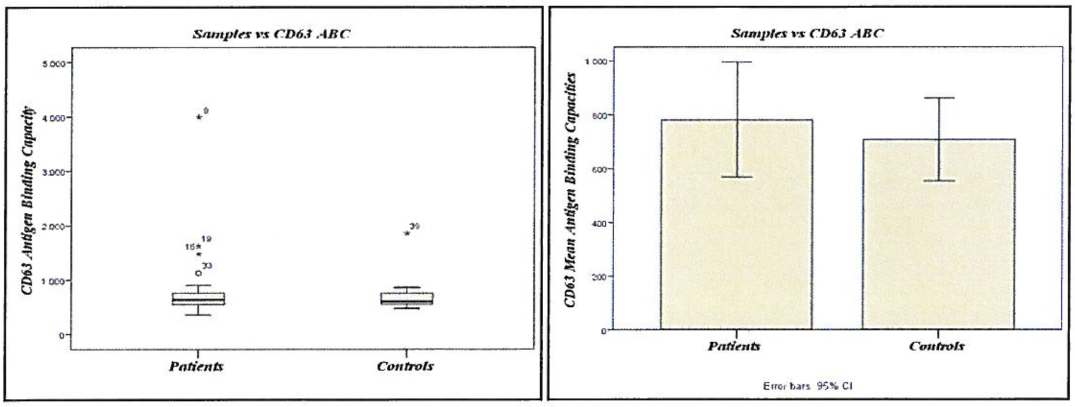


Figure 5.16. Statistical Analysis of surface CD63 ABCs of patients and controls platelet

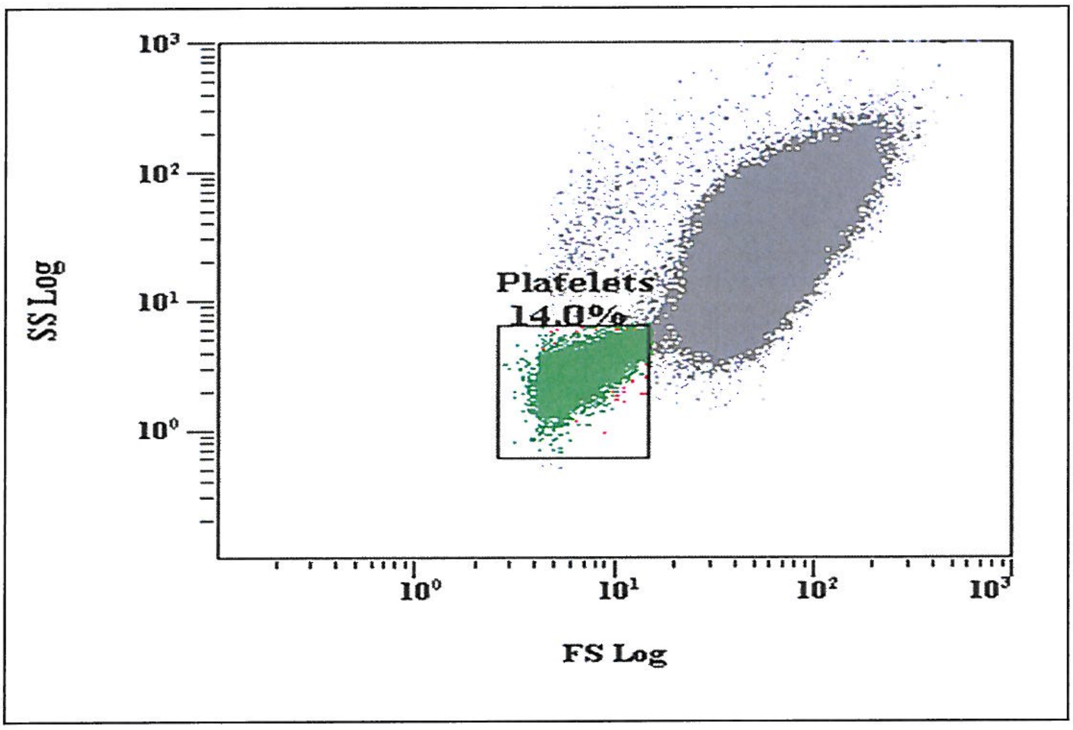


Figure 5.17. Whole Blood Platelets

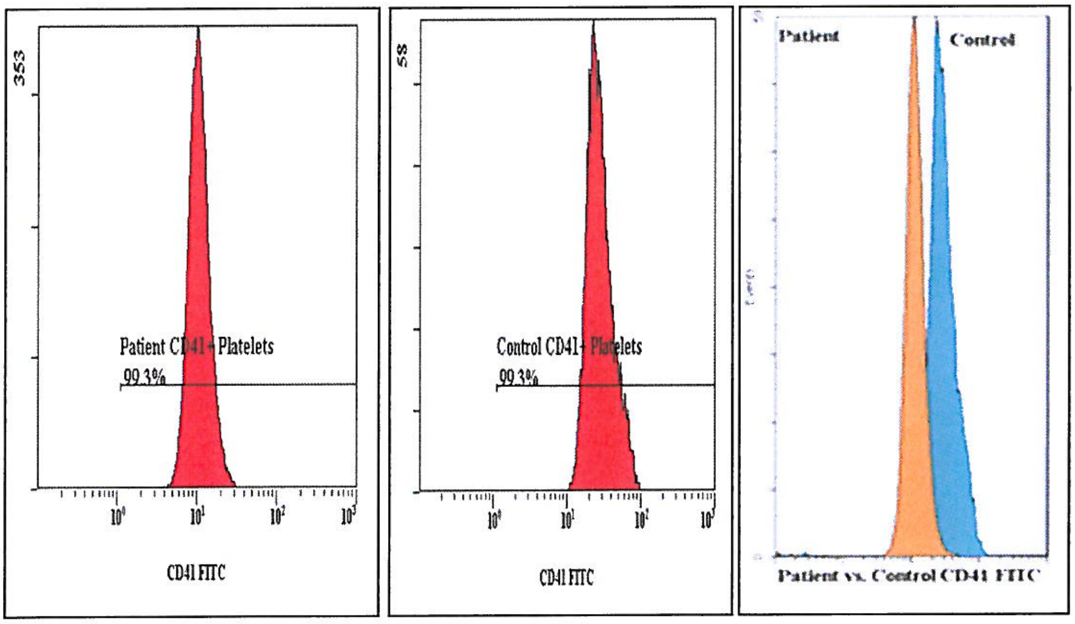


Figure 5.18. Patient vs. Control CD41 Histograms

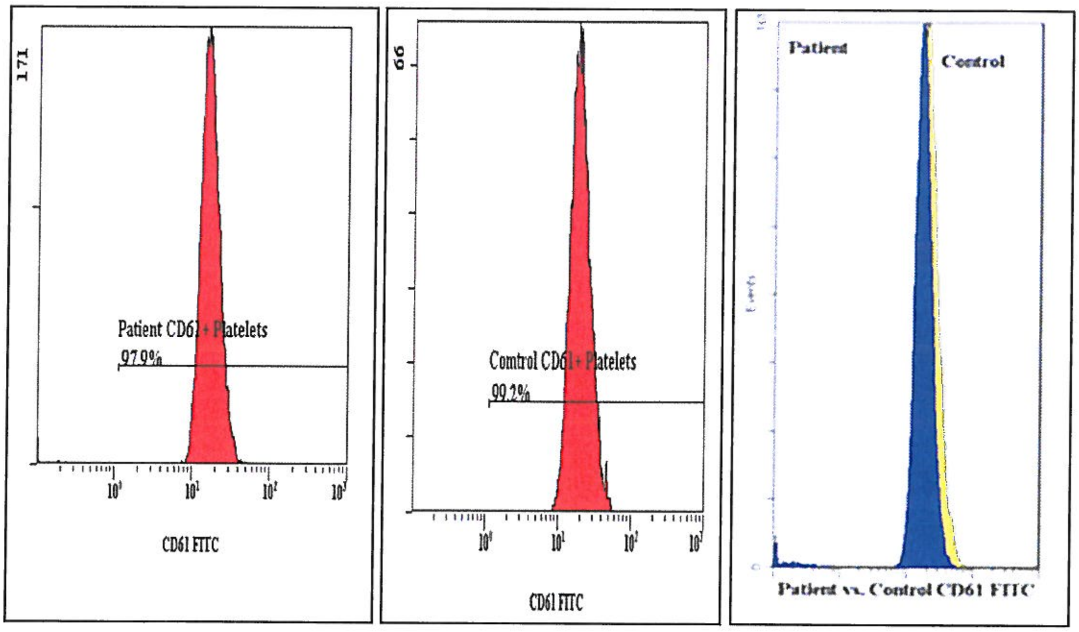


Figure 5.19. Patient vs. Control CD61 Histograms

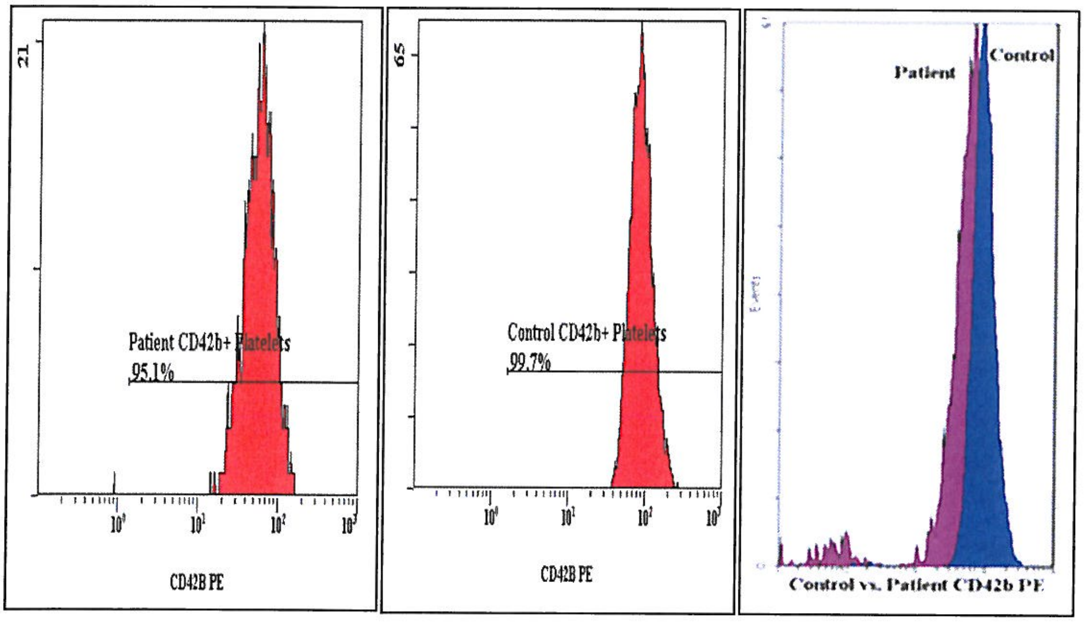


Figure 5.20. Patient vs. Control CD42b Histograms

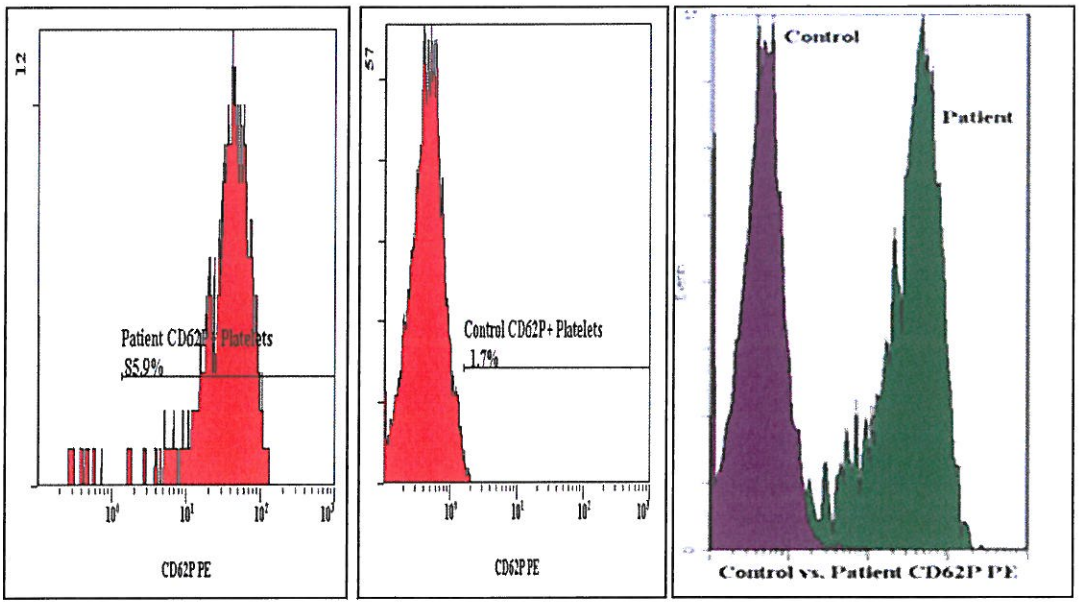


Figure 5.21. Patient vs. Control CD62P Histograms

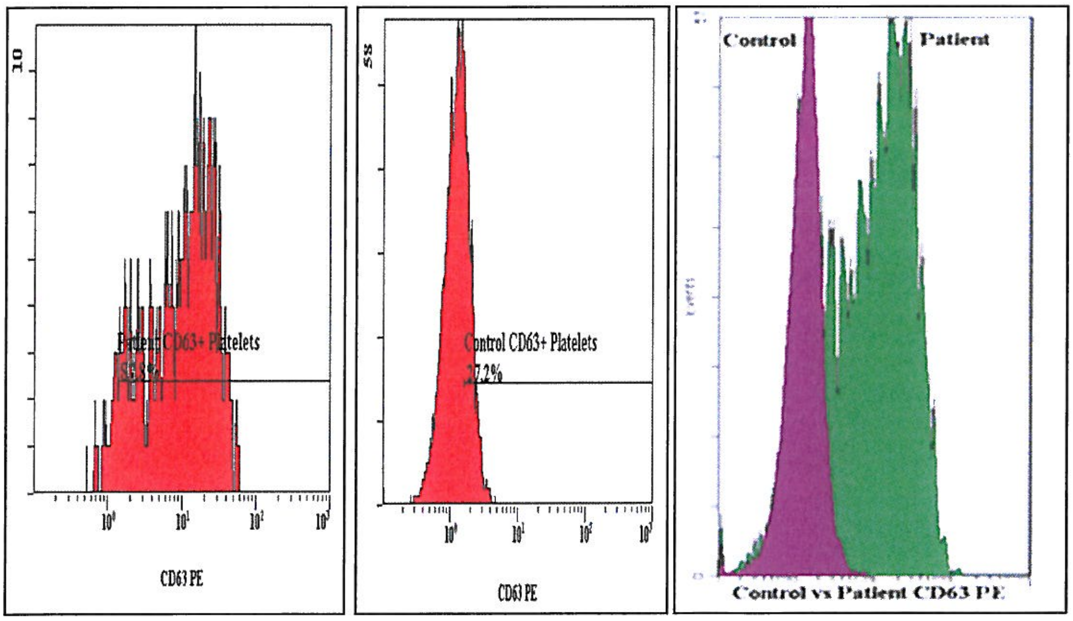


Figure 5.22. Patient vs. Control CD62P Histograms

6. DISCUSSION

In our study, all of our patients had been diagnosed with Iron Deficiency Anemia according to MCV, HCT, HGB and Ferritin levels measured by standard serological tests. All values were also measured for healthy controls. As we compared the patients with healthy controls, healthy controls had significantly higher values of MCV, HCT, HGB and Ferritin than IDA patients. Only RBCs and PLT counts were similar between these two groups. Therefore we have correctly identified our two study groups as healthy controls and IDA patients.

6.1. EFFECTS OF IDA ON PLATELETS

The effects of IDA on platelets are still an area that is being explored. There are several published studies about IDA-platelet interaction which suggest that IDA may cause thrombocytosis and/or thrombocytopenia [74, 75, 76, and 77] and aggregation dysfunctions [78, 79]. IDA can be observed in both men and women and there are variety of causes of IDA, some of which are heavy menstrual cycle, malnutrition, and disorders that cause chronic gastrointestinal bleeding and blood loss such as Helicobacter Pylori infection and colorectal cancers. In forms of IDA seen on men, parameters like cancer and bacterial infection would also have certain effects on platelets like activation and aggression and it would be impossible to determine whether the effects on platelets occur because of IDA or other parameters. That is why, in our study we have only included women patients which IDA had occurred due to malnutrition and heavy menstrual cycle.

6.2. PLATELET SURFACE RECEPTORS IN IDA-PLATELET INTERACTION

In our study, we have compared both the surface expressions and antigen binding capacities of platelet surface molecules CD41, CD61, CD42b, CD62P and CD63 between IDA patients and healthy controls and we have observed that Antigen Binding Capacities of platelet activation markers were not increased/decreased in IDA patients in comparison to healthy controls; but interestingly, the percentage of activated platelets were found to be increased in IDA patients. Evaluations of percentage and ABC of each marker is presented below.

6.2.1. cd41

CD41 [GPIIb, Integrin-α2] is one of the two subunits of GPIIb-IIIa Fibrinogen Binding Complex on platelet surface. Synergistically, CD41 non-covalently binds with CD61 to form FBC; therefore any defect on CD41 would cause platelet dysfunction. In literature, there are no studies regarding the effect of IDA on surface CD41 expression and antigen binding capacity. However in our study, we have found out that IDA patients have significantly decreased surface CD41 percentage and antigen binding capacity in comparison to healthy controls. These results show us that, by an unknown mechanism, iron may have a positive effect on platelet functions so that lack of body iron may disrupt platelet migration to inflammation and tissue damage area and may prevent platelet surface fibrinogen binding receptor complex to bind free fibrinogen released from damaged tissues with maximum efficiency in case of a tissue injury or inflammation and this may have an effect on platelet functions such as thrombus formation, growth factor secretion and tissue repair..

6.2.2. cd61

As a part of Fibrinogen Binding Complex, CD61 synergistically works with CD41 to form FBC. Therefore irregularities in surface CD61 expression may lead to conformational changes in FBC, thus causes platelet dysfunction. Although, there are no studies regarding the effects of IDA on surface CD61 expressions and antigen binding capacities in current literature, we have shown that, just as CD41, surface CD61 expression and antigen binding capacity were reduced in patients with IDA when compared to healthy controls. This can be interpreted as the surface expression and ABC of GPIIb/IIIa [CD41-CD61] FBC is reduced altogether in patients with IDA. These changes and lack of body iron may have an inhibitory effect on the efficiency of platelets to bind fibrinogen released from damaged tissues or inflammation areas and platelet functions could be reduced.

6.2.3. cd42b

CD42b, also known as the von Willebrand Factor receptor, binds to free vWF released from endothelial cells during a time of inflammation or injury and by this binding; platelets migrate to the injury area and start thrombus formation and coagulation. In previous or current literature, there are no studies regarding the effects of IDA on platelet surface CD42b expression or ABC. However, in our studies we have found out that in IDA patients, both surface expression and vWF binding capacity of CD42b were significantly reduced against healthy control samples. In contrary to other platelet activation markers, CD42b expression decreases on the surface of platelets upon activation This can mean that iron may have a positive effect on the ability of platelets to function efficiently during a vascular injury or inflammation that lack of iron during IDA may disrupt the ability of platelets to bind vWF efficiently to start coagulation process and vascular tissue repair.

6.2.4. cd62p

CD62P [P-Selectin] resides in the α-granules of platelets and during inflammation or tissue damage, translocates to the platelet surface. Therefore, surface CD62P is a potent activation marker for platelets. The main tasks of CD62P is to mediate the adhesion of platelets to the inflammation or damage area and control leukocyte trafficking on inflammation site by binding CD162 [P-Selectin Ligand] on leukocytes. Especially platelets initiate an innate immune response by activating circulating neutrophils by binding neutrophil surface CD162. In previous studies, Yıldırım et. al demonstrated that CD62P expression does not change in pediatric IDA patients against controls [85]. However, in our study we have found out that without any platelet activating agents like ADP or collagen, IDA patients showed significantly increased surface CD62P expression in comparison to healthy controls but when we studied the ABC of CD62P, we have seen that there is no significant difference between IDA patients and healthy controls. We interpreted this result as in the presence of IDA, with the lack of iron, platelets may become activated by increasing their surface CD62P expressions and due to decreased ABC, this activation may be a non-functional one, placing platelets into a futile activation cycle. In the light of these results, the question remains if platelets of IDA patients can initiate a healthy platelet dependent innate immune response and if IDA patients are more susceptible to infections.

6.2.5. cd63

CD63 is a tetraspanins family molecule that is responsible for platelet activation. In the course of studying the effects of IDA on platelet activation, Yıldırım et. al showed no change in surface CD63 expression on pediatric IDA patients [85]. In our study, we have found out that surface CD63 expression is significantly increased in IDA patients than healthy controls. However, despite this surface expression we have found no significant difference in CD63 ABC between IDA patients and healthy controls. We have interpreted this result as same as CD62P which is that the lack of iron, with an unknown mechanism, may activate platelets but due to the reduced ABC, platelets may enter a futile activation cycle, reducing platelet dependent innate immunity response.

6.3. PLATELET ACTIVATION MECHANISMS, EFFECTS ON PLATELET SURFACE MARKERS AND RELATION WITH IDA

Platelets are needed to be activated in order to show their functions. Activation can occur by several phenomenons in vivo and in vitro such as fibrinogen release, binding of ADP to surface $P2Y_1$ and $P2Y_{12}$ receptors [20]. Platelets also can be activated by hormones such as adrenaline, thrombin, serotonin, vasopressin [21]. During inflammation, release of enzymes Cyclooxygenase-1 and -2 (COX-1, COX-2), Prostaglandin E-2 Synthase (PGE-2) and Nitric Oxide and Indoleamine 2, 3-Dioxygenase also activate platelets. Platelets also can be activated by high concentrations of Ca^{+2} and Mg^{+2} ions and spontaneous activation occurring only in in vitro studies.

Upon activation, platelets undergo several changes. These are changes in shape, aggregation and secretion of soluble factors. When activated, platelets change their smooth oval or discoid morphology and become irregularly shaped and their randomly dispersed granules migrate to the cell periphery and become membrane organelle-like structures known as pseudopods [6]. After activation process, platelets form aggregates with each other by using fibrinogen and vWF as these molecules, when are bonded to a platelet, make a cross-link to an adjacent platelet by using the FBC and vWFr of that platelet [86]. Also, binding of ADP to $P2Y_1$ and $P2Y_{12}$ receptors [20], hormones like adrenaline, thrombin, serotonin, vasopressin [21], inflammatory enzymes COX-1 and COX-2, Prostaglandin E-2 Synthase (PGE-2) Nitric Oxide; Indoleamine 2, 3-Dioxygenase and high concentrations of Ca^{+2} and Mg^{+2} can cause platelet aggregation. The last change occurring in activated platelets is the secretion of soluble contents. Upon platelet activation, P-Selectin (CD62P) in alpha granules platelets translocates to cell surface [37, 38]. Secreted growth factor, cytokine and chemokine concentrations also change during platelet activation [9, 10, 11, 12, 14 and 15].

After platelets are activated, platelet surface GPIIb/IIIa (CD41-CD61) FBC and surface P-Selectin (CD62P) molecules undergo conformational changes to bind free fibrinogen and CD162 P-Selectin Ligand respectively [87, 88, 89]. In our study, we have found out that, GPIIb/IIIa surface expressions and ABCs were decreased in IDA patients in comparison to healthy controls. However we have also observed an elevated activation in IDA patients according platelet surface CD62P expressions. We interpreted this result as in patients with IDA body iron may have a positive effect on the conformational change occurring on GPIIb/IIIa FBC that is needed for platelets to bind free fibrinogen efficiently. We have also observed on IDA patients that despite the increase in total activated platelets, ABC of platelet surface CD62P did not significantly differ when compared to healthy controls, so we have suggested that body iron may have a positive effect on conformational changes that are needed on surface CD62P molecule of activated platelets to function properly. When we quantified the surface expression of platelet gp1ba (CD42b), we have found out that platelets of IDA patients have decreased expression of CD42b on their surfaces which is in correlation with the increased surface CD62P expression on platelets of IDA patients since CD42b expression inversely proportional to surface CD62P expression on activated platelets.

7. CONCLUSION AND FUTURE STUDIES

All of our findings suggest that body iron may have a positive effect on platelet functions in a molecular mechanism not yet uncovered and IDA may cause disrupted platelet mediated innate and adaptive immune response. For these mechanisms to be clearly understood we suggest studies like MALDI-TOF mass spectroscopy and/or Scanning Electron Microscopy assays to uncover if iron plays a direct role on conformational changes needed for surface GPIIb/IIIa and P-Selectin molecules and if GPIIb/IIIa and P-Selectin conformations are effected on platelet surface with the lack of iron. We also suggest that surface CXCR4 [CD184] expressions and ABCs should be analyzed on platelets of IDA patients to observe if the lack of iron causes the efficiency of platelets to receive and interpret the signals carried with pro-inflammatory chemokines released during inflammation. When induced by TLR-4 ligands such as LPS, platelets become activated and translocate intracellular CD62P to their surface and recruit circulating neutrophils by binding CD62P with P-Selectin ligand on neutrophils surface so we also suggest that surface TLR4 expression on platelets should be analyzed in IDA patients to assess if the lack of iron causes any changes to platelet surface TLR4 expression and neutrophil activation. Also $CD15^+$ neutrophil and $CD3^+CD4^+$ helper T lymphocyte and $CD19^+$ B Lymphocyte activations and chemotactic capabilities should be analyzed in IDA patients to actually observe if the lack of body iron causes any changes to platelet dependent innate and adaptive immune response, T-helper lymphocyte trafficking and T-Cell dependent B Cell activations. We also suggest that soluble growth factor and cytokine profiles and concentrations should be analyzed in platelets of IDA patients to observe the advanced effects of IDA on platelets and immune system.

As will be seen through this report, even though IDA is frequent, there is limited number of studies on relation of platelets and iron mechanism in this disorder. We have aimed to make a preliminary study in order to obtain deliberate data on the effect of IDA on platelets and vice versa. While the etiology for IDA seen in men may be due to many different factors such as chronic blood losses caused by colon cancers and/or Helicobacter Pylori infection [2] we have chosen to examine young women in fertile age in order to be able to explain that there is a direct relation of platelet activation and functions in these IDA patients and we suggest that there is need to explain these mechanisms in detail through more detailed studies.

REFERENCES

1. De Benoist, Bruno, Erin McLean, Ines Egli, and Mary Cogswell. *Worldwide Prevalence of Anaemia 1993–2005 WHO Global Database on Anaemia.* WHO Publications, 2008.

2. Liu, Ken, and Arthur J. Kaffes. "Iron Deficiency Anaemia: A Review of Diagnosis, Investigation and Management." *European Journal of Gastroenterology & Hepatology,* Vol. 24, No. 2, pp.109-116, 2012.

3. Hoffbrand, Allan V., Daniel Catovsky, Edward G. D. Tuddenham, and Anthony R. Green, eds. *Postgraduate Haematology.* 6th ed. Chichester: Wiley-Blackwell, 2010.

4. Erduran, Erol. *TÜRKİYE'DE DEMİR EKSİKLİĞİ ANEMİSİ VE GÜNCEL YAKLAŞIM.* Proceedings of 36th National Hematology Congress, Belek, Antalya. Turkish Society of Hematology, 2010.

5. Akins, Paul T., Susan Glenn, Matti M. Nemeth, and Colin P. Derdeyn. "Carotid Artery Thrombus Associated With Severe Iron-Deficiency Anemia and Thrombocytosis." *Stroke,* Vol. 27, No. 5, pp.1002-1005, 1996.

6. Michelson, Alan D. *Platelets.* 2nd ed. Amsterdam: Academic Press/Elsevier, 2006.

7. "Microscope Images - Blood." Microscope Images - Blood. Accessed May 26, 2013. http://www.mesacc.edu/~johnson/labtools/Dblood/rblood.html >.

8. Reese, Jane B., Lisa A. Urry, Michael L. Cain, Steven A. Wasserman, Peter V. Minorsky, and Robert B. Jackson. *Campbell Biology Books a La Carte Edtion.* 9th ed. Benjamin-Cummings Pub, 2012.

9. Gunsilius, Eberhard, Andreas Petzer, Günther Stockhammer, Walter Nussbaumer, Petra Schumacher, Johannes Clausen, and Günther Gastl. "Thrombocytes Are the Major Source for Soluble Vascular Endothelial Growth Factor in Peripheral Blood." *Oncology,* Vol.58, No. 2, pp.169-174, 2000.

10. Weibrich, G., W. K G Kleis, G. Hafner, and W. E. Hitzler. "Growth Factor Levels in Platelet-rich Plasma and Correlations with Donor Age, Sex, and Platelet Count." *Journal of Cranio-Maxillofacial Surgery,* Vol. 30, No. 2, pp.97-102, 2002.

11. Faude, F., E. Edel, M. Dannhauer, C. Petzel, P. Meier, and P. Wiedermann. "Autologous Thrombocyte Administration in Treatment of Idiopathic Macular Foramen." *Der Ophthalmologe: Zeitschrift Der Deutschen Ophthalmologischen Gesellschaft,* Vol. 94, No. 12, pp.877-881, 1997.

12. Boilard, E., P. Blanco, and P. A. Nigrovic. "Platelets: Active Players in the Pathogenesis of Arthritis and SLE." *Nature Reviews Rheumatology,* Vol. 8, No. 9, pp.534-542, 2012.

13. Soslau, G., D. A. Morgan, J. S. Jaffe, I. Brodsky, and Y. Wang. "Cytokine mRNA Expression in Human Platelets and a Megakaryocytic Cell Line and Cytokine Modulation of Platelet Function." *Cytokine,* Vol. 9, No. 6, pp.405-411, 1997.

14. Hartwig, D., C. Hartel, H. Hennig, M. Muller-Steinhardt, P. Schlenke, and H. Kluter. "Evidence for De Novo Synthesis of Cytokines and Chemokines in Platelet Concentrates." *Vox Sanguinis,* Vol. 82, No. 4, pp.182-190, 2002.

15. Picker, Susanne M., Alexander Steisel, and Birgit S. Gathof. "Evaluation of White Blood Cell- and Platelet-Derived Cytokine Accumulation in MIRASOL-PRT-Treated Platelets." *Transfusion Medicine and Hemotherapy,* Vol. 36, No. 2, pp.114-120, 2009.

16. Cognasse, Fabrice, Hind Hamzeh, Patricia Chavarin, Sophie Acquart, Christian Genin, and Olivier Garraud. "Evidence of Toll-like Receptor Molecules on Human Platelets." *Immunology and Cell Biology,* Vol. 83, No. 2, pp.196-198, 2005.

17. Aslam, R., E. R. Speck, M. Kim, A. R. Crow, Annie K. W. Bang, F. Ğ. Nestel, H. Ni, A. H. Lazarus, J. Freedman, and J. W. Semple. "Platelet Toll-like Receptor Expression Modulates Lipopolysaccharide-induced Thrombocytopenia and Tumor Necrosis Factor-Production in Vivo." *Blood,* Vol. 107, No. 2, pp.637-641, 2006.

18. Andonegui, G., S. M. Kerfoot, K. McNagny, K. V. J. Ebbert, K. D. Patel, and P. Kubes. "Platelets Express Functional Toll-like Receptor-4." *Blood,* Vol. 106, No. 7, pp.2417-2423, 2005.

19. Byrne, M., S. Kerrigan, P. Corcoran, J. Atherton, F. Murray, D. Fitzgerald, and D. Cox. "Helicobacter Pylori Binds Von Willebrand Factor and Interacts with GPIb to Induce Platelet Aggregation☆." *Gastroenterology,* Vol. 124, No. 7, pp.1846-1854, 2003.

20. Dorsam, Robert T., and Satya P. Kunapuli. "Central Role of the P2Y12 Receptor in Platelet Activation." *Journal of Clinical Investigation,* Vol. 113, No. 3, pp.340-345, 2004.

21. Ardlie, N.g., H.a. Cameron, and J. Garrett. "Platelet Activation by Circulating Levels of Hormones: A Possible Link in Coronary Heart Disease." *Thrombosis Research,* Vol. 36, No. 4, pp.315-322, 1984.

22. Jackson, Shaun P. "Arterial Thrombosis—insidious, Unpredictable and Deadly." *Nature Medicine,* Vol. 17, No. 11, pp.1423-1436, 2011.

23. Shattil, S. J., M. Cunningham, and J. A. Hoxie. "Detection of Activated Platelets in Whole Blood Using Activation- Dependent Monoclonal Antibodies and Flow Cytometry." *Blood,* Vol. 70, No. 1, pp.307-315, 1987.

24. Klein, Martin J. *Einstein and the Wave-particle Duality.* [Indianapolis]: Bobbs-Merrill, 1964.

25. Planck, Max. "On the Law of Distribution of Energy in the Normal Spectrum." *Annalen Der Physik,* Vol. 4, No. 553, pp.1, 1901.

26. Jablonski, A. "Emission Anisotropy and Decay of Fluorescence of Solution." *Acta Physica Polonica,* Vol. 28, pp.717, 1965.

27. Calvete, J. J. "On the Structure and Function of Platelet Integrin αIIbβ3, the Fibrinogen Receptor." *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.),* Vol. 208, No. 4, pp.346-360, 1995.

28. Shattil, S. J. "Signaling through Platelet Integrin Alpha IIb Beta 3: Inside-out, Outside-in, and Sideways." *Thrombosis and Haemostasis,* Vol. 82, No. 2, pp.318-325, 1999.

29. Nurden, A. T., and J. P. Caen. "An Abnormal Platelet Glycoprotein Pattern in Three Cases of Glanzmann's Thrombasthenia." *British Journal of Haematology,* Vol.28, No. 2, pp.253-260, 1974.

30. WolframAlpha Computatinal Knowledge Engine. Computer software. Http://www.wolframalpha.com/input/?i=+ITGA2B+protein.

31. WolframAlpha Computatinal Knowledge Engine. Computer software. Http://www.wolframalpha.com/input/?i=+ITGB3+protein.

32. McMichael, A. J., N. A. Rust, J. R. Pilch, R. Sochynsky, J. Morton, D. Y. Mason, C. Ruan, G. Tobelem, and J. Caen. "Monoclonal Antibody to Human Platelet Glycoprotein I I. IMMUNOLOGICAL STUDIES." *British Journal of Haematology,* Vol. 49, No. 4, pp.501-509, 1981.

33. Andrews, R. K., Y. Shen, E. E. Gardiner, J. F. Dong, J. A. Lopez, and M. C. Berndt. "The Glycoprotein Ib-IX-V Complex in Platelet Adhesion and Signaling." *Thrombosis and Haemostasis,* Vol.82, No. 2, pp.357-364, 1999.

34. Huizinga, E. G., S. Tsuji, R. A. P. Romijn, M. E. Schiphorst, P. G. De Groot, J. J. Sixma, and P. Gros. "Structures of Glycoprotein Ibα and Its Complex with Von Willebrand Factor A1 Domain." *Science,* Vol. 297, No. 5584, pp.1176-1179, 2002.

35. Von Hundelshausen, P., and C. Weber. "Platelets as Immune Cells: Bridging Inflammation and Cardiovascular Disease." *Circulation Research,* Vol. 100, No. 1, pp.27-40, 2007.

36. Ryan, U., and R. E. Worthington. "Cell-cell Contact Mechanisms." *Current Opinion in Immunology,* Vol. 4, No. 1. pp.33-37, 1992.

37. Johnston, G., R. G. Cook, and R. P. McEver. "Cloning of GMP-140, a Granule Membrane Protein of Platelets and Endothelium: Sequence Similarity to Proteins Involved in Cell Adhesion and Inflammation." *Cell,* Vol. 56, No. 6, pp.1033-1044, 1989.

38. McEver, R. P., and M. N. Martin. "A Monoclonal Antibody to a Membrane Glycoprotein Binds Only to Activated Platelets." *Journal of Biological Chemistry,* Vol. 259, No. 15, pp.9799-9804, 1984.

39. McEver, Rodger P. "GMP-140, a Receptor That Mediates Interactions of Leukocytes with Activated Platelets and Endothelium." *Trends in Cardiovascular Medicine,* Vol. 1, No. 4, pp.152-156, 1991.

40. WolframAlpha Computatinal Knowledge Engine. Computer software. Http://www.wolframalpha.com/input/?i=+P+Selectin+protein.

41. Maecker, H. T., S. C. Todd, and S. Levy. "The Tetraspanin Superfamily: Molecular Facilitators." *The FASEB Journal,* Vol. 11, No. 6, pp.428-442, 1997.

42. Berditchevski, Fedor, Sharon Chang, Jana Bodorova, and Martin E. Hemler. "Generation of Monoclonal Antibodies to Integrin-associated Proteins EVIDENCE THAT α3β1 COMPLEXES WITH EMMPRIN/BASIGIN/OX47/M6." *Journal of Biological Chemistry,* Vol. 272, No. 46, pp.29174-29180, 1997.

43. Rubinstein, Eric, François Le Naour, Cécile Lagaudrière-Gesbert, Martine Billard, Hélène Conjeaud, and Claude Boucheix. "CD9, CD63, CD81, and CD82 Are Components of a Surface Tetraspan Network Connected to HLA-DR and VLA Integrins." *European Journal of Immunology,* Vol. 26, No. 11, pp.2657-2665, 1996.

44. Berditchevski, Fedor, Gianfranco Bazzoni, and Martin E. Hemler. "Specific Association Of CD63 with the VLA-3 and VLA-6 Integrins." *Journal of Biological Chemistry,* Vol. 270, No. 30, pp.17784-17790, 1995.

45. Skubitz, K. M., K. D. Campbell, J. Iida, and A. P. Skubitz. "CD63 Associates with Tyrosine Kinase Activity and CD11/CD18, and Transmits an Activation Signal in Neutrophils." *The Journal of Immunology,* Vol. 157, No. 8, pp.3617-3626, 1996.

46. Nieuwenhuis, H. K., J. J. Van Oosterhaut, E. Rozemuller, F. Van Iwaarden, and J. J. Sixma. "Studies with a Monoclonal Antibody against Activated Platelets: Evidence That a Secreted 53,000-molecular Weight Lysosome-like Granule Protein Is Exposed on the

Surface of Activated Platelets in the Circulation." *Blood,* Vol. 70, No. 3, pp.838-845, 1987.

47. De Haas, M., J. M. Kerst, C. E. Van Der Schoot, J. Calafat, C. E. Hack, J. H. Nuijens, D. Roos, R. H. Van Oens, and A. E. Von Dem Borne. "Granulocyte Colony-stimulating Factor Administration to Healthy Volunteers: Analysis of the Immediate Activating Effects on Circulating Neutrophils." *Blood,* Vol. 84, No. 11, pp.3885-3894, 1994.

48. Mul, Frederik P.J., Edward F. Knol, and Dirk Roos. "An Improved Method for the Purification of Basophilic Granulocytes from Human Blood." *Journal of Immunological Methods,* Vol. 149, No. 2, pp.207-214, 1992.

49. Knol, E. F., M. Hoogerwerf, FP J. Mul, T. W. Kuijpers, and D. Roos. "Analysis Using the Myeloid Panel MAb of Purified Basophils Obtained from Human Blood : Changes in Expression during Degranulation." *Oxford University Press*, pp.1042-1043 1989.

50. Cammack, R. "“Super-reduction” of High-potential Iron-sulphur Protein in the Presence of Dimethyl Sulphoxide." *Biochemical and Biophysical Research Communications,* Vol. 54, No. 2, pp.548-554, 1973.

51. Mathews, Christopher K., K. E. Van Holde, and Kevin G. Ahern. *Bioquímica.* San Francisco: Benjamin Cummings, 1999.

52. Maton, Anthea. *Human Biology and Health.* Englewood Cliffs, NJ: Prentice Hall, 1997.

53. Lehninger, Albert L., David L. Nelson, and Michael M. Cox. *Lehninger Principles of Biochemistry.* New York: Worth Publishers, 2000.

54. White, Abraham, and Abraham White. *Principles of Biochemistry.* New York: McGraw-Hill, 1978.

55. Solomons, T. W. Graham., and Craig B. Fryhle. *Organic Chemistry*. New York: Wiley, 2000.

56. Muir, A., and U. Hopfer. "Regional Specificity of Iron Uptake by Small Intestinal Brush-border Membranes from Normal and Iron-deficient Mice." *The American Journal of Physiology,* Vol. 248, No. 3, pp.376-379, 1985.

57. Gunshin, H., B. Mackenzie, U. V. Berger, Y. Gunshin, M. F. Romero, W. F. Boron, S. Nussberger, J. L. Gollan, and M. A. Hediger. "Cloning and Characterization of a Mammalian Proton-coupled Metal-ion Transporter." *Nature,* Vol. 388, No. 6641, pp.482-488, 1997.

58. Qiu, Andong, Michaela Jansen, Antoinette Sakaris, Sang Hee Min, Shrikanta Chattopadhyay, Eugenia Tsai, Claudio Sandoval, Rongbao Zhao, Myles H. Akabas, and I. David Goldman. "Identification of an Intestinal Folate Transporter and the Molecular Basis for Hereditary Folate Malabsorption." *Cell,* Vol. 127, No. 5, pp.917-928, 2006.

59. Ferris, C. D., S. R. Jaffrey, A. Sawa, M. Takahashi, S. D. Brady, R. K. Barrow, S. A. Tysoe, H. Wolosker, D. E. Barañano, S. Doré, K. D. Poss, and S. H. Snyder. "Haem Oxygenase-1 Prevents Cell Death by Regulating Cellular Iron." *Nature Cell Biology,* Vol. 1, No. 3, pp.152-157, 1999.

60. Donovan, Adriana, Alison Brownlie, Yi Zhou, Jennifer Shepard, Stephen J. Pratt, John Moynihan, Barry H. Paw, Anna Drejer, Bruce Barut, Agustin Zapata, Terence C. Law, Carlo Brugnara, Samuel E. Lux, Geraldine S. Pinkus, Jack L. Pinkus, Paul D. Kingsley, James Palis, Mark D. Fleming, Nancy C. Andrews, and Leonard I. Zon. "Positional Cloning of Zebrafish Ferroportin1 Identifies a Conserved Vertebrate Iron Exporter." *Nature,* Vol. 403, No. 6771, pp.776-781, 2000.

61. Mckie, A. "A Novel Duodenal Iron-Regulated Transporter, IREG1, Implicated in the Basolateral Transfer of Iron to the Circulation." *Molecular Cell,* Vol. 5, No. 2, pp.299-309, 2000.

62. Walters, G. O., F. M. Miller, and M. Worwood. "Serum Ferritin Concentration and Iron Stores in Normal Subjects." *Journal of Clinical Pathology,* Vol.26, No. 10, pp.770-772, 1973.

63. Crichton, Robert R., and Mireille Charloteaux-Wauters. "Iron Transport and Storage." *European Journal of Biochemistry,* Vol. 164, No. 3, pp.485-506, 1987.

64. Shaw, George C., John J. Cope, Liangtao Li, Kenneth Corson, Candace Hersey, Gabriele E. Ackermann, Babette Gwynn, Amy J. Lambert, Rebecca A. Wingert, David Traver, Nikolaus S. Trede, Bruce A. Barut, Yi Zhou, Emmanuel Minet, Adriana Donovan, Alison Brownlie, Rena Balzan, Mitchell J. Weiss, Luanne L. Peters, Jerry Kaplan, Leonard I. Zon, and Barry H. Paw. "Mitoferrin Is Essential for Erythroid Iron Assimilation." *Nature,* Vol. 440, No. 7080, pp.96-100, 2006.

65. Chen, Wen, Prasad N. Paradkar, Liangtao Li, Eric L. Pierce, Nathaniel B. Langer, Naoko Takahashi-Makise, Brigham B. Hyde, Orian S. Shirihai, Diane M. Ward, Jerry Kaplan, and Barry H. Paw. "Abcb10 Physically Interacts with Mitoferrin-1 (Slc25a37) to Enhance Its Stability and Function in the Erythroid Mitochondria." *Proceedings of the National Academy of Sciences of the United States of America,* Vol. 106, No. 38, pp.16263-16268, 2009.

66. Zhang, De-Liang, Robert M. Hughes, Hayden Ollivierre-Wilson, Manik C. Ghosh, and Tracey A. Rouault. "A Ferroportin Transcript That Lacks an Iron-Responsive Element Enables Duodenal and Erythroid Precursor Cells to Evade Translational Repression." *Cell Metabolism,* Vol. 9, No. 5, pp.461-473, 2009.

67. Soe-Lin, S., S. S. Apte, B. Andriopoulos, M. C. Andrews, M. Schranzhofer, T. Kahawita, D. Garcia-Santos, and P. Ponka. "Nramp1 Promotes Efficient Macrophage Recycling of Iron following Erythrophagocytosis in Vivo." *Proceedings of the National Academy of Sciences,* Vol. 106, No. 14, pp.5960-5965, 2009.

68. Hentze, Matthias W., Martina U. Muckenthaler, Bruno Galy, and Clara Camaschella. "Two to Tango: Regulation of Mammalian Iron Metabolism." *Cell,* Vol. 142, No. 1, pp.24-38, 2010.

69. Hoffman, Ronald, Edward Benz, Sanford Shattil, Bruce Furie, and Harvey Cohen. *Hematology: Basic Principles and Practice, 4e (HEMATOLOGY: BASIC PRINCIPLES & PRACTICE.* 4th ed. Philadelphia, PA: Churchill Livingstone, 2005.

70. Akan, H., N. Güven, T. Aydogdu, M. Arat, M. Beksaç, and K. Dalva. "Thrombopoietic Cytokines in Patients with Iron Deficiency Anemia with or without Thrombocytosis." *Acta Haematologica,* Vol. 103, No. 3, pp.152-156, 2000.

71. Bilic, Ernest, and Ervina Bilic. "Amino Acid Sequence Homology of Thrombopoietin and Erythropoietin May Explain Thrombocytosis in Children with Iron Deficiency Anemia." *Journal of Pediatric Hematology/oncology,* Vol. 25, No. 8, pp.675-676 2003.

72. Geddis, Amy E., and Kenneth Kaushansky. "Cross-Reactivity Between Erythropoietin and Thrombopoietin at the Level of Mpl Does Not Account for the Thrombocytosis Seen in Iron Deficiency." *Journal of Pediatric Hematology/Oncology,* Vol. 25, No. 11, pp.919-920, 2003.

73. Racke, Frederick K. "EPO and TPO Sequences Do Not Explain Thrombocytosis in Iron Deficiency Anemia." *Journal of Pediatric Hematology/Oncology,* Vol. 25, No. 11, pp.919, 2003.

74. Schloesser, L. L., M. A. Kipp, and F. J. Wenzel. "Thrombocytosis in Iron- Deficiency Anemia." *Journal of Laboratory and Clinical Medicine,* Vol. 66, pp.107-114, 1965.

75. Perlman, Meryl K., Joel G. Schwab, James B. Nachman, and Charles M. Rubin. "Thrombocytopenia in Children with Severe Iron Deficiency." *Journal of Pediatric Hematology/oncology,* Vol. 24, No. 5, pp.380-384, 2002.

76. Sonneborn, D. "Letter: Thrombocytopenia and Iron Deficiency." *Annals of Internal Medicine,* Vol. 80, No. 1, pp.111, 1974.

77. Kadikoylu, Gurhan, Irfan Yavasoglu, Zahit Bolaman, and Taskin Senturk. "Platelet Parameters in Women with Iron Deficiency Anemia." *Journal of the National Medical Association,* Vol. 98, No. 3, pp.398-402, 2006.

78. Akay, Olga Meltem, Enver Akin, Fezan Sahin Mutlu, and Zafer Gulbas. "Effect of Iron Therapy on Platelet Function among Iron-Deficient Women with Unexplained Menorrhagia." *Pathophysiology of Haemostasis and Thrombosis,* Vol. 36, No. 2, pp.80-83, 2007.

79. Kürekçi, A. E., A. A. Atay, S. Ü Sarici, C. Zeybek, V. Köseoğlu, and O. Özcan. "Effect of Iron Therapy on the Whole Blood Platelet Aggregation in Infants with Iron Deficiency Anemia*." *Thrombosis Research,* Vol. 97, No. 5, pp. 281-285, 2000.

80. Chan, A., and G. Deveber. "Prothrombotic Disorders and Ischemic Stroke in Children." *Seminars in Pediatric Neurology,* Vol. 7, No. 4, pp.301-308, 2000.

81. Greer, M. "Uncommon Causes of Stroke. Part 2. Changes in Blood Constituents and Hemodynamic Factors." *Geriatrics,* Vol. 33, No. 1, pp.51-56, 1978.

82. White, J. G. "The Morphology of Platelet Function." *Methods in Hematology,* Vol. 8, pp.1-25, 1983.

83. Oliveira, Gabriel Lima, Giuseppe Lippi, Gian Luca Salvagno, Martina Mortagnana, Giovanni Poli, Giovanni Pietro Soleno, Geraldo Picheth, and Gian Cesare Guidi. "K(3)EDTA Vacuum Tubes Validation for Routine Hematological Testing." *ISRN Hematology,* Vol. 2012, pp.1-5, 2012.

84. Okano, K., M. Araki, Y. Mimura, H. Nogaki, and K. Ichihara. "Simultaneous Assay of Activated Platelet Count and Platelet-activating Capacity by P-selectin Detection Using K2-EDTA-treated Whole Blood for Antiplatelet Agents." *International Journal of Laboratory Hematology,* Vol. 34, No. 6, pp.621-629, 2012.

85. Yıldırım, Zuhal K., Mehmet F. Orhan, and Mustafa Büyükavcı. "Platelet Function Alterations and Their Relation to P-selectin (CD62P) Expression in Children with Iron Deficiency Anemia." *Blood Coagulation & Fibrinolysis: An International Journal in Haemostasis and Thrombosis,* Vol. 22, No. 2, pp.98-101, 2011.

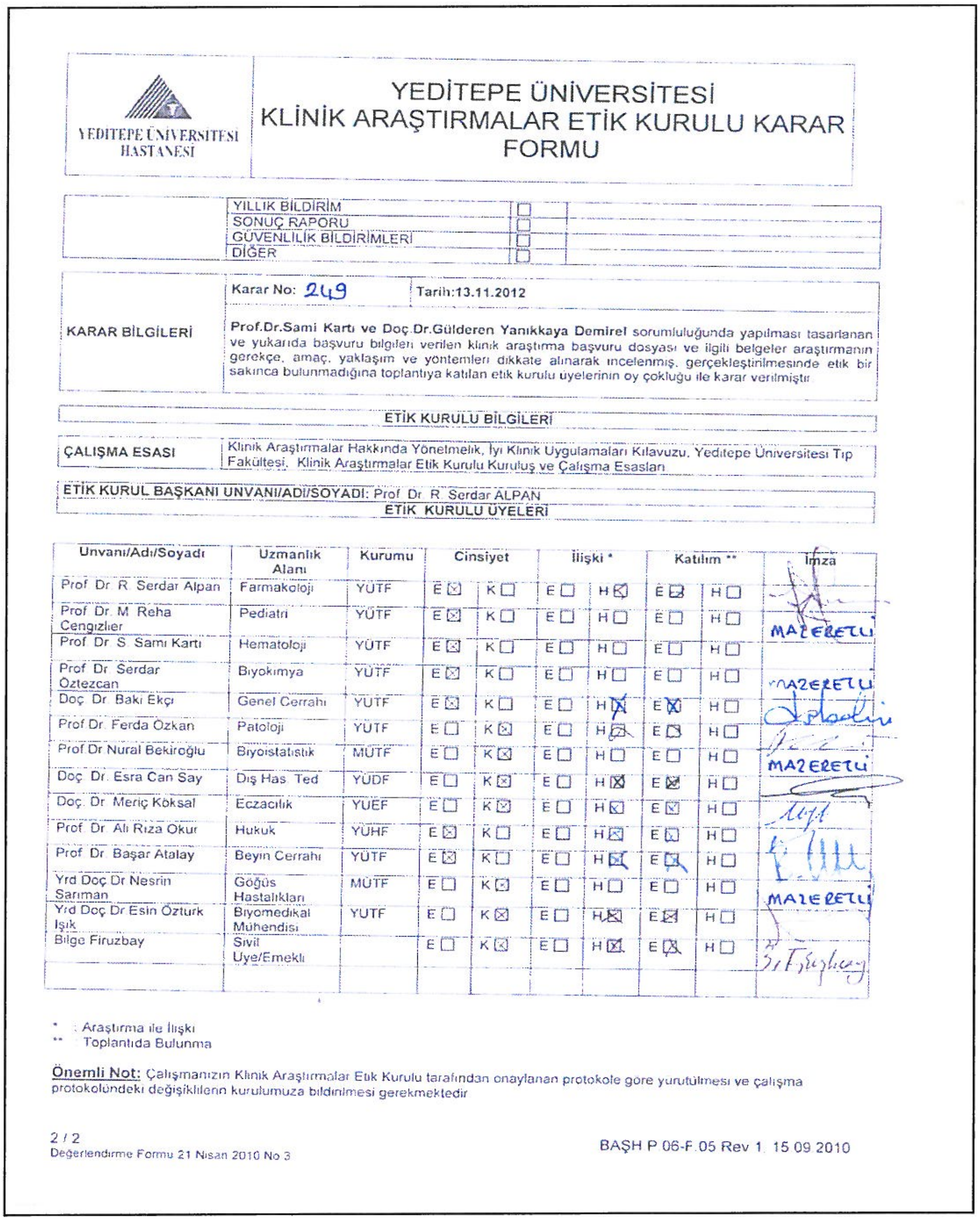
86. McManama, G., J. N. Lindon, M. Kloczewiak, M. A. Smith, J. A. Ware, J. Hawiger, E. W. Merrill, and E. W. Salzman. "Platelet Aggregation by Fibrinogen Polymers Crosslinked across the E Domain." *Blood,* Vol. 66, No. 2, pp.363-371, 1986.

87. Shattil, S. J., J. A. Hoxie, M. Cunningham, and L. F. Brass. "Changes in the Platelet Membrane Glycoprotein IIb.IIIa Complex during Platelet Activation." *Journal of Biological Chemistry,* Vol. 260, No. 20, pp.11107-11114, 1985.

88. Thomas, W. "For Catch Bonds, It All Hinges on the Interdomain Region." *The Journal of Cell Biology,* Vol. 174, No. 7, pp.911-913, 2006.

89. Vickers, John D. "Binding of Polymerizing Fibrin to Integrin Alpha IIb Beta3 on Chymotrypsin-treated Rabbit Platelets Decreases Phosphatidylinositol 4,5-bisphosphate and Increases Cytoskeletal Actin." *Platelets,* Vol. 10, No. 4, pp.228-237, 1999.

APPENDIX A: ETHICS COMMITTEE APPROVAL FORM



YEDİTEPE ÜNİVERSİTESİ HASTANESİ

YEDİTEPE ÜNİVERSİTESİ
KLİNİK ARAŞTIRMALAR ETİK KURULU KARAR FORMU

☐ YILLIK BİLDİRİM
☐ SONUÇ RAPORU
☐ GÜVENLİLİK BİLDİRİMLERİ
☐ DİĞER

KARAR BİLGİLERİ

Karar No: 249 **Tarih:13.11.2012**

Prof.Dr.Sami Kartı ve Doç.Dr.Gülderen Yanıkkaya Demirel sorumluluğunda yapılması tasarlanan ve yukarıda başvuru bilgileri verilen klinik araştırma başvuru dosyası ve ilgili belgeler araştırmanın gerekçe, amaç, yaklaşım ve yöntemleri dikkate alınarak incelenmiş, gerçekleştirilmesinde etik bir sakınca bulunmadığına toplantıya katılan etik kurulu üyelerinin oy çokluğu ile karar verilmiştir.

ETİK KURULU BİLGİLERİ

ÇALIŞMA ESASI: Klinik Araştırmalar Hakkında Yönetmelik, İyi Klinik Uygulamaları Kılavuzu, Yeditepe Üniversitesi Tıp Fakültesi, Klinik Araştırmalar Etik Kurulu Kuruluş ve Çalışma Esasları

ETİK KURUL BAŞKANI UNVANI/ADI/SOYADI: Prof. Dr. R. Serdar ALPAN

ETİK KURULU ÜYELERİ

Unvanı/Adı/Soyadı	Uzmanlık Alanı	Kurumu	Cinsiyet		İlişki *		Katılım **		İmza
Prof. Dr. R. Serdar Alpan	Farmakoloji	YÜTF	E ☒	K ☐	E ☐	H ☒	E ☒	H ☐	
Prof. Dr. M. Reha Cengizler	Pediatri	YÜTF	E ☒	K ☐	E ☐	H ☐	E ☐	H ☐	MAZERETLİ
Prof. Dr. S. Sami Kartı	Hematoloji	YÜTF	E ☒	K ☐	E ☐	H ☐	E ☐	H ☐	
Prof. Dr. Serdar Öztezcan	Biyokimya	YÜTF	E ☒	K ☐	E ☐	H ☐	E ☐	H ☐	MAZERETLİ
Doç. Dr. Bakı Ekçi	Genel Cerrahi	YÜTF	E ☒	K ☐	E ☐	H ☒	E ☒	H ☐	
Prof. Dr. Ferda Özkan	Patoloji	YÜTF	E ☐	K ☒	E ☐	H ☒	E ☒	H ☐	
Prof. Dr. Nural Bekiroğlu	Biyoistatistik	MÜTF	E ☐	K ☒	E ☐	H ☐	E ☐	H ☐	MAZERETLİ
Doç. Dr. Esra Can Say	Diş Has. Ted.	YÜDF	E ☐	K ☒	E ☐	H ☒	E ☒	H ☐	
Doç. Dr. Meriç Köksal	Eczacılık	YÜEF	E ☐	K ☒	E ☐	H ☒	E ☒	H ☐	
Prof. Dr. Ali Rıza Okur	Hukuk	YÜHF	E ☒	K ☐	E ☐	H ☒	E ☒	H ☐	
Prof. Dr. Başar Atalay	Beyin Cerrahi	YÜTF	E ☒	K ☐	E ☐	H ☒	E ☒	H ☐	
Yrd. Doç. Dr. Nesrin Sarıman	Göğüs Hastalıkları	MÜTF	E ☐	K ☒	E ☐	H ☐	E ☐	H ☐	MAZERETLİ
Yrd. Doç. Dr. Esin Öztürk Işık	Biyomedikal Mühendisi	YÜTF	E ☐	K ☒	E ☐	H ☒	E ☒	H ☐	
Bilge Firuzbay	Sivil Üye/Emekli		E ☐	K ☒	E ☐	H ☒	E ☒	H ☐	

* : Araştırma ile İlişki
** : Toplantıda Bulunma

Önemli Not: Çalışmanızın Klinik Araştırmalar Etik Kurulu tarafından onaylanan protokole göre yürütülmesi ve çalışma protokolündeki değişikliklerin kurulumuza bildirilmesi gerekmektedir.

2 / 2
Değerlendirme Formu 21 Nisan 2010 No 3

BAŞH P 06-F.05 Rev 1. 15.09.2010

Figure A1. Ethics Committee Approval Form