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# Platelet Plasma Membrane Comparison between Pregnant and Non-pregnant Females

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# Platelet Plasma Membrane Comparison between Pregnant and Non-pregnant Females

By

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## Thesis

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# Platelet Plasma Membrane Comparison between Pregnant and Non-pregnant Females

## Abstract

Hemostasis is dependent on the functioning of three systems: blood vessels, platelets, and plasma proteins. During vessel injury, platelets adhere, secrete the contents of granules and aggregate. Each event in platelet activation is dependent upon plasma membrane proteins and a deficiency in any one of the plasma membrane proteins leads to excessive bleeding. Pregnancy is described as a hypercoagulable state during which frequent bleeding abnormalities are observed. The mechanism of hypercoagulability is not yet known. In an attempt to shed light on the hypercoagulable state, this study compares the plasma membrane proteins between pregnant and non-pregnant females.

# Acknowledgments

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## Introduction

### Background

Platelets are an essential mediator of hemostasis along with the blood vessels and proteins of the coagulation system. Platelets are crucial in allowing the body's hemostatic system to function properly through the formation of a platelet aggregation and to provide the surface for coagulation to occur. These anuclear cell fragments derived from bone marrow's megakaryocytes are not just cellular pieces designed to plug "leaky" blood vessels. These complex cell fragments contain different granules that release their contents upon stimulation through a signaling mechanism using receptors on the platelet surface. The contents of the granules recruit more platelets and also activate the other systems of coagulation. The stimulation and response is graded and goes from a normal platelet not sticking to the endothelium lining the vascular system to a "platelet plug" when the subendothelial connective tissue is exposed and if the stimulus is strong enough the platelets will form a "viscous metamorphasis"—an irregular mass of degenerated platelet material without membranes (Harmening, 1997). The graded response is dependent on the extent of damage with which differing amounts of a variety of substances such as ADP, epinephrine, $Ca^{2+}$, collagen, thrombin, and thromboxane are released to stimulate the platelets. This platelet system is held in check to keep the vascular system intact but not to have the platelets overactivate occluding blood vessels yielding the surrounding tissues hypoxic.

During pregnancy the female body changes to accommodate the developing fetus within a placenta. This includes alterations in the hemostatic mechanism and its

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parameters. Coagulation factors that increase during pregnancy include factors VII, VIII, X, XII, and vWF. There also seems to be a suppression of fibrinolysis, an increase of soluble fibrin, and an increase in fibrin degradation products (FDPs) (O'brien, 1986). The platelet count during pregnancy is controversial with some studies demonstrating no change and others indicating a slight decrease in platelet number (Perry, 1992). With the hemostatic changes described above, pregnancy has been referred to as a state hyper-coagulation.

## Problem Statement

Pre-eclampsia is a complication related to pregnancy characterized by hypertension, proteinuria, and edema. Pre-eclampsia affects between 1-5% of pregnancies. When the triad of symptoms is accompanied by convulsions the term eclampsia is used. This occurs in 0.05% of the pre-eclamptic gravids and the damage to mother and fetus is obvious. While the diagnosis is quite clear the cause and treatment are not. Though thrombocytopenia is clinically seen in only about 15% of pre-eclamptic patients, the platelet has been hypothesized as the source of the problem. Many studies have shown that the platelet is hyperactivated during pre-eclampsia (ie. Norris et al. 1993 and Kilby et al. 1993). As to date, there is no known cardiovascular or hemostatic predictor, including platelet factors or markers, that are able to predict whether pre-eclampsia will occur in a patient. The platelet is hypothesized to be the cause of pre-eclampsia primarily in the fact that aspirin treatment lessens the damaging effects of pre-eclampsia. Aspirin inhibits the cyclooxygenase enzyme on the platelet membrane which

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is responsible for converting certain membrane phospholipids into thromboxane, a potent platelet aggregator and vasoconstrictor.

How the platelet becomes hyperactivated during pre-eclampsia is unknown. It is not even known if and how the platelet changes during normal pregnancies. Before the platelet changes are compared between normal pregnant and pre-eclamptic patients it must first be established whether the platelet changes normally occur during pregnancy along with the other hemostatic parameters.

## Purpose

The purpose of this study is to establish what changes occur to the platelet during normal pregnancy. Platelets will be examined from two populations: nonpregnant females and normal pregnant females. Normal pregnancy in this context refers to pregnancy uncomplicated by hypertension, thrombocytopenia, or pre-eclampsia. A compete blood count will be run to evaluate platelet count, platelet distribution width (PDW) and any CBC abnormality revealed. Flow cytometry will then be used to study the changes to different receptors on the platelet membrane including the receptors for platelet adhesion, platelet aggregation, and different receptors indicating activation.

This study will discern whether pre-eclampsia is most likely due to an overcompensation of the platelet to the added pressures on the body during pregnancy or whether it is a disease state unlike any changes that occur to the platelet.

## Research Hypothesis

During pregnancy, the female's body changes to compensate for the growing fetus. Changes in the platelet membrane receptors are also included in the overall changes in the female's body during pregnancy.

## Hypothesis

1) During normal pregnancy, there are increases in platelet proteins which are essential for platelet function (GP Ib, GP IX, GP IV, GP IIb, and GP IIIa).

2) During pregnancy there is an increase in circulating activated platelets.

# Chapter 2
## Literature Review

### Introduction

The three components of hemostasis, the arrest of bleeding, are the blood vessels, platelets, and the plasma proteins. Blood vessels play a role by vasoconstricting during vascular injury. Platelets provide primary hemostasis, which is described as the aggregation of platelets forming a platelet plug at the site of injury. The third component, plasma proteins, provides secondary hemostasis by forming a strong fibrin clot, reinforcing the platelet plug.

### Vascular system

The vascular system helps to prevent bleeding by two mechanisms. First, it contracts the damaged vessels, vasoconstriction, and secondly it relaxes the surrounding vessels which diverts blood flow around the damaged vasculature. The vascular system not only reroutes blood flow around damaged vasculature it initiates primary and secondary hemostasis. Only when the integrity of the endothelial cells lining the blood vessels is comprimised do platelets begin aggregation and thrombin begins converting fibrinogen into the fibrin clot. Thus, the vascular system prevents indiscriminate activation of the other hemostatic mechanisms.

### Platelet

### Origin/ lifecycle

The platelet or thrombocyte is a cell fragment of the largest hematopoietic cell, the megakaryocyte. An understanding of the platelet- megakaryocyte system is crucial in understanding the clinical significance of pre-eclampsia in which thrombocytopenia and other changes in the platelet are observed.

All hematopoietic cells begin as a pleuripotent stem cell in the bone marrow. These pleuripotent stem cells have the capacity for both continuous replication and differentiation (Harmening, 1997). The differentiaition occurs via different actions of cytokines, which are soluble mediators of cell communication. Two groups of cytokines involved in megakaryopoiesis are colony stimulating factors (CSFs) and interleukins (ILs). Through the actions of the different CSFs and ILs these pleuripotent stem cells differentiate into a CFU-GEMM, colony forming unit- granulocyte erythroid, monocyte/macrophage, megakaryocyte. The CFU-GEMM is a semicommitted stem cell which is the progenitor cell of the myeloid family of blood cells (Harmening, 1997). Under the directions of certain specific cytokines the CFU-GEMM differentiates into a megakaryoblast.

The megakaryoblast is a premature form of the megakaryocyte. A staging system has been established to follow the maturation of the megakaryoblast to the megakaryocyte using four defined criteria. These four criteria, nuclear to cytoplasmic ratio, nuclear shape, basophilia, and cytoplasmic granularity, conveniently divide the maturation process of the megakaryocyte into four stages (Rifkin, 1998). It is important to note that during the maturation process the megakaryocytic cells are able to undergo endomitosis. Endomitosis refers to the ability of a cell to undergo mitotic divisions without cytoplasmic divisions generating very large multinucleated or polyploid cell (Rifkin, 1998). The megakaryocytic cells can therefore have 1, 2, 4, 8, 16, or even 32 nuclei. Each nucleus will contain the diploid set of chromosomes and therefore the average megakaryocyte, which contains 8 lobes, will have 16 single sets of chromosomes, referred to as 16N.

The four stages of the megakaryocyte maturation include the megakaryoblast (stage I), the promegakaryocyte (stage II), the granular megakaryocyte (stage III) and finally the megakaryocyte. The cell size increases during this maturation process from about 15-50 um in diameter for the megakaryoblast all the way up to megakaryocyte, which can be 100 um in diameter. During the maturation, endomitosis is also occurring and the nuclear/ cytoplasm ratio is changing from about 4/1 to between 1/1- 1/12 in the megakaryocyte. The cytoplasm becomes more granular as maturation is occurring, mostly due to an increase in the numbers of mitochondria and polyribosomes, both of which are important structural constants needed to fuel the cell and make the proteins that will be shedded into platelets. During this maturation a network known as the demarcating membrane system (DMS) is forming via invaginations of the plasma membrane (Rifkin, 1998). The DMS becomes the future plasma membrane system of the megakaryocyte offspring—the platelet. By the time the megakaryocyte matures the DMS has become very extensive and through here platelets will bud off.

The platelets shed from the megakaryocyte usually near a venous sinus which facilitates the shedding (Rifkin, 1998). The platelets do not leave the marrow through a fenestrae but rather the proplatelets squeeze through the endothelial cell bodies. Each megakaryocyte can shed between 1,000 and 5,000 platelets. Once the megakaryocyte is exhausted it undergoes pyknosis and is phagocytized by neighboring macrophages. The platelets that are shed are between 1 and 4 um in diameter and 0.5 and 1.0 um in thickness. They may be round, oval, or rod shaped and appear as dense blue to purple particles with granules that stain with graded intensity during Romanowsky stain preparation (Rifkin, 1998). Once in the peripheral circulation, platelets are thought to

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circulate between 8 and 12 days. If one assumes a normal platelet count of 150,000 and 450,000 / ul a steady state turnover would require 1.2-1.5 *10 11 platelets shed/ day (Rifkin, 1998). Of the total population of platelets in the body one-third are found sequestered in the spleen and these become available for use upon need.

## Structure of the platelet

A description of the platelet anatomy is important to discuss in order to more fully understand what changes occur in the platelet during pregnancy and preeclampsia. "Structure dictates function," is a quote often told to beginning physiology students and in this case a discussion of the structure will clarify later sections on function of platelets and the mechanism by which this occurs. There are three areas of which the platelet anatomy can be divided: the plasma membrane, the cytoskeleton (sol gel zone), and the organelles. Each will be discussed separately.

## Plasma membrane

The platelet's plasma membrane is approximately 7.5 nm thick and has the typical phospholipid trilaminar structure. Overlying the plasma membrane is a surface coat of glycocalyx that is between10 and 50 nm thick. There are a number of glycoproteins present in this layer including: Ia, Ib, Ic, IIa, IIb, III, IV, V, and IX (Rifkin 1998). These glycoproteins are receptors essential for normal platelet function: adhesion, aggregation, and secretion, which will be discussed later. The glycocalyx also provides a surface to which some factors of the coagulation system may adhere. The underlying plasma membrane is also very important for platelet function. The ionic pumps within the membrane maintain the ionic gradient. The phospholipid constituents provide a source of fatty acids used by certain enzymes to generate prostaglandins, which are potent

vasoactive agents (Moncada, 1979). The platelet plasma membrane also contains some of the factors of the coagulation system and is also a place where certain coagulation enzyme complexes are assembled. The platelet has a series of channels throughout it called the open cannalicular system (OCS) which are extensions of the plasma membrane. Though the platelet is usually referred to as "discoid a more useful mental image of platelets may be of small sponges." (Ault, 1993) The OCS increase platelet surface absorption allowing increased numbers of plasma procoagulants to be collected increasing fibrin formation and platelet stored products can also be released more rapidly through here (Harmening, 1997).

## Sol Gel Zone

The cytoskeleton or sol gel zone is the part of the platelet that is responsible for the structural arrangement of the platelet. Microtubules and microfilaments are the two main components of the cytoskeleton. The microtubules form a circumferential band around the periphery of the cytoplasm maintaining the platelets discoid shape (Rifkin, 1998). During platelet stimulation the microtubules contract toward the center of the platelet "bringing the organelles towards the interior and their reorganization which facilitates the secretory process" (Harmening, 1997). The microtububules will also later appear in the pseudopods. During stimulation the microtubules appear to prevent secretion in response to a minimal stimulus. The microfilaments are responsible for the pseudopod formation in the stimulated platelet. Two proteins associated with the microfilaments, actin and myosin, are contractile proteins by nature and facilitate the quick response of the activated platelet. Though it is not clear how this occurs certain facts are known. Platelet activation via an agonist increases intracellular $Ca^{2+}$, which

activates certain microfilament binding proteins. One example is gel solin which when

activated by the increased $Ca^{2+}$, severs actin filaments which is thought to be important in

microfilament reorganization (Williams, 1999).

The cytoskeleton is also important in that it is tied in with other platelet structures.

The GPIIb/IIIa fibrinogen receptor is an example. It is known that a portion of the

receptor is functionally and structurally linked to the cytoskeleton. Experiments have

shown that if the microfilaments cannot polymerize partial inhibition is seen with the

induced activation via the GPIIb/IIIa receptor (Williams, 1999). In the unstimulated

platelet, though, the microfilaments are scattered throughout the platelet ready to contract

with the proper stimulus.

<u>Organelles</u>

The organelles constitute the major portion of the platelet cytoplasm. These

structures include: dense bodies, $\alpha$ granules, and mitochondria. Other structures are less

prevalent but are still required for proper platelet function and include lysosomes,

perioxisomes, and glycogen granules (Harmening, 1997).

| PLATELET GRANULE CONTENTS |
| --- |
| **DENSE BODIES** |
| ADP, ATP |
| GDP, GTP |
| Calcium |
| Magnesium |
| Serotonin |
| |
| $\alpha$-GRANULES |
| Platelet factor 4 (PF4) |
| $\beta$-Thromboglobulin |
| Fibrinogen |
| von Willebrand Factor |
| Fibronectin |
| Thrombospondin |
| Factors V and XI |
| Protein S |
| Plt. Derived Growth Factor |
| Transforming Growth Factor $\beta$ |
| Endothelial cell Growth Factor |
| Epidermal growth factor |
| $\alpha_2$-Plasmin inhibitor |
| Plasminogen activator inhibitor-1 |

Table 1

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The α-granules are the most numerous granule in the cytoplasm numbering about 20-200/ platelet. The α-granules contain mainly coagulation factors and proteins involved with platelet aggregation. Table 1 shows many of the different contents of the α-granule. Some of these factors will be discussed in the upcoming sections because of their importance in magnifying or changing the platelet activity or the coagulation/ vascular system's activity. Two examples of the importance of secretion of the α-granule is in the factors, platelet factor 4 (PF4) and β-thromboglobulin. Both these substances bind heparin and neutralize it (Rifkin, 1998). Heparin is the primary inhibitor of thrombin, which stabilizes a clot and therefore the roles of these factors is obvious.

The dense granules are less numerous than the α-granules (2-10/ platelet) and do not contain as many different substances. Table 1 also lists the contents of the dense bodies. Probably the most important substance released from the dense bodies is ADP. This is a platelet aggregator but it's functioning time is short lived. After it's rapid breakdown it is converted into adenosine which indirectly decreases aggregation (Rifkin, 1998). The $Ca^{2+}$ and $Mg^{2+}$ released are important in activating different mechanisms of the platelet. A more detailed discussion of some of these factors and substances is forthcoming.

The review by Rifkin (1998) states that "the significance of their (the lysosome) release is unclear, however during platelet secretion lysosomal contents are released more slowly and incompletely than other granules." The lysosomes may digest substances that the platelet endocytoses but the "platelets have limited ability to phagocytize any constituent" (Rifkin, 1998). An article by Sternberg (Rifkin, 1998) adds that the lysosomes require stronger agonists for release than α-granules, and this would suggest

that lysosomes have a greater role in lysis of thrombin than in mediating a hemostatic response.

A description of platelet origin and structure has been put forth. The role of platelets and the plasma coagulation system will be discussed next along with how the platelet is involved in this system. An overview of the coagulation system with the platelets role will be important to future sections which will be going into very detailed descriptions of specific receptors and factors involved in the platelets role in hemostasis. This study is examining whether changes occur with the platelet receptors during pregnancy and therefore such a description is necessary.

<div align="center"><u>Primary Hemostasis</u></div>

After damage to a blood vessel subendothelial connective tissue is exposed. The platelet will reversibly adhere to the subendothelium via a plasma protein, von Willebrand Factor (vWF), and a receptor on the platelet called glycoprotein Ib (GPIb). The platelet can also rapidly adhere and be activated by collagen directly. This initial adhesion acts to plug the site of bleeding. If the damage is more severe platelet aggregation and the release reaction will occur. It is difficult to separate these two events since they are closely tied together. These two processes occur if a stronger stimulus is present leading to granule release. In vivo, a platelet usually experiences the action of several agonists (ie. collagen, ADP, $TXA_2$, adrenalin, and thrombin) and it is the synergy that determines the extent of platelet activation. The release reaction occurs with the dense granules first dumping their contents into the extracellular plasma followed by the $\alpha$-granules. Within the granules are many different substances, two of which are ADP and $Ca^{2+}$. These two serve to amplify the process, making this a positive feedback loop.

The platelets go through an irreversible shape change with the pseudopod formation. A

third factor released, fibrinogen, binds to it's receptor on the platelet surface (GPIIb/IIIa)

with $Ca^{2+}$ aiding in the ligand formation.

### Secondary hemostasis

The goal of the coagulation system is the formation of fibrin. Through a cascade

of biochemical events thrombin is activated and then converts fibrinogen into fibrin,

which stabilizes the initial platelet plug that has formed at the site of vascular injury.

Most factors involved with the coagulation system normally circulate in an

inactive state. Once one is activated the others follow like a waterfall or cascade. There

are three divisions of this system an intrinsic, an extrinsic, and a common pathway where

the intrinsic and extrinsic meet. Figure 1 illustrates these systems.
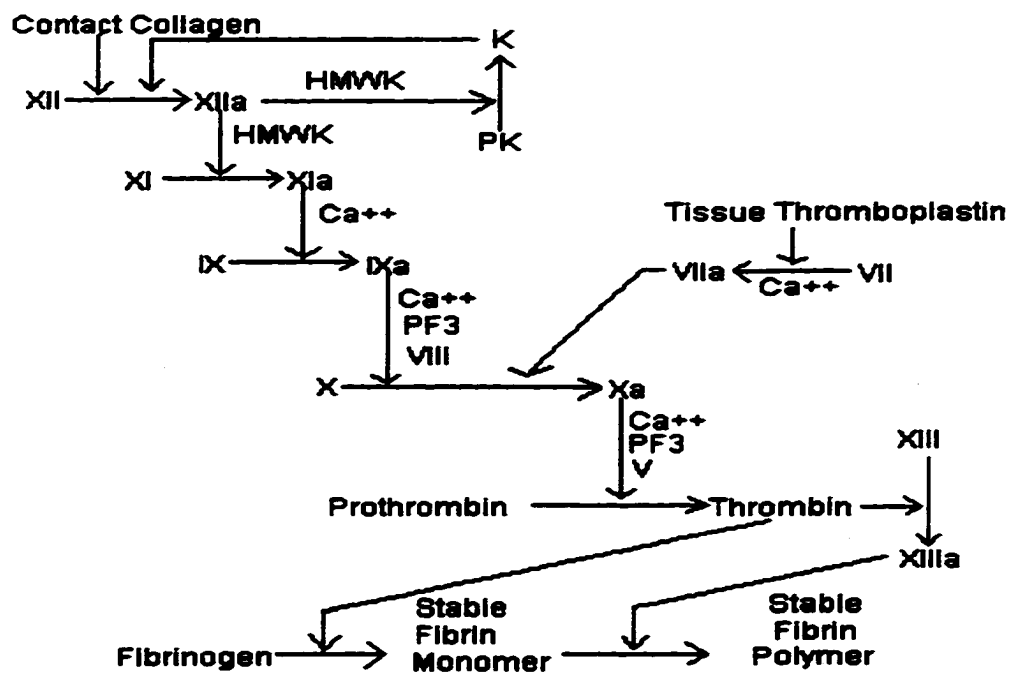


FIGURE 1

The activation of the intrinsic pathway occurs when blood is exposed to collagen

or the endothelial basement membrane. Factor XII, normally present in plasma,

undergoes a conformational change when bound to these substances and becomes activated. Factor XIIa hydrolyzes prekallekrein and Factor XI, if HMWK, a cofactor, is also present. Prekallekrein is cleaved into kallekrein. Kallekrein is an enzyme, which can form bradykinin, a potent substance in the inflammatory process and secondarily it feeds back and potentiates Factor XIIa. The other factor, XIa, will activate Factor IX in the presence of $Ca^{2+}$, the next step in the cascade. Factor IXa will participate in the formation of the Xase complex on the activated platelet's membrane. The other participants are Factor VIII, $Ca^{2+}$, and PF3. PF3 is not a specific factor but rather the state of the activated platelet surface. "The activated platelet surface accelerates these complexes (Xase and prothrombinase) several thousand fold by increasing proximity and concentration of reactants and provides the appropriate phospolipid moieties needed for both reactions... this was formally referred to as PF3 activity (Rifkin, 1998).

The Xase complex is the beginning of the common pathway and will activate Factor X. Factor X can be activated by the extrinsic pathway, which begins with Factor VII. When a vessel wall is damaged, tissue factor (also referred to as Factor III or thromboplastin) is released. If any $Ca^{2+}$ is present Factor VII will be activated to Factor VIIa via a conformational change rather than a peptide bond cleavage.

Factor Xa will join with $Ca^{2+}$ and Factor V to form the prothrombinase complex on the activated platelet membrane (PF3). Prothrombin is now converted to thrombin. Thrombin will convert the fibrinogen into fibrin monomers. Thrombin, though, has other functions to aid in the clot formation. It feeds back to increase the amount of Factors V and VIII thereby amplifying the intrinsic cascade. Thrombin also activates Factor XIII which crosslinks the fibrin monomers into fibrin polymers. The crosslinked clot is now

stronger and less likely to rapidly be dissolved by the fibrinolytic system. Thrombin has

the function to activate parts of the fibrinolytic system aiding in the lysis of the fibrin

clot.

## Mechanism of platelet activation

$Ca^{2+}$ plays a critical role in platelet activation. The mechanism by which this

occurs is shown in figure 2. The extracellular agonist will activate a G protein and this G

protein activates phospholipase C (PLC). PLC cleaves phosphatidyl inositol 4, 5-

bisphosphate, $PIP_2$, into two signaling molecules- one is membrane bound,

diacylglycerol, DG, and the one that is cytosolic is inositol 1, 4,5- trisphosphate, $IP_3$.



FIGURE 2

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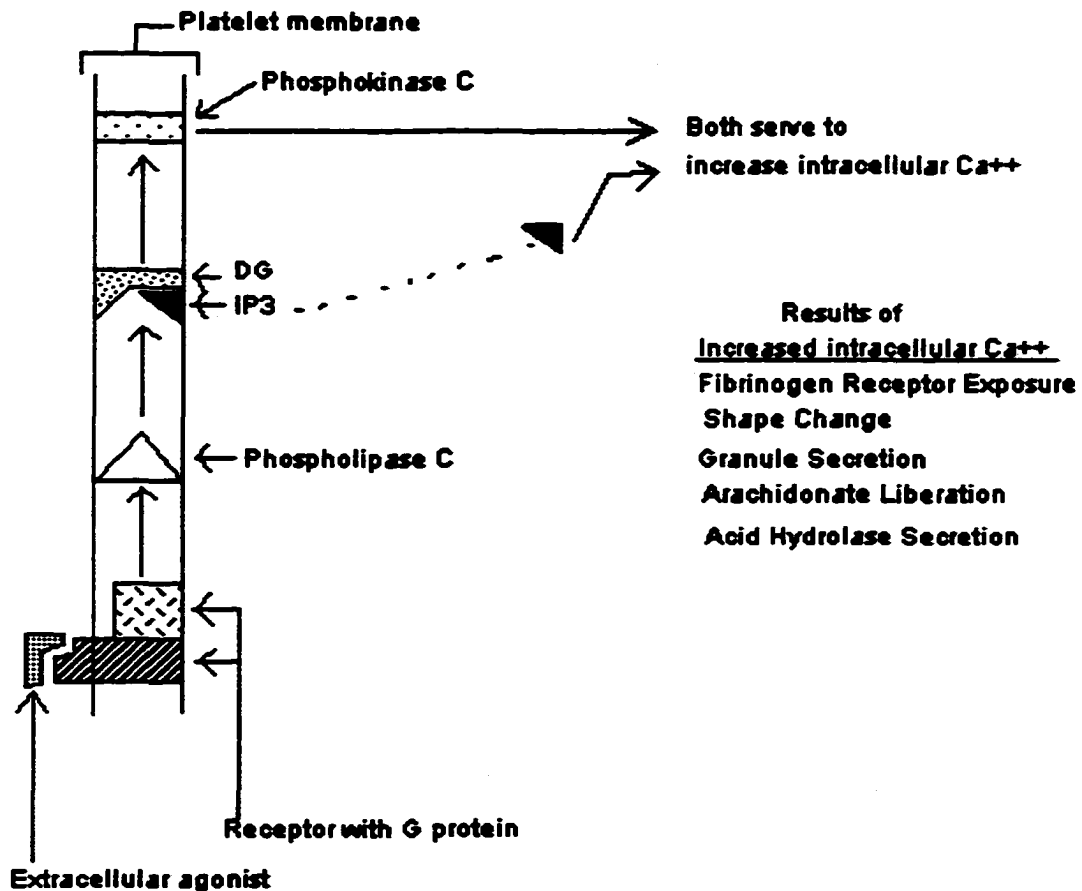
Both of these serve to elevate cytosolic $Ca^{2+}$, $IP_3$ (via $Ca^{2+}$ sensitive channels within the platelet) and DG (via activating other kinases, protein kinase C (PKC)). Platelet response is executed by the concentration of $Ca^{2+}$ in the cytosol with shape change requiring the smallest amount and aggregation requiring a higher amount (Holmsen, 1994). Figure 2 also indicates that the $Ca^{2+}$ will lead to granule release providing proof that the positive feedback loop does occur.

<u>Thromboxane synthesis</u>

In addition to increases in $Ca^{2+}$ during platelet activation, thromboxane is synthesized and released. Thromboxane ($TXA_2$) is a potent platelet aggregator and vessel wall constrictor. The pathway by which $TXA_2$ is made and how it is regulated is important in a discussion of platelet activation. Platelet agonists such as collagen, thrombin, and ADP activate phospholipase $A_2$, which is an enzyme that hydrolyzes arachidonate in the membrane to free it from the membrane. Free arachidonate is oxygenated by two different mechanisms, the cyclooxygenase and the lipoxygenase pathways, though the roles of the byproducts of the lipoxygenase pathways are unclear (Holmsen, 1994).

The cyclooxygenase pathway takes arachidonate and converts it into different products, one being $TXA_2$. $TXA_2$ induces platelet aggregation via decreasing the levels of cAMP in the platelet. Aspirin, which is a cyclooxygenase inhibitor, can be used to prevent thrombotic phenomenon and therefore the importance of $TXA_2$ in platelet aggregation can be seen (Moncada, 1979). $TXA_2$ activity is short lived: it is an unstable molecule with a half life of only 30 seconds and the byproducts do not affect platelet aggregation (Moncada, 1979). The potency of $TXA_2$ action needs to be regulated,

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otherwise major thrombotic events will occur. A second product of the cyclooxygenase

pathway is prostacyclin (PGI₂), which has anti platelet aggregatory properties along with

vasodilatory properties of vessel walls. Figure 3 shows the balance between the two

prostaglandins. Prostacyclin is not made in the platelet but rather within the endothelial

cells lining the blood vessels. Prostacyclin serves to activate adenylate cyclase increasing

cAMP levels so under normal conditions TXA₂ and PGI₂ counteract each other's actions

to maintain homeostasis with respect to platelet function (Walsch, 1998). During a

pregnancy complicated by pre-eclampsia and/ or pregnancy induced hypertension (PIH)

it is hypothesized that platelets increase their production of TXA₂ and therefore the

increased hypertension and platelet activation would be seen. This study will be looking

at whether increased platelet activation is occurring during pregnancy and therefore the

results of this are obvious.
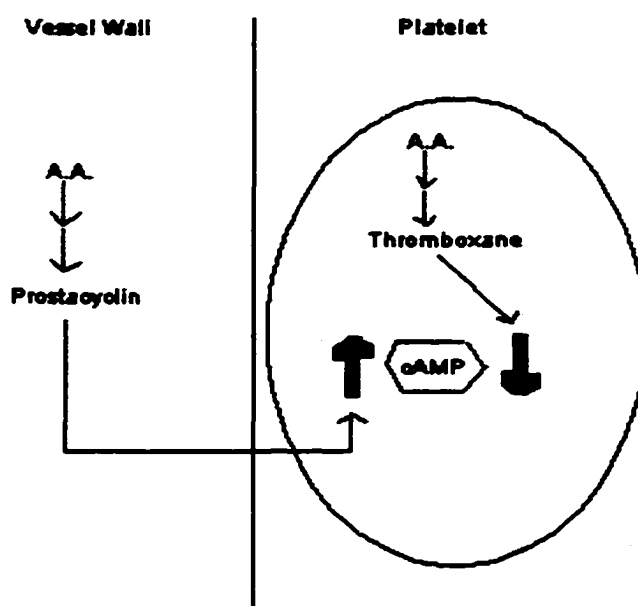


FIGURE 3

## GPIb

Glycoprotein Ib is the platelet receptor responsible for initial platelet adhesion via

a plasma protein called vWF. The receptor is a tetramer of four different units each

having a function in the cell signaling. While vWF is the primary ligand for this receptor

it is not the only one. Thrombin also uses one type of GPIb to initiate its strong

activation properties. The signaling mechanism once GPIb is activated is partially

understood and it is known that vWF and thrombin use different mechanisms. vWF

changes its conformation according to the shear stress within the blood vessel and these

different conformations have different platelet activation potentials. Looking at these

points in depth will give a greater understanding what GPIb does and how it interacts

with the platelet and hemostasis. This discussion will clarify why changes in its number

or density could be responsible for the hypercoagulable state seen in pre-eclampsia.

The platelet glycoprotein Ib is formed from four homologous polypeptide

subunits, GPIbα, GPIbβ, GPIX, and GPV, and each of these is encoded by a unique gene

(Dong, 1997). There are about 25,000 GPIb complexes/ platelet and this complex can be

seen in figure 4 below. Though the vast majority of receptors exist on the platelet surface
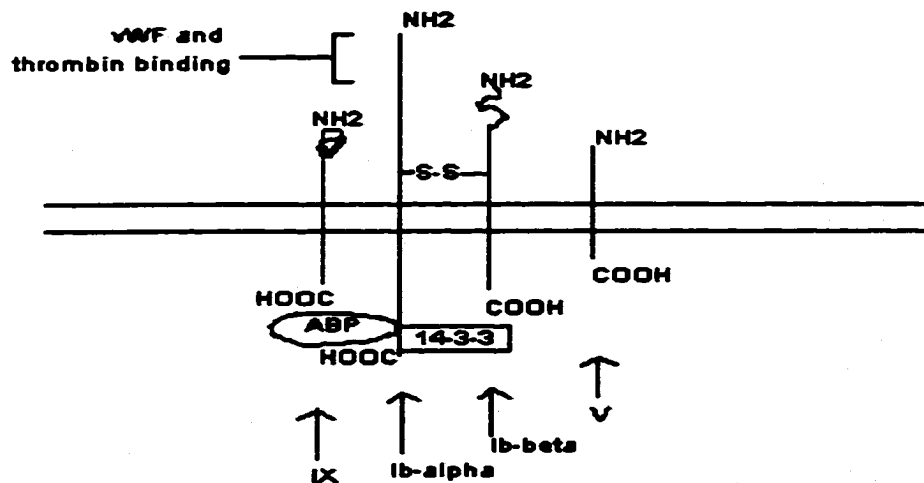
certain studies have shown GPIb receptor localization to vacuolar structures, but the researchers are not able to differentiate whether these are true vacuoles or parts of the OCS (Drake, 1986). GPIbα is by far the largest subunit, with over half of the total mass in it and it contains the longest cytoplasmic domain, totaling about 100 amino acids. This region was shown to mediate associations with the cortical cytoskeleton of the platelet by directly binding actin binding proteins (ABP) and this association may modulate ligand binding (Dong, 1997). This subunit is also responsible for the vWF and thrombin binding. The Dong article also demonstrated through truncated receptors that GPIbα helps anchor the receptor complex in place to provide the optimal conformation to bind vWF.

The GPIbβ subunit is said to serve as a link between GPIbα and GPIX even though there is a disulfide link between GPIbα and GPIbβ but only a noncovalent association between the other subunits. GPV's role in this complex is mostly unknown but studies have indicated that GPV is required for efficient surface expression of GPIbα and GPIX (Parisse, 1999).

The mechanism by which GPIb signals the platelet to activate is also complex. In general, activation via vWF causes the breakdown of $PIP_2$ in the platelet membrane leading to an increase in cytosolic $Ca^{2+}$. Figure 4 also showed a 14-3-3 protein attached to GPIbα. This is thought to be the human isoform of $PLA_2$ which is the enzyme known to produce $TXA_2$ (Parisse, 1999). The third signaling mechanism associated with this receptor is the ABP. This protein is cleaved by a protein called calpain which then allows it to depolarize the platelet cytoskeleton so actin filament reorganization can occur which is part of platelet activation discussed previously. An article by Yuan et al. (1997)

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showed that vWF activation of GPIb can induce cytoskeletal translocation in aggregating and spreading platelets. This can occur independent of GPIIb/IIIa, which is the receptor known to play a central role in cytoskeletal signaling.

vWF is the primary factor that activates GPIb. It induces adhesion, aggregation, and spreading of activated platelets. vWF circulates in plasma at 7ug/ml and is also present in $\alpha$-granules and weibel palade bodies of endothelial cells (Siedlecki, 1996). Upon blood vessel injury vWF is exposed and mediates platelet adhesion. vWF changes its conformation depending on the shear force in the surrounding circulation so it is more active in microcirculation and stenosed arteries. It was not until 1997 when Siedlecki et al. proved the hypothesis and demonstrated that inactive vWF was a globular protein and as shear force was increased protein unfolding occurred and vWF took an extended conformation. They also showed vWF binds to any hydrophobic surface and being such a large molecule (260 kDa) the greater the shear the more hydrophobic contacts between it and the underlying connective tissue, artificial cardiac devices, or components of arthesclerotic plaques. A study by Chow et al. (1992) demonstrated that platelets in a high shear environment will have an increased cytosolic $Ca^{2+}$. The important point they made was that this was entirely from a transmembrane influx of $Ca^{2+}$. The kinetics of this response is slower than say $Ca^{2+}$ release via thrombin or ADP which releases $Ca^{2+}$ from internal stores but this transmembrane influx of $Ca^{2+}$ preceded ADP release. They showed that vWF binding to GPIb is absolutely essential for shear stress induced $Ca^{2+}$ influx which lead to aggregatory response while vWF binding to GPIIb/IIIa potentiates the shear response but is insufficient to do this without GPIb (the mechanism by which this is mediated is still unknown) (Chow, 1992).

While vWF is the primary ligand for GPIb, thrombin is another. Thrombin is known to have greater than one receptor on platelets and one is known to be on the GPIb receptor. The GPIb thrombin receptor is known to be a high affinity receptor for thrombin (~50/ platelet) and is associated with other membrane proteins (molecular weight ~900kDa as opposed to normal GPIb ~190kDa). It was demonstrated that the high affinity thrombin receptor mediates its response through phospholipase $A_2$ as opposed to a moderate affinity thrombin receptor acting through a G protein (Greco, 1996). There are several lines of proof for this: 1) the moderate affinity receptor has been shown to activate a G protein, 2) the 14-3-3 signal transducing protein ($PLA_2$ isoform) has been shown to be associated with GPIb and 3) $PLA_2$ itself has been shown to be activated by the binding of vWF to GPIb. Though the high affinity thrombin receptors are described here, vWF is the primary ligand in platelet adhesion via GPIb. vWF can bind to GPIIb/IIIa in certain situations. GPIIb/IIIa is an integrin receptor and the most numerous receptor on the platelet membrane. vWF has an amino acid sequence (RGD) which can sometimes bind to GPIIb/IIIa and aid in adhesion. GPIIb/IIIa will be discussed in detail in the next section.

## GPIIb/IIIa

GPIIb/IIIa (also referred to as αIIbβ3) is the primary receptor responsible for aggregation, primarily binding to fibrinogen. It is a member of the integrin family, which is a class of receptors binding cells to the extracellular matrix. After reviewing its structure its unique signaling mechanism will be examined. The receptor's function and ligands will be thoroughly discussed in the signaling mechanism outline.
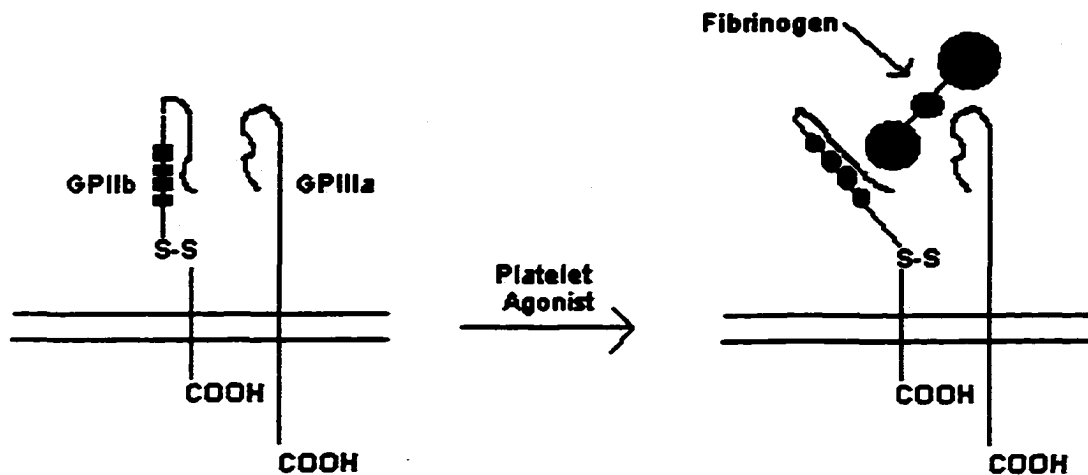
FIGURE 5

GPIIb/IIIa is composed of noncovalently linked $\alpha$ and $\beta$ subunits. The $\alpha$ and $\beta$

monomers form a one to one ratio of $Ca^{2+}$ and heterodimer complex and it is the

extracellular $Ca^{2+}$ that affects the receptor's structure and function. $Ca^{2+}$ helps maintain

the physical integrity that is required for activation induced expression of the fibrinogen

(Fb) receptors and Fb binding (Sims, 1991). The bulk of the integrin is extracellular with

the short intracellular portions being responsible for regulating the conformation of the

receptor. (see figure 5) The structure of GPIIb/IIIa, explained basically is very complex

and "its structure and binding properties indicated the presence of domains involved in a

number of major functions including: subunit association, conformational changes

exposing the ligand binding pocket, extracellular ligand recognition and binding, ligand

induced expression of neoepitopes, membrane associated receptor clustering, and

cytoskeletal interactions" (Calvette, 1995). The GPIIb/IIIa receptor does not bind to

fibrinogen unless the platelet is stimulated through a process referred to as inside-out

induction of fibrinogen binding.

In an unstimulated platelet GPIIb/IIIa is maintained in an inactive conformation and serves only as a low affinity adhesion receptor for surface coated fibrinogen (Calvette, 1995). Through a system of intracellular signals, which occur after platelet stimulation, the extracellular globular portion undergoes a change in quaternary structure opening up the Fb binding site. An amino acid sequence of RGD, which is found on fibrinogen, vWF, vitronectin, thrombospondin, and other binding proteins, binds onto this newly exposed site. The Calvette review states that if the RGD sequence extends between 11-32 angstroms from polyacrylnitrile beads it can be bound by GPIIb/IIIa on the unactivated platelet and this sort of "RGD extension" is only available when the binding proteins are insoluble which prevents premature aggregation. The mechanism by which the binding site is exposed is unknown but an article by Sims et al. puts forth some ideas. Other studies have shown that through $TXA_2$ or thrombin Fb receptors can be induced by G proteins and their cascade involving PKC. Other platelet activators, such as ADP and epinephrine, expose Fb receptors without PKC activation and "the metabolic pathways involved in this circumstance as well as the event ultimately responsible for Fb receptor exposure remain to be characterized" (Sims, 1991). One signaling mechanism that is hypothesized by Savage involves vWF and GPIb. Savage states that "vWF interaction with GPIb is coupled to a signal transduction mechanism leading to PKC activation. This in turn may activate GPIIb/IIIa allowing it to support irreversible platelet adhesion to immobilized vWF..." A study by Du et al. (1993) discerned that long distance conformational changes occurred with this inside out signaling. They found that the "intracellular binding site was 16nm from the Fb binding site and near the membrane

spanning domain... such (long distance conformational) changes are likely to be involved in bi-directional signaling by this receptor (Du, 1993).

Du et al. referred to bi-directional signaling which means that not only is there inside out signaling but also outside in signaling. The Du article makes reference that this pair of receptors also acts in concert so each increases binding affinity of the other. The outside in binding refers to the Fb binding to the extracellular domain of the GPIIb/IIIa receptor. This binding causes conformational changes on both Fb and GPIIb/IIIa which expresses neoepitopes called LIBS (ligand induced binding sites). The exposure of the LIBS correlates with cytoskeletal independent clustering of occupied GPIIb/IIIa complexes in the plane of the membrane followed by platelet aggregation and attachment of GPIIb/IIIa patches to the cytoskeletal network (Calvetti, 1995). He goes on to state that "though the physiological significance of LIBS expression remains to be established it is tempting to speculate that the exposure of the neoepitopes in the C-terminus of IIIa extracellular domain could be related to expression of interacting surfaces for other platelet and/or plasma components" (Calvetti, 1995).

Through the extracellular- intracellular binding GPIIb/IIIa also becomes attached to the platelet cytoskeleton. This attachment is necessary for clot retraction to occur and appears to trigger a major cytoskeletal reorganization. Through activated GTP binding proteins, PKCs, src tyrosine kinases, and other signaling mechanisms the cytoskeleton is reorganized in different steps "leading to the assembly of a multimeric signal transduction complex, which may explain the causal relationship between GpIIb/IIIa occupancy and a number of intracellular events elicited by ligand binding such as

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increase in $Na^+/H^+$ exchange, intracellular pH, phosphatidyl inositol metabolism, calpain activation, and thrombin induced phosphotyrosine dephosphorylation" (Calvetti, 1995).

One final function of the GPIIb/IIIa receptor has to do with an antithrombotic mechanism. Wencel-Drake et al. (1996) were able to show that after platelets had been stimulated, a certain time passed and then the platelets became nonadhesive even though Fb binds irreversibly to GPIIb/IIIa. This occurred because the receptor which bound Fb was being internalized via receptor mediated endocytosis. "This observation that in vivo older platelets exhibit a loss of membrane as well as $\alpha$-granule contents supports the concept of recurrent cycles of circulating platelet activation and recovery" (Wencel-Drake, 1996). The study that showed that GPIb was being internalized also showed that "a major diffuse intracellular pool of GPIIb/IIIa was seen and that the antigen to the receptor was localized to a granule membrane" (Wencel- Drake, 1996).

Though GPIb and GPIIb/IIIa are the primary receptors involved with platelet activity, others do play a role in platelet function. One is GPIV, a collagen receptor. During exocytosis of the $\alpha$-granule the previously internal granular membrane is everted on the extracellular surface revealing receptors that had been confined internally. The high affinity thrombin receptors were discussed but moderate affinity receptors use a different signaling mechanism.

## GPIV

GPIV (CD36) is an 88 kDa platelet membrane protein and normal platelets have about 30,000 GPIV/ platelet. The function of this receptor has largely been discerned form the NaK phenotype, people with this phenotype completely lack this receptor (Parisse, 1999). GPIV is the receptor for collagen V and NaK deficient individuals have

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no clinical bleeding disorders and no problems binding to any other type of collagen (I, III, IV) except for collagen V (Saelman, 1994). Type V collagen differs from the other collagens in many respects especially in that though it is present in all extra cellular matrices its concentration is far below those of other collagens, primarily I and III (Kehrel, 1993). The Kehrel article did point out that though collagen V was in low concentration in normal patients, it was present in abnormally high levels in patients with inflamed fibrotic tissue.

The Kehrel study showed that GPIV deficient platelets aggregated normally to the major platelet agonists. They hypothesized that GPIV is a direct receptor for collagen V and is important in mediating a signal arising from collagen V. The Saelman study goes a step farther and states that, "collagen type V as fibrillary collagen may be present in mixed fibrils together with collagens I and III and it is conceivable that interactions with GPIV may enhance the interaction of platelets with such fibrils" (Saelman, 1994).

## GMP-140

GMP-140 is an adhesion molecule known as a LECAM or selectin. This molecule is normally sequestered on the inner surface of the $\alpha$ granules and only appears on the surface of activated platelets (Ault, 1993). This makes it a perfect marker for use in detecting activated platelets. The previous discussion of platelet activation demonstrated that granular release occurred during activation, and a study by Tschoepe et al. (1990) reported that GMP-140 expression on the platelet surface of a thrombin activated platelet paralleled with polymerization of the cytoskeletal protein actin. An important distinction between the secretion of the $\alpha$-granule and lysosomal granules involves the completeness of the release. "Only 30-60% of the hydrolases are secreted

from the lysosomes in contrast to near 100% from the other ($\alpha$ and dense) granules"

(Holmsen, 1994). This means that the presence of GMP-140 on a platelet indicates that a

platelet has been activated whereas using a lysosomal marker would only yield 30-60%

of the activated platelets.

## Thrombin receptor

Thrombin is the most potent activator of Fb binding, platelet activation, and clot

retraction. Previously it was discussed that thrombin acts through different types of

receptors- the high affinity thrombin receptors are a subclass of GPIb. The importance of

thrombin to platelet activation demands a look at the moderate affinity receptors.

The thrombin receptor is a single chain seven transmembrane spanning G protein

coupled receptor. The mechanism for G proteins activation was discussed previously

(see figure 2). The moderate affinity thrombin receptor acts through a novel mechanism.

Instead of thrombin binding to a receptor to activate it, it enzymatically cleaves its

receptor after arginine-41 in the amino terminal domain exposing a new amino terminus

that functions as an agonist and activates the receptor. Figure 6 diagrams this.
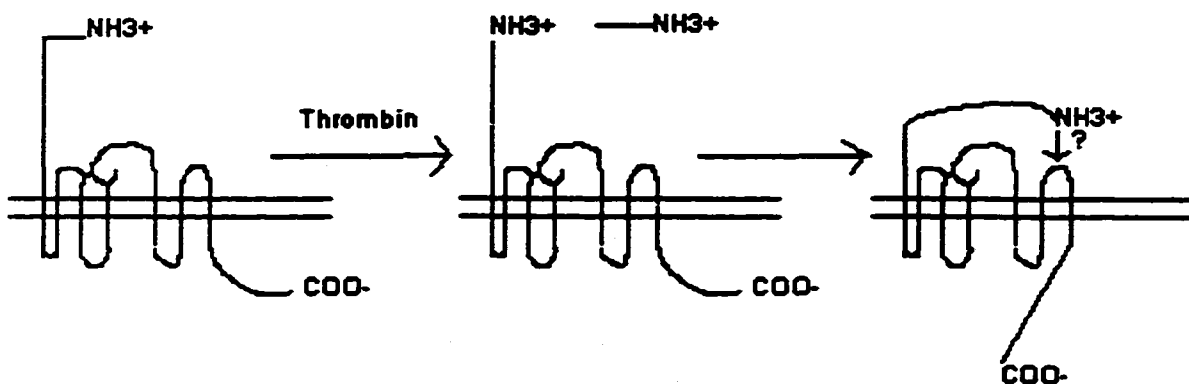


FIGURE 6

This activation turns on the G protein cascade but this novel mechanism does not allow for deactivation via the ligand releasing- the ligand is part of the receptor. A study by Molino et al. (1997) looked at the fate of the activated thrombin receptors. The thrombin receptors are partially internalized while others are shed into microparticles along with other integral membrane proteins. They could not account for the fate of the 40% of thrombin receptors still located on the platelet surface after activation had ceased. It is logical, though, that platelets cannot restore the thrombin receptor function. Platelets only need to respond to thrombin once, afterwards the viscous metamorphosis that is left is incorporated into the growing hemostatic plug.

# Pre-eclampsia

## Hemostatic changes during normal pregnancy

During pregnancy, the body goes through changes to accommodate the growing fetus in the uterus. Part of these changes involve the coagulation system. During normal pregnancy, there are various effects on the coagulation system that have been compared to a chronic state of compensated DIC (Perry, 1992, 340). Factors that increase with pregnancy include fibrinogen, VII, VIII, vWF, X, and XII. These factors can elevate up to 200% and remain elevated during pregnancy. Other factors, such as XI and XIII, decrease during pregnancy. Platelet counts during uncomplicated pregnancies show considerable individual variation with some maintaining normal counts, some having slight decreases, and some having a larger decreases nearing thrombocytopenic ranges (Frohlich, 1998).

## Pre-eclampsia Introduction

Pre-eclampsia is diagnosed with the triad of symptoms that include hypertension, proteinuria, and peripheral edema. Some suggest that pre-eclampsia is an exaggerated response to the normal changes seen in pregnancy. Platelets have been described as the source of the problems with pre-eclampsia. Thrombocytopenia is seen in pre-eclampsia and its hypothesized causes will also be discussed and some theories about why it occurs. Thromboxane ($TXA_2$)/ prostacyclin ($PGI_2$) imbalance has also been implicated as the primary cause of pre-eclampsia and this theory will be examined. Finally, there is only minimal literature about the predictors of pre-eclampsia and these will be discussed. Other flow studies looking at platelets and pre-eclampsia have been done but their conclusions were different from what this study is examining.

## Hemostatic changes in pre-eclampsia

A study by Higgins et al. (1998) looked at hemostasis in normotensive and pre-eclamptic pregnant females. They found that there were significantly raised fibrin degradation products (FDPs) during normotensive pregnancies. FDPs are markers for fibrinolysis and therefore the authors suggest that fibrinolysis is enhanced during normal pregnancy. The authors suggest that this could be due to a reactive response to increased fibrin production. During pre-eclampsia, changes occur in excess of the changes already seen in normotensive pregnancies. The Perry review (1992) suggests "that the intrinsic pathway may be altered by pre-eclampsia." This was demonstrated by an increased PTT seen in pre-eclampsia and he also says that the common pathway may be hypercoagulable. Previous studies had demonstrated higher levels of thrombin-antithrombin III, soluble fibrin, and FDP's in pre-eclampsia compared to normotensive pregnancies. The Higgins et al. (1998) study, though, contradicted this data when they found that there was no significant difference in these factors between pre-eclamptic and normotensive pregnancies. They account for the difference in that the previous studies had all used small sample groups and the pre-eclamptic patients all had severe pre-eclampsia. The Higgins study, on the other hand, was a prospective observational study originally following 1,000 primagravid females. Also, some of the other studies used different laboratory methods between the two test groups while this one used only one, ELISA.

## Platelet counts in Pre-eclampsia

In pre-eclampsia, 15% of patients present with thrombocytopenia. There are several possible mechanisms, but some are more likely than others. An immune

mechanism has been implicated in causing the thrombocytopenia via increased platelet bindable Ig and complement. A study by O'Neill (1985) showed that a reduction in platelet count can be seen 6-12 hours after mating in mice. This was also seen in mice from syngenic matings and transfer of parthenogenetically active eggs producing the same result. He suggests that this was due to a response in PAF, not to an immune mechanism.

Another mechanism for pre-eclampsia associated thrombocytopenia may be due to the increase in Factor X from increased immune complexes and vascular disruption, but the Higgins study did not find an increase in Factor X between the two sample groups. The most likely reason for thrombocytopenia is due to an increase in platelet activation followed by the activated platelets being removed from the circulation by the RE system. Norris et al. (1993) showed that in vitro response to platelet aggregating agents in patients with severe pre-eclampsia was significantly decreased. Patients with moderate pre-eclampsia also showed less response with some of the aggregators, collagen and arachidonic acid. If the platelets are activated, granule release would have occurred and platelet exhaustion would ensue, yielding the results seen. One would also expect to see increased platelet granule contents within the plasma. A study by Gujarti et al. (1994) showed an increase in 5-HT in plasma while others have shown increased ADP in plasma from patients with severe pre-eclampsia confirming the theory that platelets are activated during pre-eclampsia.

A study by Graves et al. (1992) showed that there is an increase in the platelet angiotensin II receptor in females with pregnancy induced hypertension (PIH), a disease closely related to pre-eclampsia. They were able to conclude that angiotensin binding on

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this receptor caused changes in cytosolic $Ca^{2+}$ indicating that this is a receptor 1 subtype. This receptor subtype has previously been shown to be coupled with changes in platelet cellular $Ca^{2+}$. A small study by Kilby et al. (1993) saw that increased platelet cytosolic $Ca^{2+}$ was related to essential hypertension during pregnancy. This could be related to pre-eclampsia and platelet activation, see figure 2, where $PIP_2$ is stimulated to break down and in the end, cytosolic $Ca^{2+}$ will increase activating the platelet.

### $TXA_2/PGI_2$ imbalance

The imbalance of two prostaglandins, thromboxane ($TXA_2$) and prostacyclin ($PGI_2$), has been implicated in being responsible for the pathogenesis of pre-eclampsia. The general description of prostaglandins is found on figure 3.

During normal pregnancy the production of $TXA_2$ is increased and this is consistent with the increased platelet activation of normal pregnancy. $PGI_2$ levels also increase during normal pregnancy. "However, if altered prostaglandin metabolism is the major cause of vasodilation in pregnancy and there are no changes in receptor number or function one must postulate that the increments in $TXA_2$ will be less than $PGI_2$, the result being an increase in the ratio of vasodilating to vasoconstricting prostaglandins and this does seem to be the case" (August, 1995). The increased $TXA_2$ leads to vasoconstriction and platelet aggregation if it remains unchecked by $PGI_2$, which are the main symptoms of pre-eclampsia. It was shown that platelet sensitivity to prostacyclin is reduced by 49% (Briel, 1984). This would lead to increased platelet coagulability and aggregation. This is thought to happen in pre-eclampsia. The increase in $PGI_2$ is not as large as in normal pregnancy, which leads to the imbalance in the ratio of the two prostaglandins.

Researchers have identified the placenta as the source for the imbalance in the prostaglandins. A study by Fitzgerald et al. (1986) demonstrated that the increased $TXA_2$ in pre-eclampsia is mainly derived from platelets. Aspirin irreversibly suppresses the cyclooxygenase pathway in the platelets and reversibly inhibits $TXA_2$ production from the placenta, which is hypothesized to be a second source of $TXA_2$. Their results showed a decrease in $TXA_2$ metabolites in the urine with a subsequent increase with stopping the aspirin treatment, which mimicked the normal platelet turnover in the vascular system.

This data does not provide any proof that the placenta is not producing some other factor involved with pre-eclampsia. $PGI_2$ is primarily synthesized in the endothelial cells and is thought to exert its effects in a paracrine mechanism between the endothelial cells and the vascular smooth muscle cells. But there could be a hormonal factor, which exerts the effects to the endothelial cells. Smarson et al. (1995) demonstrated that endothelial cell proliferation is suppressed by plasma but not serum from females with pre-eclampsia. They hypothesized that certain cells form the placenta, syncytiotrophoblast, produce this toxic factor and that in pre-eclampsia this substance is shed into the maternal circulation in abnormal amounts. This would disrupt the increase in $PGI_2$ synthesized by endothelial cells leaving the ratio favoring $TXA_2$ and therefore hypercoagulability.

## Predictors of Pre-eclampsia

Though the exact cause of pre-eclampsia remains unknown, there are certain conditions, which increase the chances of developing pre-eclampsia. Women with pregestational insulin requiring diabetes, chronic hypertension, multifetal gestation, and in females with pre-eclampsia in previous pregnancies are more apt to develop pre-eclampsia. The risk in each of these groups is further increased by elevated MAP in the

second trimester with the risk being approximately 3.3 times greater in females with

MAP greater than 85mm Hg. compared to below 75mm Hg (Caritis, 1998). Increase

MAP in the second trimester maybe an early marker for the disease but it could also

merely be a risk factor for pre-eclampsia. Until a biochemical or physiologic marker is

discovered that can be used as a screening tool the at-risk population needs to be

identified.

### Other Platelet- Pre-eclampsia Flow studies

The use of flow cytometry in identifying platelet changes in pregnancy has been

reported. A study by Konijnenberg et al. (Feb. 1997) used markers for platelet activation

(P-selectin, CD63, and PAC-1) and certain extracellular adhesion receptors (annexin-V

and platelet endothelial cell adhesion molecule-1). They found that during platelet

activation (via increased $\alpha$ and lysosomal granule markers) there was an increase in the

endothelial cell adhesion molecule-1 indicating that besides granule release there were

"other hirethro unknown mechanisms of increased surface antigen exposure involved

with pre-eclampsia" (Konijnenberg, 1997). A second study by Konijnenberg (August,

1997) examined whether platelet activation early in pregnancy could predict the

occurance of pre-eclampsia. They found that only first trimester CD63 expression could

be a predictor of pre-eclampsia and this was not a very strong predictor (correlation

coefficient of 0.63).

Janes and Goodall (1994) also examined changes that occur during pregnancy.

They found that there was an increase in the CD63 marker in normal pregnant versus non

pregnant women but the fibrinogen binding was the same between the two groups.

Platelet responsiveness to ADP in vitro showed a heightened degranulation response

(CD63 expression) in normal pregnancy compared with the non-pregnant contol group. This responsiveness to was heightened with both non-proteinuric and proteinuric pre-eclampsia but this response was not accompanied by an increase in fibrinogen binding to GP IIb/IIIa.

# Chapter 3

## Methods

### Design

This was a cross sectional study comparing several platelet functional parameters between two groups of females (non-pregnant vs. normal pregnant). This study was done with the cooperation of West Michigan OB/Gyn and Spectrum Health. The proposal was approved by Spectrum Health (Appendix A). A copy of the approved informed consent form is present in Appendix B.

Variables

    1. Independent variables

        The independent variables of the study are nonpregnant and normal pregnant females.

    2. Dependent variables

        a. platelet number

        b. mean platelet volume

        c. flow cytometric analysis of

            1. GP Ib, a cell surface glycoprotein necessary for adhesion of platelets.

            2. GP IIb, a cell surface glycoprotein expressed on the surface of platelets which is coupled with GPIIIa.

            3. GP IIIa, a cell surface glycoprotein expressed on the surface of platelets which is coupled with GPIIb.

4. Activated GP IIb/ IIIa, upon platelet activation the IIb/IIIa

receptor changes conformation and exposes a "RGD" binding

site necessary for interactions with fibrinogen.

5. GP IV, a platelet cell surface glycoprotein responsible for

collagen V binding.

6. GMP-140, a granular membrane protein expressed on the

platelet surface indicative of platelet granule secretion.

7. CD29, a general attachment receptor or integrin.

## Subjects

The control group of non-pregnant females was obtained from two nursing classes

taught at Grand Valley State University's Eberhard center. The students were recruited on

a voluntary basis and informed consent was obtained. After informed consent was

obtained a demographic sheet was completed by each subject (Appendix C). The group of

pregnant females was obtained from the practice of Dr. Jelesma, M.D. Normal pregnancy

is defined as normotensive females who are pregnant with no reported complications and

who are not taking anti-platelet medications. Inclusion criteria for both groups were

females of childbearing years without hypertension. Exclusion criteria included non-

menstrating females of childbearing years, menopausal, post-menopausal, and hypertensive

females.

## Blood Collection

Two 5 ml tubes of EDTA blood was collected from each participant through

venipuncture using a 21 gauge or larger needle. To avoid thrombin generation, the first 5

ml tube of blood was discarded. Within two hours of collecting the blood 100ul was added

to 1 ml of 1% formaldehyde solution (at 2-8 C) and mixed, preventing nonspecific activation of the platelets. This blood was subsequently used in the flow cytometric analysis of the platelets while the remaining EDTA blood was used to measure platelet count and mean platelet volume.

### Platelet size and mean platelet volume

The blood collected in the EDTA tube was used to determine platelet count and mean platelet volume using a Sysmex K-1000.

### Preparation of platelets for flow analysis

The 100 ul of blood that was placed in 1 ml of 1% formaldehyde solution was incubated at least thirty minutes, to allow fixing to occur. Following the incubation, the blood was centrifuged at 2500 RPM (1200*G) for 5 minutes at room temperature (20-25 C). The supernatant was aspirated and 2 ml of PBS with 0.1% sodium azide was added to the pelleted blood. The pellet was resuspended by vortexing and then this was centrifuged at 2500 RPM for 5 minutes at room temperature. The supernatant was aspirated and the pellet resuspended in 1 ml PBS with 1% fetal calf serum and 0.1% sodium azide. The resuspended blood was subsequently incubated with antibodies to various platelet proteins.

### Analysis of platelet membrane proteins

Platelet analysis was performed by a "direct" flow cytometric method with a variety of mouse monoclonal antibodies or goat polyclonal antibodies directly conjugated to one of two fluorescent fluorochrome labels called fluorescein isothiocynate (FITC) or phycoerythrin (PE). Prior to application to the flow cytometer, the blood was fixed and washed according to the method described above. A 50 ul aliquot of the resuspended blood was incubated with 20 ul of commercially available antibodies reactive to:

*GP Ib (CD42b, FITC, F0802, DAKO, Denmark),

*GP IX (CD42a, FITC, 348083, Becton/ Dickson, San Jose, CA),

*Integrin B1 (CD29, FITC, 66003109, Coulter, Hialeah, FL),

*GP IV (CD36, PE, P-9312, Sigma, St. Louis, MO),

*GP IIb (CD41, FITC, F7088, DAKO, Denmark) and

*GP IIb (CD41, PE, R7058, DAKO, Denmark),

*GP IIIa (CD61, FITC, F0803, DAKO, Denmark),

*GMP-140 (CD62, PE, 348107, Becton/ Dickson, San Jose, CA), and

*activated IIb/ IIIa (PAC-1, FITC, 340507, Becton/ Dickson, San Jose, CA).

After incubation for 20 minutes at room temperature without stirring and isolation from the light, 2 ml of PBS with 0.1% sodium azide was added. After vortexing this was centrifuged at 2500 RPM for 5 minutes. When the supernatant was aspirated this wash cycle was repeated. The pellet was then resuspeneded in 1 ml PBS with 0.1% sodium azide and was then ready for flow cytometric analysis.

## Flow Cytometric Analysis

### Data Acquisition

The platelets were analyzed on a Becton- Dickson FACSort flow cytometer (Becton-Dickson Immunocytometry Systems, San Jose, CA) equipped with Lysis II software. The dot plot of forward angle (FSC) and side angle (SSC) scatter of laser light was collected in log scale to establish electronic gates on the platelets. The threshold was established on FSC to exclude debris. Fluorescent compensation was established by Calbrite beads (Becton- Dickson). The first part of the flow cytometric analysis consisted of the data acquisition. After selecting into the Lysis program the acquisition mode was

selected. To get optimal results the flow cytometer was set according to the folloing

parameters. FSC and SSC were collected on a log scale. The FACSort detectors were set

to: FSC (E00), SSC (322), FL1 (749), and FL2 (690). The compensation was set at 1.8 %

for FL1, 14.9% for FL2, and 0% for the other detectors. The compensation threshold was

set according to the FSC-H and was set at 112. The machine was selected to collect

results from 10,000 cells in either the medium or high collection modes. The results

consisted of scatter plots of the cells and the histograms of the amount of fluorescence/

cell. Each sample was saved to a file for later analysis.

## Data Analysis

The second part of the flow cytometric analysis consisted of the data analysis.

Each sample was analyzed while obtaining certain information. Figure 7 shows an

example of an analysis. The upper left diagram shows a dot plot of the forward vs side

scatters of the 10,000 cells collected. This was collected of a log vs log scale to obtain

proper separation of the platelets from the other cells. A gate was then established

according to where platelets land on the scatter plot. Fluourescence 1 (FL1) for FITC vs

fluourescence 2 (FL2) for PE were examined on the upper right plot. Quadrants were set

at about $10^1$ on both FL1 and FL2. This value was chosen to differentiate between the

platelets with Ab binding vs. non-binding. This value allowed platelets with nonspecific

binding to not influence the results. The quandrant statisitics were printed to the right of

this plot and showed the different percentages of platelets within each quadrant. The FL1

and FL2 were examined more closely on a histogram, which are the two boxes on the

lower half of the page. The histogram statistics provided the peak and mean channels of

each Ab, which is directly indicative of the peak and mean values of the platelet

membrane proteins examined in the study. These are the values used in the Results

section, chapter 4.

# Chapter 4
## Results

### Number of Subjects

Blood was collected on a total of forty-four subjects. Included in the study were twenty-two nonpregnant and eighteen pregnant females. Four females were excluded from the study due to the following reasons: menopausal (2), spontaneous abortion one week after blood draw (1), and previous hysterectomy (1). The demographic data of the two groups is located in table 2.

### Demographic Data

The average age of the nonpregnant females was 27.7 years while the pregnant group's average age was 29.4 years, which showed no statistically significant difference when a t-test was run with a cutoff at 2.750 (two-sided value at 1% significance). The subjective data identified that three of the pregnant females reported smoking during pregnancy, between two and twenty cigarettes/ day, while none of the nonpregnant females smoked. Eleven (50%) of the nonpregnant subjects reported being on contraceptives and the only significant medication reported by the pregnant subjects were prenatal vitamins. Significant in the context of this paper refers to medications that would affect platelets or platelet activation. Both groups had similar results to both numbers of prior pregnancies and numbers of live births (t-values of -1.04 and -0.69, respectively).

### Platelet Count and Mean Platelet Volume

Table 3 shows the average platelet count and mean platelet volume (MPV) of the two groups. A significance level of 1% was used which gives a t-value of +/- 2.750.

## Table 2- Patient Demographic Data

**Non pregnant**

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Patient # | 1 | 2 | 3 | 4 | 5 | 9 | 10 | 11 | 12 | 13 | 14 |
| AGE | 23 | 22 | 22 | 21 | 22 | 23 | 40 | 37 | 24 | 23 | 23 |
| Prior Pregnancies | 0 | 0 | 0 | 1 | 0 | 0 | 5 | 3 | 0 | 0 | 0 |
| # of live births | 0 | 0 | 0 | 1 | 0 | 0 | 3 | 3 | 0 | 0 | 0 |
| Allergies? | Sulfa | | PCN | | | | | | | | |
| Smoker? | | | | | | | | | | | |
| Medications? | Triphasil-28 | Orthocept | Ortho-tricyclene | Imitrex | Paxil/Nordette | | Survent/Albutero | Levoxil/ OrthoNovum | Orthocyclin | | BCP-Orthocept |
| Cardiovasular dx/ Hypertension | | | | | | | | | | | |
| Significant medical history | | | | | | | Asthma | | | | |

**Non pregnant**

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Patient # | 15 | 16 | 21 | 22 | 23 | 24 | 26 | 27 | 28 | 31 | 32 |
| AGE | 23 | 41 | 35 | 24 | 30 | 43 | 25 | 26 | 29 | 28 | 27 |
| Prior Pregnancies | 0 | 3 | 4 | 0 | 7 | 2 | 0 | 0 | 0 | 0 | 0 |
| # of live births | 0 | 3 | 2 | 0 | 4 | 2 | 0 | 0 | 0 | 0 | 0 |
| Allergies? | | | | PCN | | | environmental | codeine | seasonal | seasonal | |
| Smoker? | | | | | | | | | | | |
| Medications? | BCP-Trilevlin | Zyrtec/Depoprovera | Alesse | | Prozac | Claritin/Orthonovum | | | Dyazide/ Nor | Tylenol/ oral contr. | |
| Cardiovasular dx/ Hypertension | | Family Hx | | | | | | | | | |
| Significant medical history | Eczema | | | Asthma | | | | | controlled HTN | | |

**Pregnant**

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Patient # | 34 | 35 | 36 | 38 | 39 | 40 | 41 | 42 | 43 | 44 | 46 |
| AGE | 37 | 29 | 27 | 33 | 26 | 22 | 35 | 36 | 23 | 26 | 31 |
| Gestation (weeks) | 11 | 12 | 8 | 11 | 13 | 13 | 11 | 18 | 8 | 8 | 31 |
| Prior Pregnancies | 0 | 0 | 0 | 2 | 0 | 2 | 5 | 5 | 3 | 1 | 0 |
| # of live births | 0 | 0 | 0 | 2 | 8 | 1 | 3 | 4 | 2 | 0 | 0 |
| Allergies? | | Bactrim | | | | sudafed | PCN | | | Erythromycin | |
| Smoker? | | n/a | 12/d/10y | | 2/d/3y | | | | | | |
| Medications? | synthroid | SN+ | | Pherganwi | SN+ | | | | | | |
| Cardiovasular dx/ Hypertension | | hrt murmur | | | | | | | | | |
| Significant medica | ZIFT | abnor PAP | | gest DM | abnor PAP | | | | | kid stones/abnor PAP | |

**Pregnant**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Patient # | 49 | 50 | 51 | 55 | 56 | 57 | 67 |
| AGE | 31 | 20 | 34 | 29 | 31 | 29 | 27 |
| Gestation (weeks) | 25 | 25 | 11 | 36 | 11 | 29 | 21 |
| Prior Pregnancies | 5 | 2 | 3 | 1 | 3 | 0 | 0 |
| # of live births | 3 | 1 | 3 | 1 | 3 | 0 | 0 |
| Allergies? | | Cephlerin | | | Contact c | Seasonal | |
| Smoker? | | | 20/d/14y | | | | |
| Medications? | | | SN+ | | | | |
| Cardiovasular dx/ Hypertension | | | | | SVT | | |
| Significant medical history | | | c-sect | | OGTT 146 | | |

Since the t-values were zero or very close to zero the platelet counts and MPV can be considered equal.

Table 3. Platelet count and MPV in non pregnant and pregnant samples

|  | Nonpregnant (n=22) | Pregnant (n=18) | t-value | *S/NS |
|---|---|---|---|---|
| Platelet count ($*10^3$/ul) | 240.3+/- 45.0 | 235+/- 45.7 | 0.368 | NS |
| M. P. V. (fl) | 9.66+/- 0.77 | 9.70+/- 0.73 | 0 | NS |

* Two-sided 99% confidence interval

## Negative Controls

Negative controls were run with each series of nonpregnant and pregnant samples. Less that 1% of the events in the negative controls were positive for binding. The negative controls were mouse $IgG_1$ antibodies conjugated to FITC or PE. Figures 7 and 8 are examples of two controls run, figure 7 for nonpregnant females and figure 8 for pregnant females.

## CD29

CD29 is an integrin functioning as a general attachment protein for the platelet to the extracellular matrix (ECM.). Figures 9 and 10 are examples of the dot plots and histograms of a nonpregnant (figure 9) and pregnant (figure 10) subject. The statistics of the histogram show a peak channel of 78.36 for the nonpregnant subjects and a peak channel of 123.24 for pregnant subjects. The t-test confirmed a statistically significant difference between the two groups (t-value of $-5.34$). The mean channels for the non pregnant and pregnant groups were not significantly different (values of 119.64 and 147.88, respectively and a t-value of $-2.24$) Table 4

44

DATE: 3-MAR-99

TIME: 11:00:07

SELECTED PREFERENCES: Arithmetic/Linear
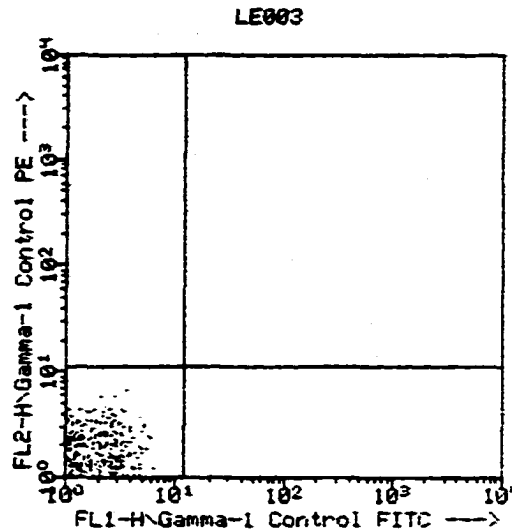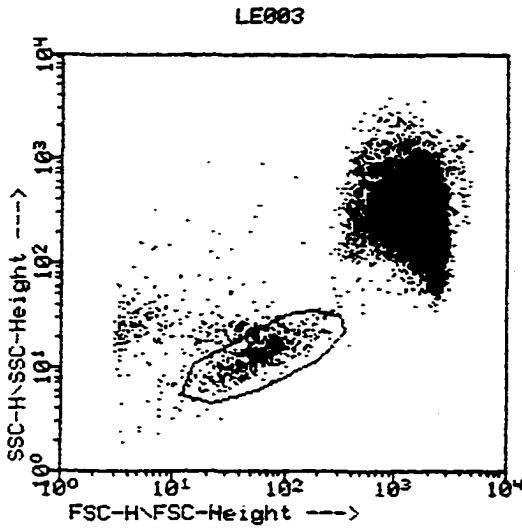

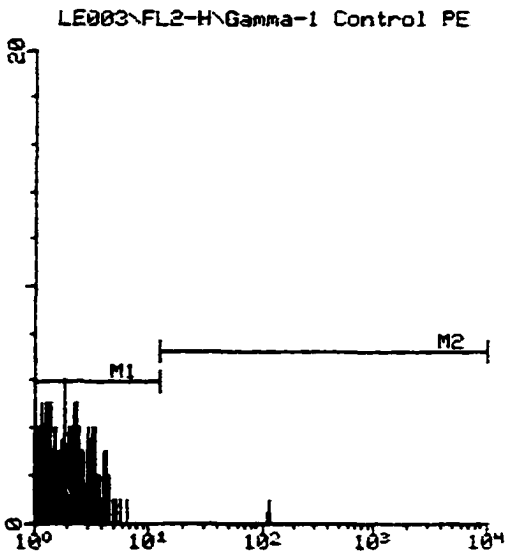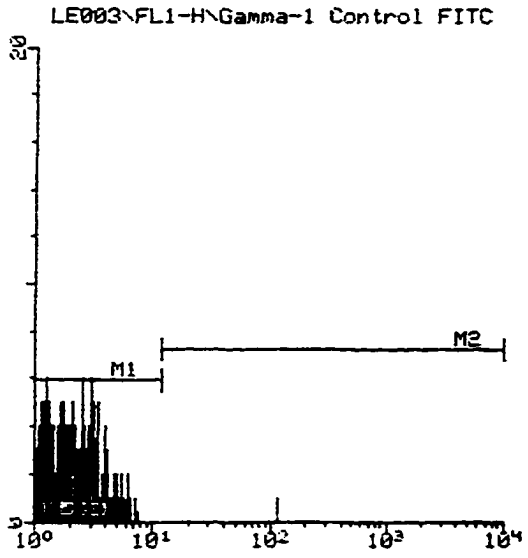
LE003

LE003

LE003

```
------ Quad Stats ------
File: LE003  Sample:    186
Date:  1/27/99  Gate G1= R1
Selected Preference: Arithmetic/Linear
Parameters: FL1-H(LOG),FL2-H(LOG)  Qua>
Total=    10000  Gated=       466
Quad    Events  % Gated  % Total    Xm>
```

| Quad | Events | % Gated | % Total | Xm> |
|---|---|---|---|---|
| 1 UL | 0 | 0.00 | 0.00 | — |
| 2 UR | 1 | 0.21 | 0.01 | 111 |
| 3 LL | 465 | 99.79 | 4.65 | 2 |
| 4 LR | 0 | 0.00 | 0.00 | — |

LE003\FL1-H\Gamma-1 Control FITC

LE003\FL2-H\Gamma-1 Control PE

LE003\FL1-H\Gamma-1 Control FITC

—— Arithmetic Histogram Statistics for LE003 ——
Selected Preferences: Arithmetic/Linear
Parameter FL1-H Gamma-1 Control FITC    Gate G1= R1

| M | Left,Right | Events | % | Peak | PkChl | Mean |
|---|---|---|---|---|---|---|
| 0 | 1.00, 9910 | 466 | 100.00 | 78 | 1.00 | 2.40 |
| 1 | 1.00,12.08 | 465 | 99.79 | 78 | 1.00 | 2.16 |
| 2 | 12.08, 9910 | 1 | 0.21 | 1 | 114.44 | 114.44 |

LE003\FL2-H\Gamma-1 Control PE

—— Arithmetic Histogram Statistics for LE003 ——
Selected Preferences: Arithmetic/Linear
Parameter FL2-H Gamma-1 Control PE    Gate G1= R1

| M | Left,Right | Events | % | Peak | PkChl | Mean |
|---|---|---|---|---|---|---|
| 0 | 1.00, 9910 | 466 | 100.00 | 129 | 1.00 | 2.01 |
| 1 | 1.00,12.30 | 465 | 99.79 | 129 | 1.00 | 1.76 |
| 2 | 12.86, 9910 | 1 | 0.21 | 1 | 114.44 | 114.44 |

Figure 7. Non-pregnant negative control flow cytometric results

LYSYS II  Ver 1.1  2/6/92

DATE: 3-APR-99                                          TIME: 13:49:25

SELECTED PREFERENCES: Arithmetic/Linear



MB003\FL1-H\Gamma-1 Control FITC

MB003\FL2-H\Gamma-1 Control PE

Quad Stats summary:

| | MB003 | | | |
|---|---|---|---|---|
| File: MB003 Sample: 027 | | | | |
| Date: 3/18/99  Gate Q1= R1 | | | | |
| Selected Preference: Arithmetic/Linear | | | | |
| Parameters: FL1-H(LOG),FL2-H(LOG)  Quad L | | | | |
| Total= 10000  Gated= 252 | | | | |
| Quad | Events | % Gated | % Total | Xmean |
| 1 UL | 0 | 0.00 | 0.00 | — |
| 2 UR | 0 | 0.00 | 0.00 | — |
| 3 LL | 252 | 100.00 | 2.52 | 2.24 |
| 4 LR | 0 | 0.00 | 0.00 | — |
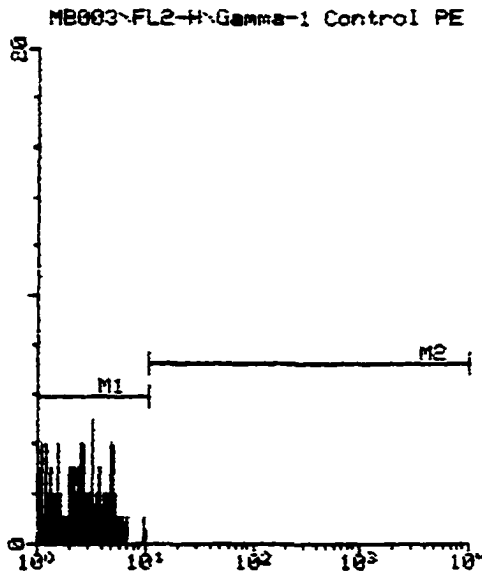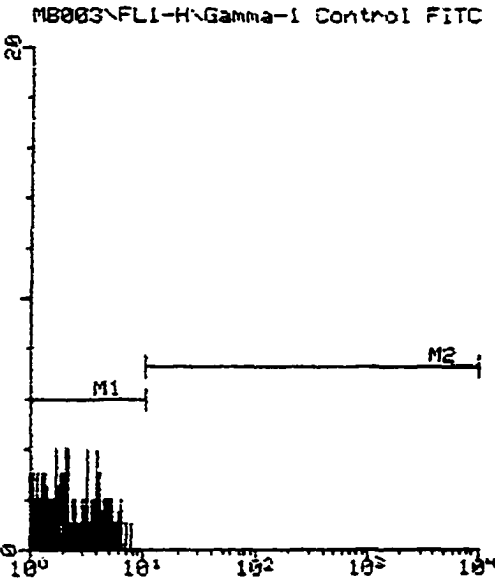
MB003\FL1-H\Gamma-1 Control FITC
--- Arithmetic Histogram Statistics for MB003 ---
Selected Preferences: Arithmetic/Linear
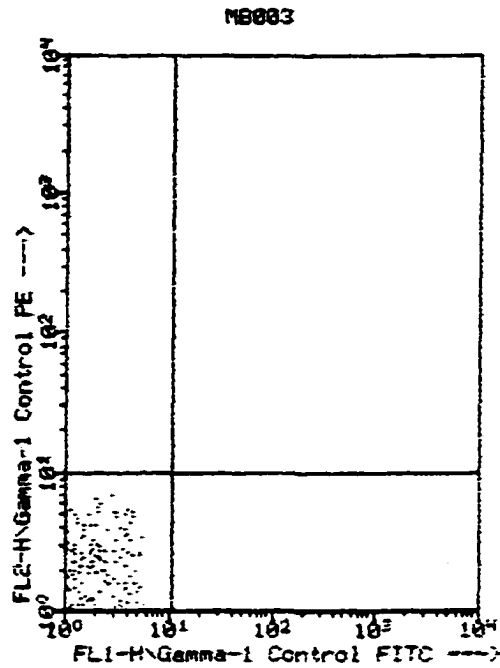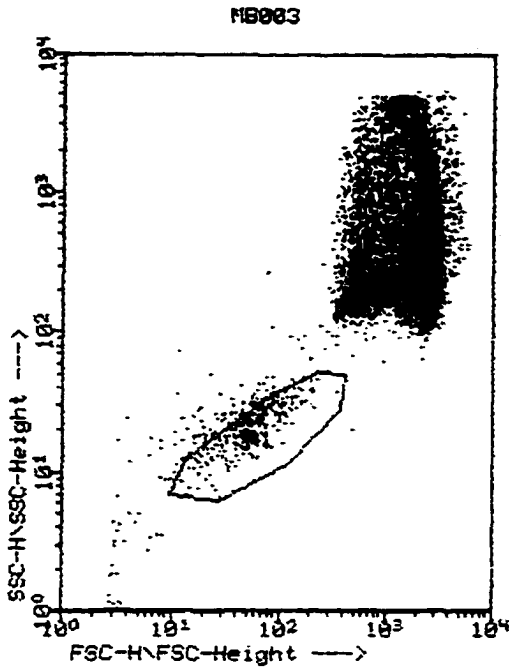Parameter FL1-H Gamma-1 Control FITC    Gate G1= R1

| M | Left,Right | Events | % | Peak | PkChl | Mean |
|---|---|---|---|---|---|---|
| 0 | 1.00, 9910 | 252 | 100.00 | 56 | 1.00 | 2.27 |
| 1 | 1.00,10.55 | 252 | 100.00 | 56 | 1.00 | 2.27 |
| 2 | 10.55, 9910 | 0 | 0.00 | 0 | — | — |

MB003\FL2-H\Gamma-1 Control PE
--- Arithmetic Histogram Statistics for MB003 ---
Selected Preferences: Arithmetic/Linear
Parameter FL2-H Gamma-1 Control PE    Gate G1= R1

| M | Left,Right | Events | % | Peak | PkChl | Mean |
|---|---|---|---|---|---|---|
| 0 | 1.00, 9910 | 252 | 100.00 | 56 | 1.00 | 2.27 |
| 1 | 1.00,10.94 | 252 | 100.00 | 56 | 1.00 | 2.27 |
| 2 | 10.94, 9910 | 0 | 0.00 | 0 | — | — |

Figure 8. Pregnant negative control flow cytometric results

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LYSYS II  Ver 1.1  2/6/92

DATE: 3-MAR-99                                          TIME: 11:01:28
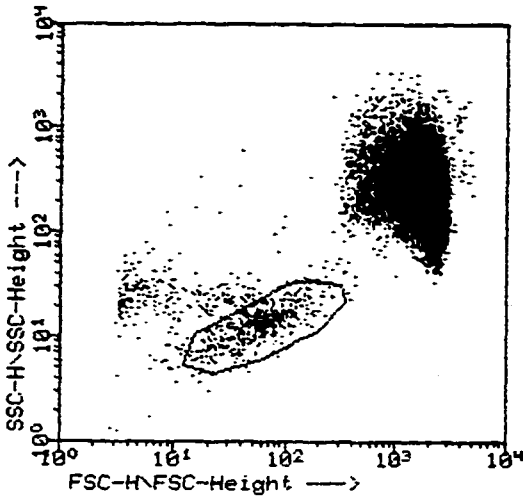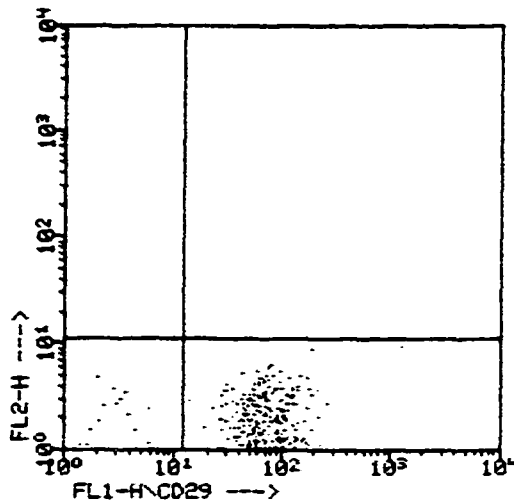
SELECTED PREFERENCES: Arithmetic/Linear

LE004                    LE004                    LE004
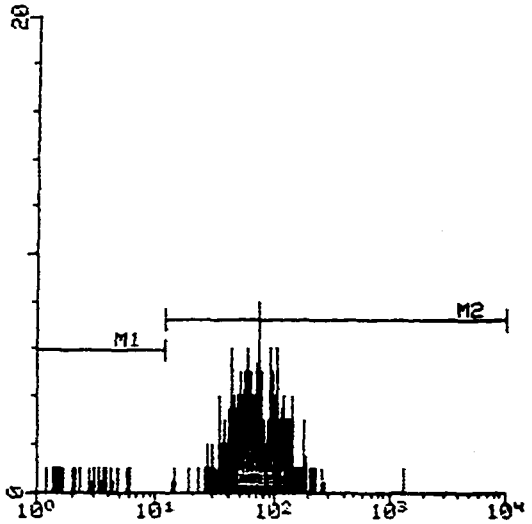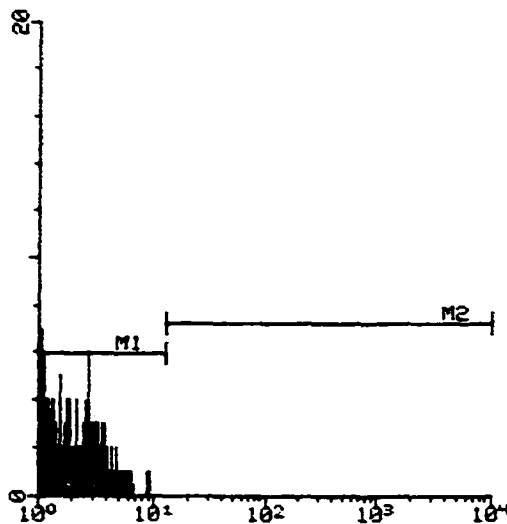
```
---- Quad Stats ----
File: LE004  Sample:  187
Date:  1/27/99  Gate G1= R1
Selected Preference: Arithmetic/Linear
Parameters: FL1-H(LOG),FL2-H(LOG)  Quad
Total=   10000  Gated=      409
Quad    Events  % Gated  % Total      Xm
```

| Quad | Events | % Gated | % Total | Xm |
|---|---|---|---|---|
| 1 UL | 0 | 0.00 | 0.00 | — |
| 2 UR | 0 | 0.00 | 0.00 | — |
| 3 LL | 19 | 4.65 | 0.19 | 2. |
| 4 LR | 390 | 95.35 | 3.90 | 79. |

LE004\FL1-H\CD29              LE004\FL2-H\

LE004\FL1-H\CD29              LE004\FL2-H\

--- Arithmetic Histogram Statistics for LE004 ---        --- Arithmetic Histogram Statistics for LE004 ---
Selected Preferences: Arithmetic/Linear                  Selected Preferences: Arithmetic/Linear
Parameter FL1-H CD29    Gate G1= R1                       Parameter FL2-H    Gate G1= R1

| M | Left,Right | Events | % | Peak | PkChl | Mean | | M | Left,Right | Events | % | Peak | PkChl | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 1.00, 9910 | 409 | 100.00 | 8 | 71.05 | 76.66 | | 0 | 1.00, 9910 | 409 | 100.00 | 112 | 1.00 | 1.92 |
| 1 | 1.00,12.08 | 19 | 4.65 | 1 | 1.00 | 2.83 | | 1 | 1.00,12.30 | 409 | 100.00 | 112 | 1.00 | 1.92 |
| 2 | 12.08, 9910 | 390 | 95.35 | 8 | 71.05 | 80.25 | | 2 | 12.86, 9910 | 0 | 0.00 | 0 | -- | -- |

Figure 9. Non-pregnant CD29 flow cytometric results

47
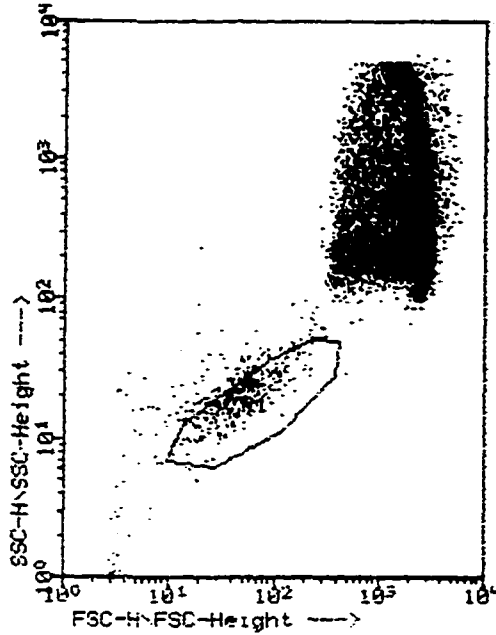
MB004                                    MB004
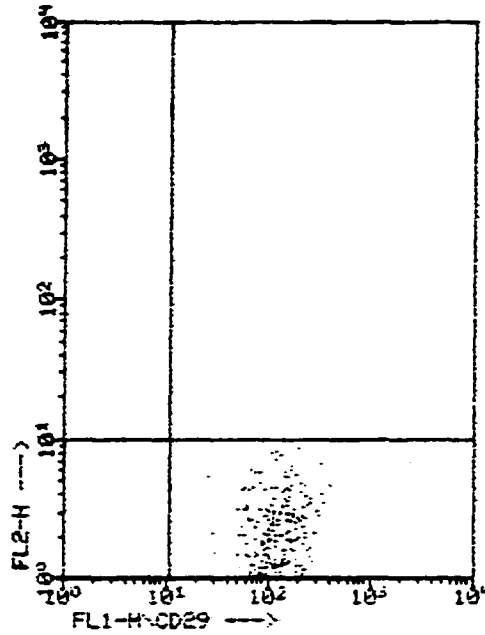
MB004
----- Quad Stats -----
File: MB004  Sample:  828
Date: 3/18/99  Gate G1= R1
Selected Preference: Arithmetic/Linear
Parameters: FL1-H(LOG),FL2-H(LOG)  Quad L
Total=   10000  Gated=     284
Quad   Events   % Gated  % Total    Xmean

| Quad | Events | % Gated | % Total | Xmean |
|------|--------|---------|---------|-------|
| 1 UL | 0 | 0.00 | 0.00 | — |
| 2 UR | 0 | 0.00 | 0.00 | — |
| 3 LL | 0 | 0.00 | 0.00 | — |
| 4 LR | 284 | 100.00 | 2.84 | 136.36 |

SSC-H\SSC-Height --->          FL2-H --->

FSC-H\FSC-Height --->          FL1-H\CD29 --->

MB004\FL1-H\CD29                MB004\FL2-H\

--- Arithmetic Histogram Statistics for MB004 ---        --- Arithmetic Histogram Statistics for MB004 ---
Selected Preferences: Arithmetic/Linear                   Selected Preferences: Arithmetic/Linear
Parameter FL1-H CD29    Gate G1= R1                        Parameter FL2-H    Gate G1= R1
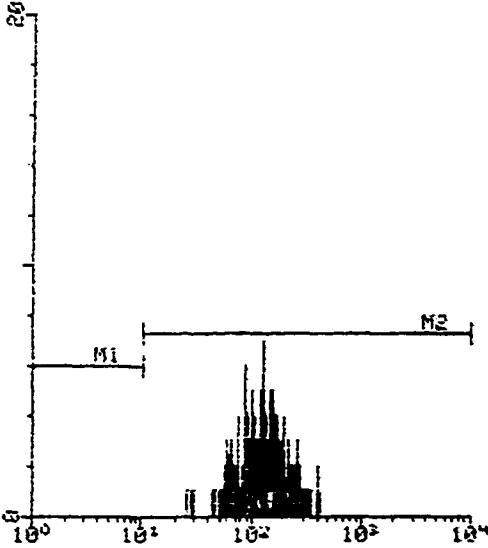
| M | Left,Right | Events | % | Peak | PkChl | Mean |
|---|-----------|--------|---|------|-------|------|
| 0 | 1.00, 9910 | 284 | 100.00 | 7 | 128.64 | 138.14 |
| 1 | 1.00,10.55 | 0 | 0.00 | 0 | — | — |
| 2 | 10.55, 9910 | 284 | 100.00 | 7 | 128.64 | 138.14 |

| M | Left,Right | Events | % | Peak | PkChl | Mean |
|---|-----------|--------|---|------|-------|------|
| 0 | 1.00, 9910 | 284 | 100.00 | 57 | 1.00 | 2.29 |
| 1 | 1.00,10.94 | 284 | 100.00 | 57 | 1.00 | 2.29 |
| 2 | 10.94, 9910 | 0 | 0.00 | 0 | — | — |

Figure 10. Pregnant CD29 flow cytometric results

summarizes the results of CD29. Again, a t-value of 2.750 was used as a cutoff (two-sided t-value with a 1% significance level for this sample size).

Table 4. CD29 Binding Results in nonpregnant and pregnant samples

|  | Nonpregnant (n=22) | Pregnant (n=17) | t-value | *S/NS |
|---|---|---|---|---|
| Peak channel CD29 | 78.36+/- 22.9 | 123.24+/-30.1 | -5.34 | S |
| Mean channel CD29 | 119.64+/- 46.77 | 147.88+/- 28.57 | -2.24 | NS |

* Two-sided 99% confidence interval

## CD36

CD36 is an antibody that recognizes GPIV, a platelet surface collagen receptor. It is the only identified receptor for collagen V at this time. Though collagen V is less prevalent than the other collagens, types I, III, and IV, it is important in mediating a signal from collagen V. Also important to reiterate is that patients who lack GPIV have no clinical bleeding signs (Saelman, 1994). Figures 11 and 12 are examples of the results obtained from the two groups for CD36. The mean for the peak channels of the nonpregnant and pregnant samples was 92.45 and 84.0, respectively, while the average of the mean channels was 110.77 and 145.22, respectively. Neither of the two t-tests showed significance at the 1% level. Table 5 sums up the statistics for the two groups. Both t-values are within the +/- 2.750 range and therefore no statistical difference existed between the two groups for CD36.

DATE: 10-FEB-99                                TIME: 12:20:38

SELECTED PREFERENCES: Arithmetic/Linear

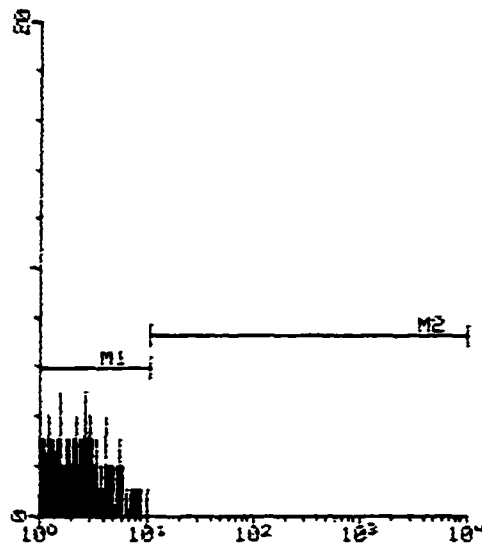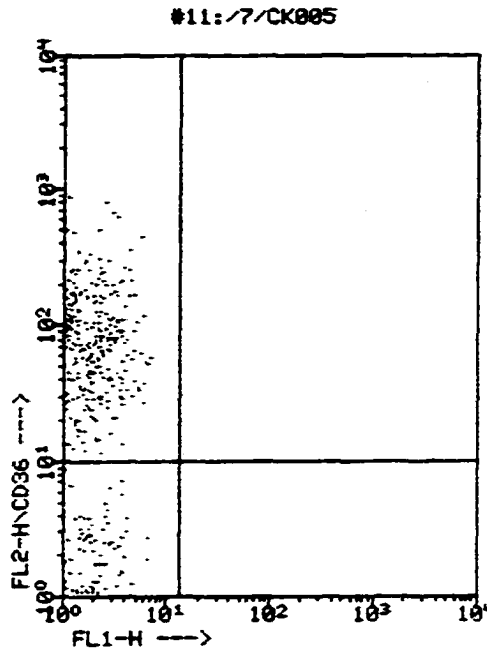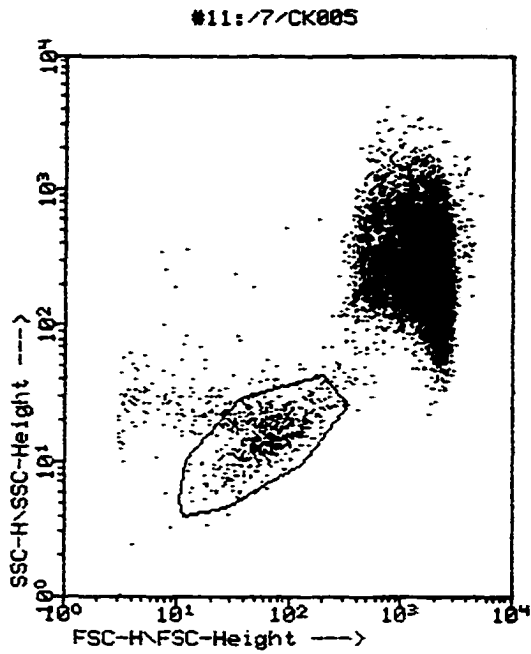#11:/7/CK005

#11:/7/CK005

#11:/7/CK005

```
------ Quad Stats ------
File: #11:/7/CK005  Sample:  091
Date:  1/27/99  Gate G1= R1
Selected Preference: Arithmetic/Linear
Parameters: FL1-H(LOG),FL2-H(LOG)  Quad
Total=  10000  Gated=    522
```
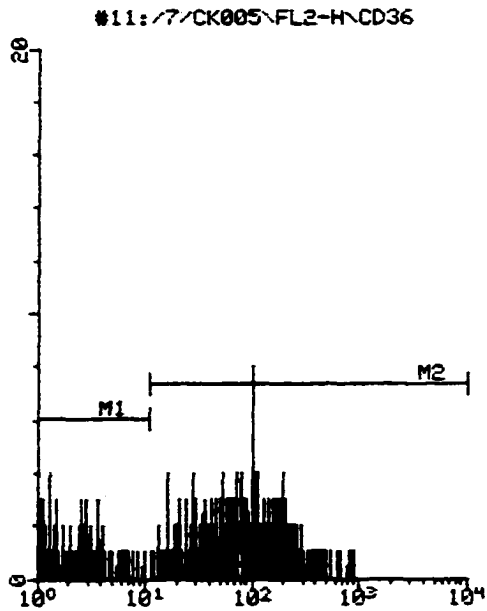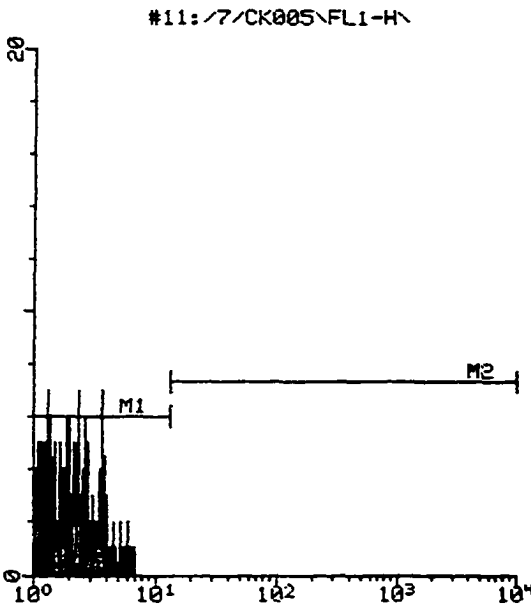
| Quad | Events | % Gated | % Total | Xme |
|------|--------|---------|---------|-----|
| 1 UL | 395 | 75.67 | 3.95 | 1. |
| 2 UR | 0 | 0.00 | 0.00 | — |
| 3 LL | 127 | 24.33 | 1.27 | 1. |
| 4 LR | 0 | 0.00 | 0.00 | — |

#11:/7/CK005\FL1-H\

#11:/7/CK005\FL2-H\CD36

#11:/7/CK005\FL1-H\

```
---- Arithmetic Histogram Statistics for #11:/7/CK00
Selected Preferences: Arithmetic/Linear
Parameter FL1-H    Gate G1= R1
```

| M | Left,Right | Events | % | Peak | PkCh1 | Mean |
|---|-----------|--------|-----|------|-------|------|
| 0 | 1.00, 9910 | 522 | 100.00 | 134 | 1.00 | 1.96 |
| 1 | 1.00,13.58 | 522 | 100.00 | 134 | 1.00 | 1.96 |
| 2 | 13.58, 9910 | 0 | 0.00 | 0 | — | — |

#11:/7/CK005\FL2-H\CD36

```
---- Arithmetic Histogram Statistics for #11:/7/CK00
Selected Preferences: Arithmetic/Linear
Parameter FL2-H CD36   Gate G1= R1
```

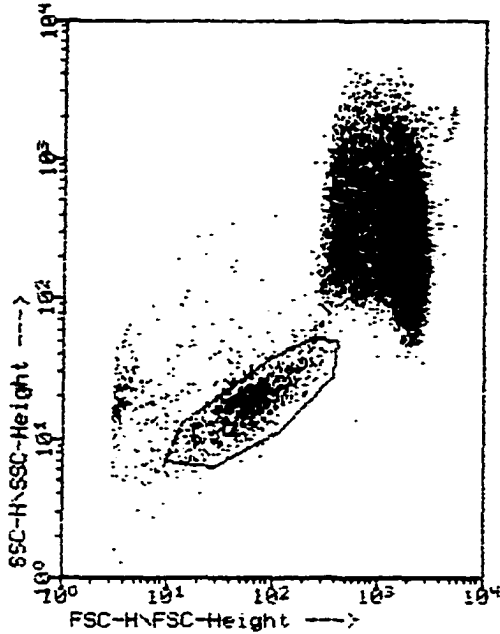| M | Left,Right | Events | % | Peak | PkCh1 | Mean |
|---|-----------|--------|-----|------|-------|------|
| 0 | 1.00, 9910 | 522 | 100.00 | 20 | 1.00 | 86.77 |
| 1 | 1.00,11.24 | 127 | 24.33 | 20 | 1.00 | 2.34 |
| 2 | 11.24, 9910 | 395 | 75.67 | 8 | 103.66 | 113.92 |

Figure 11. Non-pregnant CD36 flow cytometric results

DATE: 3-APR-99                                    TIME: 12:46:12
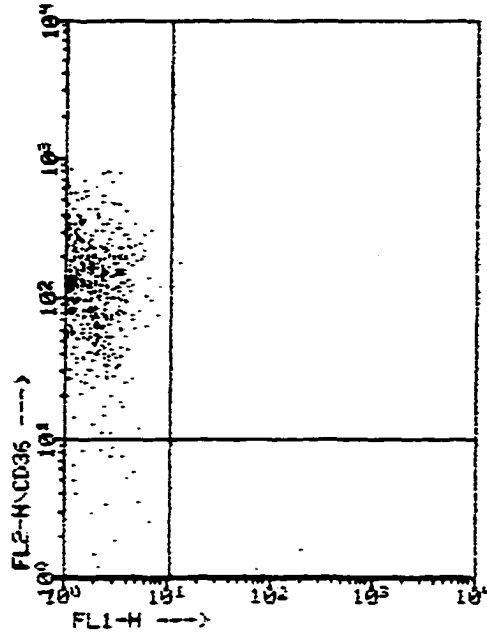
SELECTED PREFERENCES: Arithmetic/Linear

MK005                              MK005



MK005

----- Quad Stats -----
File: MK005  Sample:   049
Date:  3/19/99  Gate G1= R1
Selected Preference: Arithmetic/Linear
Parameters: FL1-H(LOG),FL2-H(LOG)  Quad L
Total=   10000  Gated=     726
Quad    Events  % Gated   % Total      Xmean

1 UL      696    95.87     6.96       1.85
2 UR        1     0.14     0.01      13.34
3 LL       27     3.72     0.27       2.34
4 LR        2     0.28     0.02     131.72

MK005\FL1-H\                    MK005\FL2-H\CD36



MK005\FL1-H\                    MK005\FL2-H\CD36
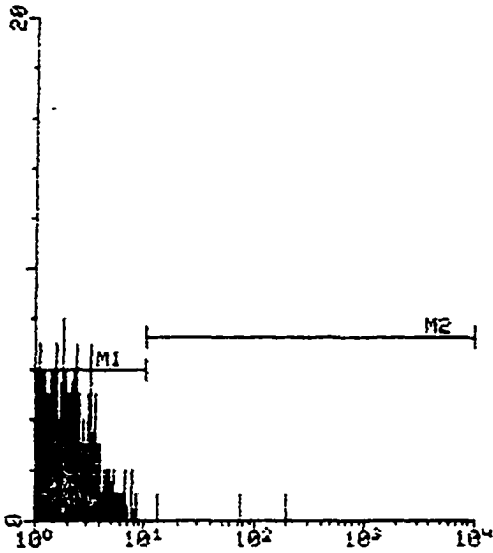
--- Arithmetic Histogram Statistics for MK005 ---      --- Arithmetic Histogram Statistics for MK005 ---
Selected Preferences: Arithmetic/Linear                 Selected Preferences: Arithmetic/Linear
Parameter FL1-H    Gate G1= R1                           Parameter FL2-H CD36   Gate G1= R1
M Left,Right  Events    %    Peak  PkChl   Mean          M Left,Right  Events    %    Peak  PkChl   Mean

0 1.00, 9910    726  100.00   189   1.00   2.28          0 1.00, 9910    726  100.00    10  96.47  156.42
1 1.00,10.55    723   99.59   189   1.00   1.96          1 1.00,10.94     29    3.99     3   1.00    4.11
2 10.55, 9910     3    0.41     1  13.34  94.44          2 10.94, 9910    697   96.01    10  96.47  162.76

Figure 12. Pregnant CD36 flow cytometric results

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Table 5. CD36 Binding Results in nonpregnant and pregnant samples

|  | Nonpregnant (n=22) | Pregnant (n=18) | t-value | *S/NS |
|---|---|---|---|---|
| Peak Channel CD36 | 92.45+/- 98.4 | 84.00+/- 53.7 | 0.328 | NS |
| Mean Channel CD36 | 110.77+/- 25.1 | 145.22+/- 54.2 | -2.65 | NS |

* Two-sided 99% confidence interval

<u>CD41</u>

CD41 is an antibody, which binds to the cell surface glycoprotein GPIIb. In this study CD41 was examined in three different t-tests. The first one comprised of FITC bound Ab to just CD41. The next two were double labeled using CD41 as a platelet marker while assessing for activation using activation specific antibodies. One included FITC Abs to CD41 with a PE Ab to CD62 (see figures 21 and 22) and the third test used FITC Abs to PAC-1 (see figures 23 and 24) while the CD41 Ab was coupled to PE. Figures 13 and 14 show the results of the first t-test while table 6 summarizes all of the results for CD41. The mean of the peak channels for CD41 for the two groups was 781.14, 791.73 (coupled with CD62), and 1574.55 (coupled with PAC-1) for the nonpregnant group and 934.28, 966.72, and 2395.28, respectively, for the pregnant group. The average of the mean channel for nonpregnant group was 787.09, 788.00 (coupled with CD62), and 1781.14 (coupled with PAC-1) while the pregnant group had values of 994.33, 1081.72, and 2700.33, respectively. Four of these showed statistically significant differences between the two groups. The CD41 FITC mean channel had a t-value of -3.37. The CD41 FITC mean channel with CD62 double labeling had a t-value of -4.77, while both peak and mean channels for

DATE: 10-FEB-99

TIME: 11:24:11

SELECTED PREFERENCES: Arithmetic/Linear
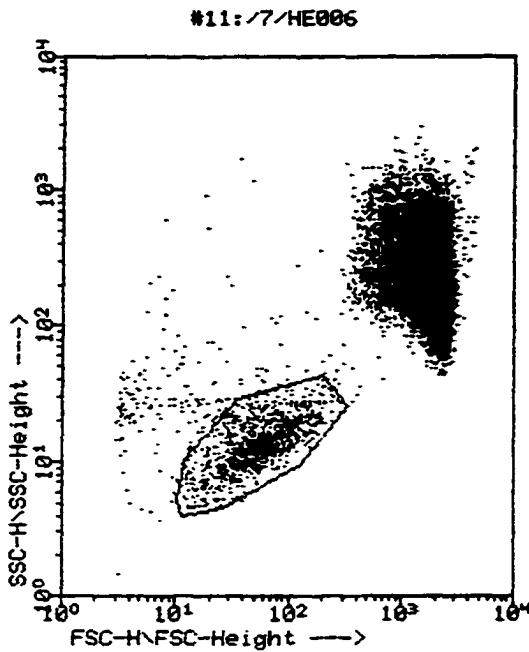
#11:/7/HE006

#11:/7/HE006

#11:/7/HE006
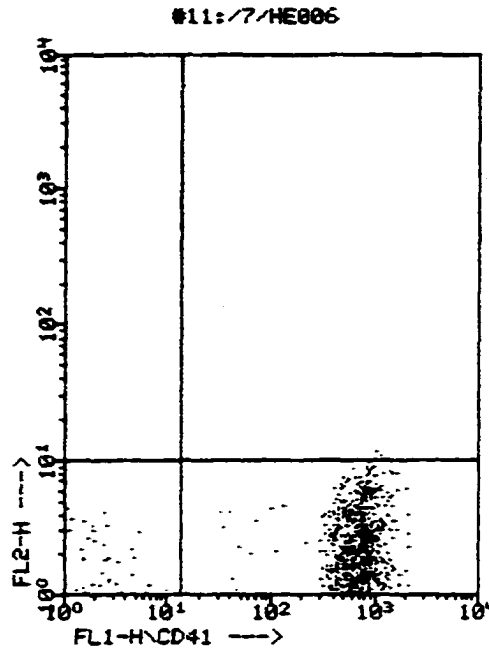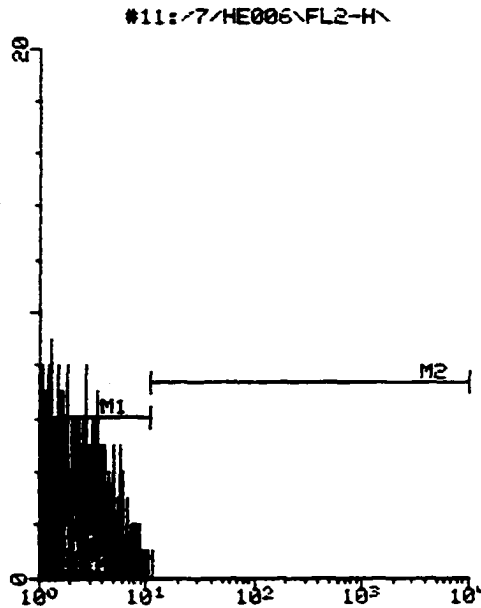
```
—— Quad Stats ——
File: #11:/7/HE006  Sample:  056
Date: 1/27/99  Gate G1= R1
Selected Preference: Arithmetic/Linear
Parameters: FL1-H(LOG),FL2-H(LOG)  Quad
Total=   10000  Gated=     939
Quad  Events  % Gated  % Total    Xme:
```

| Quad | Events | % Gated | % Total | Xme: |
|------|--------|---------|---------|------|
| 1 UL | 0 | 0.00 | 0.00 | — |
| 2 UR | 2 | 0.21 | 0.02 | 1056.9 |
| 3 LL | 53 | 5.64 | 0.53 | 2.4 |
| 4 LR | 884 | 94.14 | 8.84 | 718.9 |

#11:/7/HE006\FL1-H\CD41
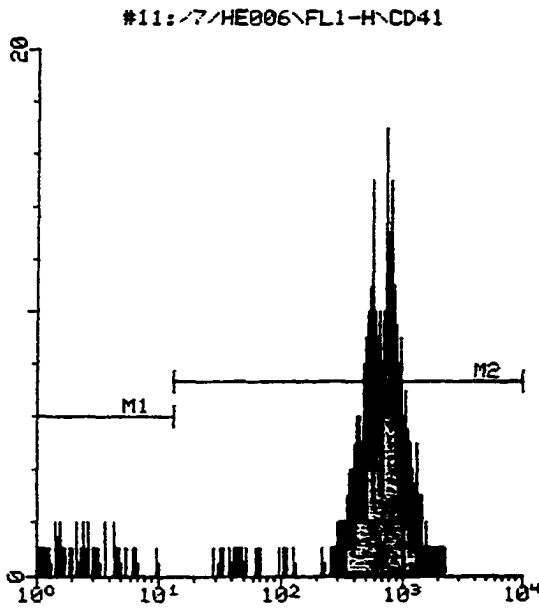
#11:/7/HE006\FL2-H\

#11:/7/HE006\FL1-H\CD41

—— Arithmetic Histogram Statistics for #11:/7/HE00
Selected Preferences: Arithmetic/Linear
Parameter FL1-H CD41    Gate G1= R1

| M | Left,Right | Events | % | Peak | PkChl | Mean |
|---|-----------|--------|------|------|--------|--------|
| 0 | 1.00, 9910 | 939 | 100.00 | 17 | 743.18 | 688.41 |
| 1 | 1.00,13.58 | 53 | 5.64 | 9 | 1.00 | 2.49 |
| 2 | 13.58. 9910 | 886 | 94.36 | 17 | 743.18 | 729.44 |

#11:/7/HE006\FL2-H\

—— Arithmetic Histogram Statistics for #11:/7/HE00
Selected Preferences: Arithmetic/Linear
Parameter FL2-H    Gate G1= R1

| M | Left,Right | Events | % | Peak | PkChl | Mean |
|---|-----------|--------|------|------|--------|--------|
| 0 | 1.00, 9910 | 939 | 100.00 | 227 | 1.00 | 2.34 |
| 1 | 1.00,11.24 | 938 | 99.89 | 227 | 1.00 | 2.33 |
| 2 | 11.24. 9910 | 1 | 0.11 | 1 | 11.65 | 11.65 |

Figure 13. Non-pregnant CD41 FITC flow cytometric results

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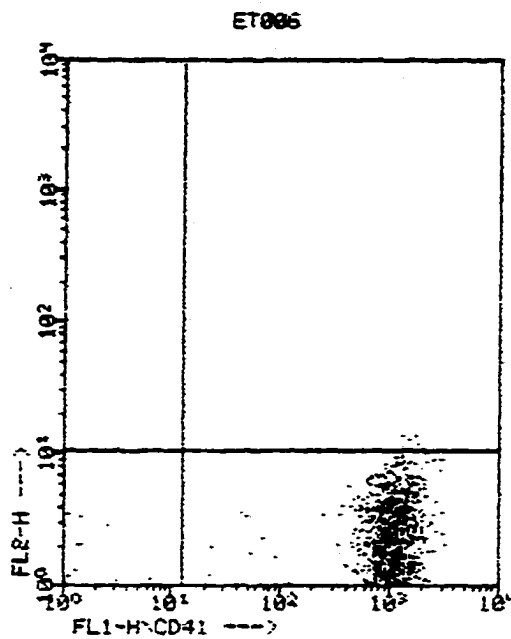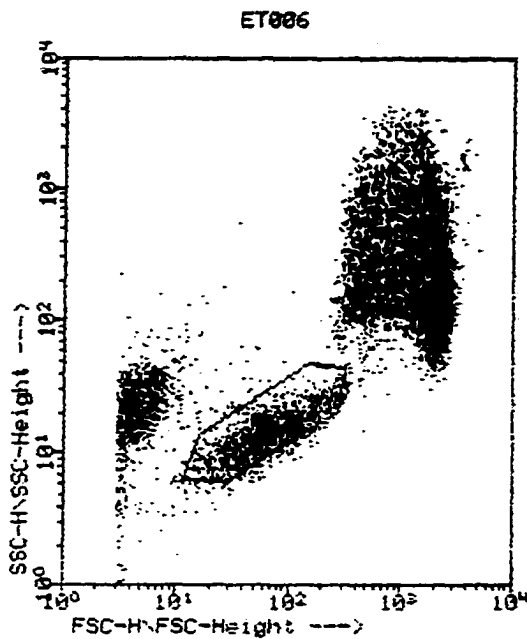
**ET006**

| Quad Stats |
| --- |

File: ET006  Sample:   017
Date:  3/25/99  Gate G1= R1
Selected Preference: Arithmetic/Linear
Parameters: FL1-H(LOG),FL2-H(LOG)  Qua
Total=   10000  Gated=    1187

| Quad | Events | % Gated | % Total | Xr |
| --- | --- | --- | --- | --- |
| 1 UL | 0 | 0.00 | 0.00 | — |
| 2 UR | 5 | 0.42 | 0.05 | 149E |
| 3 LL | 17 | 1.43 | 0.17 | 2 |
| 4 LR | 1165 | 98.15 | 11.65 | 1111 |

— Arithmetic Histogram Statistics for ET006 —
Selected Preferences: Arithmetic/Linear
Parameter FL1-H CD41    Gate G1= R1

| M | Left,Right | Events | % | Peak | PkChl | Mean |
| --- | --- | --- | --- | --- | --- | --- |
| 0 | 1.00, 9910 | 1187 | 100.00 | 18 | 1046 | 1111.38 |
| 1 | 1.00,11.14 | 17 | 1.43 | 3 | 1.00 | 2.83 |
| 2 | 11.14, 9910 | 1170 | 98.57 | 18 | 1046 | 1127.48 |

— Arithmetic Histogram Statistics for ET006 —
Selected Preferences: Arithmetic/Linear
Parameter FL2-H    Gate G1= R1

| M | Left,Right | Events | % | Peak | PkChl | Mean |
| --- | --- | --- | --- | --- | --- | --- |
| 0 | 1.00, 9910 | 1187 | 100.00 | 253 | 1.00 | 2.62 |
| 1 | 1.00,10.55 | 1181 | 99.49 | 253 | 1.00 | 2.57 |
| 2 | 10.55, 9910 | 8 | 0.67 | 2 | 10.55 | 11.86 |

Figure 14. Pregnant CD41 FITC flow cytometric results

CD41 PE (coupled with PAC-1) were statistically different, t-values of -5.38 and -8.52 respectively.

Table 6. CD41 Binding Results in nonpregnant and pregnant samples

|  | Nonpregnant (n=22) | Pregnant (n=18) | t-value | *S/NS |
|---|---|---|---|---|
| Peak Channel CD41 FITC | 781.14+/- 134.4 | 934.28+/- 337.7 | -1.95 | NS |
| Mean Channel CD41 FITC | 787.09+/- 64.4 | 994.33+/- 279.8 | -3.37 | S |
| Peak Channel CD41 FITC w/ CD62 | 791.73+/- 129.7 | 966.72+/- 270.6 | -2.68 | NS |
| Mean Channel CD41 FITC w/ CD62 | 788.00+/- 55.6 | 1081.72+/- 282.7 | -4.77 | S |
| Peak Channel CD41 PE w/ PAC-1 | 1574.55+/- 320.4 | 2395.28+/- 622.0 | -5.38 | S |
| Mean Channel CD41 PE w/ PAC-1 | 1781.14+/- 271.5 | 2700.33+/- 408.0 | -8.52 | S |

* Two-sided 99% confidence interval

CD42a

CD42a is an antibody to GPIX. This is a member of the larger receptor known as GPIb, which initiates primary platelet adhesion to vWF during hemostasis. The peak channel for CD42a had a mean of 288.59 for nonpregnant group and 253.11 for the pregnant group, while the average of the mean channels was 324.45 and 274.50 respectively. Figures 15 and 16 are examples of the dot plot and histograms obtained for CD42a while table 7 summarizes the results and statistics for CD42a. Neither the peak nor mean channels showed any statistical difference.

Table 7. CD42a Binding Results in nonpregnant and pregnant samples

|  | Nonpregnant (n=22) | Pregnant (n=18) | t-value | *S/NS |
|---|---|---|---|---|
| Peak Channel CD42a | 288.59+/- 57.6 | 253.11+/- 88.5 | 1.52 | NS |
| Mean Channel CD42a | 324.45+/- 39.1 | 274.50+/- 77.3 | 2.65 | NS |

* Two-sided 99% confidence interval

LYSYS II  Ver 1.1  2/6/92

DATE: 3-MAR-99                                    TIME: 11:26:17
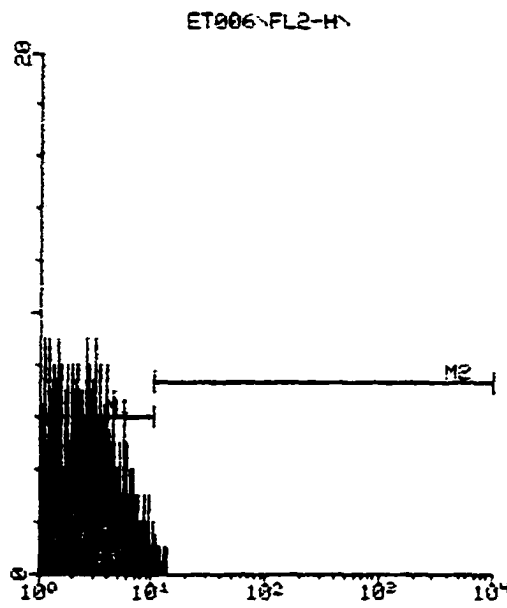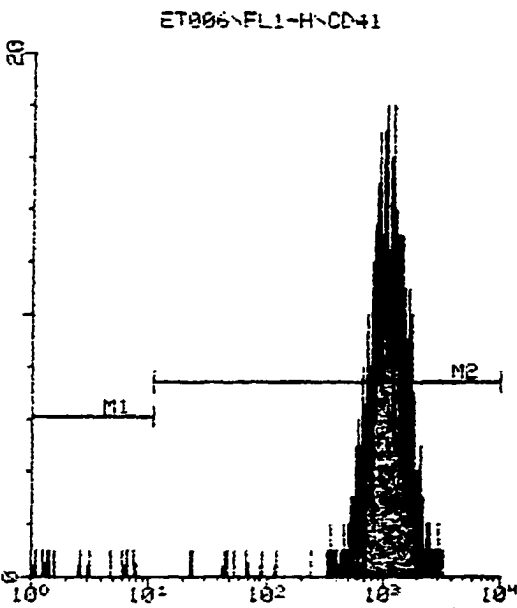
SELECTED PREFERENCES: Arithmetic/Linear



LE009
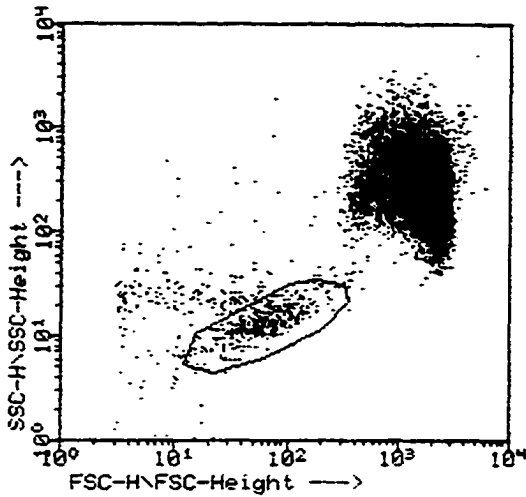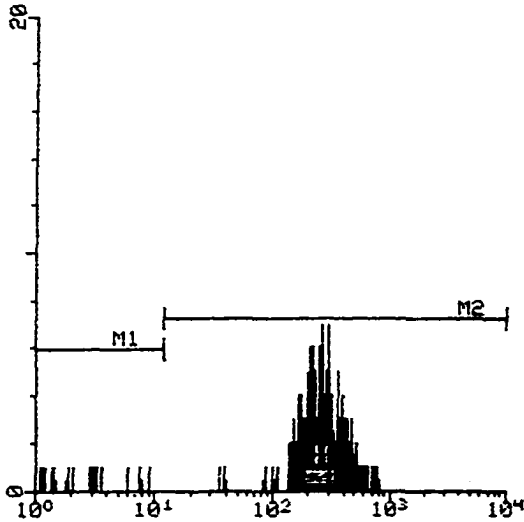
Quad Stats
File: LE009  Sample:  192
Date:  1/27/99  Gate G1= R1
Selected Preference: Arithmetic/Linear
Parameters: FL1-H(LOG),FL2-H(LOG)  Qua
Total=    10000  Gated=    331
Quad    Events  % Gated  % Total    Xm

| Quad | Events | % Gated | % Total | Xm |
|------|--------|---------|---------|-----|
| 1 UL | 1 | 0.30 | 0.01 | 1 |
| 2 UR | 0 | 0.00 | 0.00 | — |
| 3 LL | 23 | 6.95 | 0.23 | 2 |
| 4 LR | 307 | 92.75 | 3.07 | 270 |

—— Arithmetic Histogram Statistics for LE009 ——
Selected Preferences: Arithmetic/Linear
Parameter FL1-H CD42A    Gate G1= R1

| M | Left,Right | Events | % | Peak | PkChl | Mean |
|---|-----------|--------|-----|------|-------|------|
| 0 | 1.00, 9910 | 331 | 100.00 | 7 | 254.83 | 254.34 |
| 1 | 1.00,12.08 | 24 | 7.25 | 4 | 1.00 | 2.70 |
| 2 | 12.08, 9910 | 307 | 92.75 | 7 | 254.83 | 274.01 |
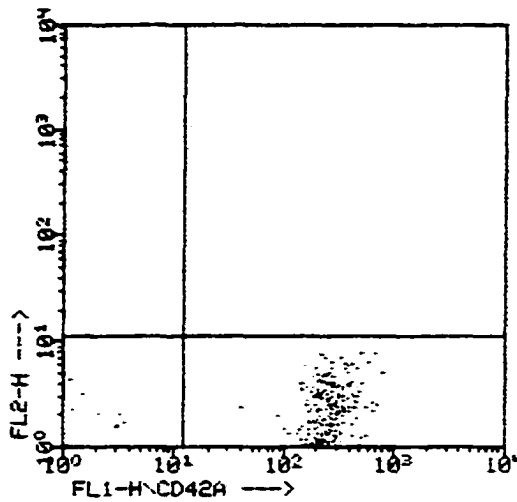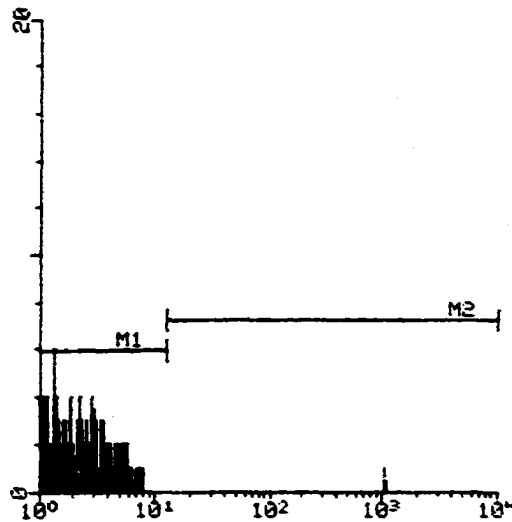
—— Arithmetic Histogram Statistics for LE009 ——
Selected Preferences: Arithmetic/Linear
Parameter FL2-H    Gate G1= R1

| M | Left,Right | Events | % | Peak | PkChl | Mean |
|---|-----------|--------|-----|------|-------|------|
| 0 | 1.00, 9910 | 331 | 100.00 | 87 | 1.00 | 5.35 |
| 1 | 1.00,12.30 | 330 | 99.70 | 87 | 1.00 | 2.17 |
| 2 | 12.86, 9910 | 1 | 0.30 | 1 | 1055 | 1055.45 |

Figure 15. Non-pregnant CD42a flow cytometric results

DATE: 1-APR-99

TIME: 22:47:18

SELECTED PREFERENCES: Arithmetic/Linear
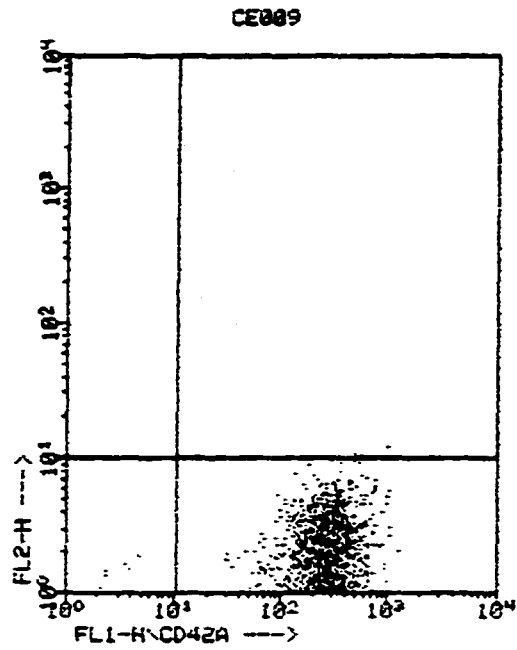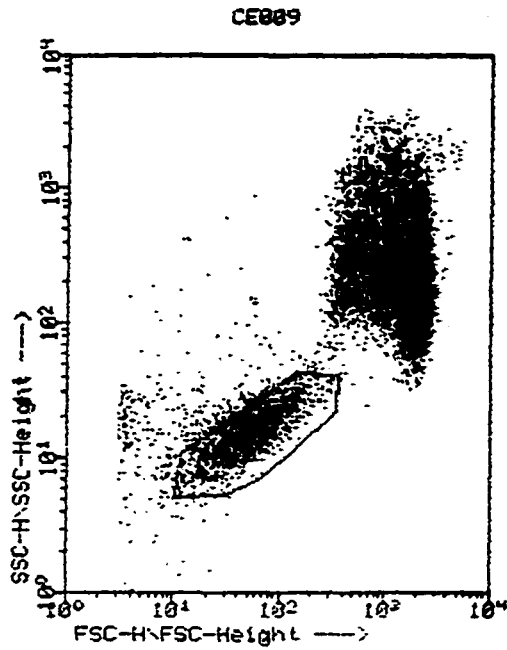


CE009

```
----- Quad Stats -----
File: CE009  Sample:    031
Date:  3/19/99   Gate GI= RI
Selected Preference: Arithmetic/Linear
Parameters: FL1-H(LOG),FL2-H(LOG)  Quad L
Total=    10000  Gated=      1428
Quad   Events  % Gated  % Total     Xmean
```

| Quad | Events | % Gated | % Total | Xmean |
|------|--------|---------|---------|-------|
| 1 UL | 0 | 0.00 | 0.00 | --- |
| 2 UR | 2 | 0.14 | 0.02 | 725.81 |
| 3 LL | 11 | 0.77 | 0.11 | 3.68 |
| 4 LR | 1415 | 99.89 | 14.15 | 288.42 |

--- Arithmetic Histogram Statistics for CE009 ---
Selected Preferences: Arithmetic/Linear
Parameter FL1-H CD42A    Gate GI= R1

| M | Left,Right | Events | % | Peak | PkChl | Mean |
|---|-----------|--------|---|------|-------|------|
| 0 | 1.00, 9910 | 1428 | 100.00 | 19 | 259.46 | 290.71 |
| 1 | 1.00, 10.55 | 11 | 0.77 | 1 | 1.05 | 3.93 |
| 2 | 10.55, 9910 | 1417 | 99.23 | 19 | 259.46 | 292.94 |

--- Arithmetic Histogram Statistics for CE009 ---
Selected Preferences: Arithmetic/Linear
Parameter FL2-H    Gate GI= R1

| M | Left,Right | Events | % | Peak | PkChl | Mean |
|---|-----------|--------|---|------|-------|------|
| 0 | 1.00, 9910 | 1428 | 100.00 | 381 | 1.00 | 2.14 |
| 1 | 1.00, 10.84 | 1427 | 99.93 | 381 | 1.00 | 2.13 |
| 2 | 10.94, 9910 | 1 | 0.07 | 1 | 12.08 | 12.09 |

Figure 16. Pregnant CD42a flow cytometric results

## CD42b

CD42b is also known as GPIbα, which is another member, besides CD42a, of

the GPIb complex, which again is responsible for primary adhesion of the platelet to

vWF. The peak channel and mean channel was 265.50 and 348.50 respectively for

the nonpregnant group while the pregnant group had values of 260.83 and 305.94,

respectively. Figures 17 and 18 show the results of CD42b and table 8 sums up the

statistics and average values for peak and mean channels of the two groups of

subjects. Like CD42a, CD42b did not show any statistically significant differences.

Table 8. CD42b Binding Results in nonpregnant and pregnant samples

|  | Nonpregnant (n=22) | Pregnant (n=18) | t-value | *S/NS |
|---|---|---|---|---|
| Peak Channel CD42b | 265.50+/- 78.4 | 260.83+/- 157.8 | 1.23 | NS |
| Mean Channel CD42b | 348.50+/- 112.5 | 305.94+/- 154.9 | 1.01 | NS |

* Two-sided 99% confidence interval

## CD61

CD61 is an antibody to GPIIIa, which is a member of the platelet receptor

more commonly known as GPIIb/ IIIa. CD41 is the Ab to GPIIb while the

noncovalently linked second subunit GPIIIa has CD61 as the Ab to it. 796.23 and

797.77 are the peak and mean channel averages for the nonpregnant group, while

811.44 and 861.06 are the means of the pregnant group. Figures 19 and 20 are

examples of the results obtained for CD61. Table 9 shows the averages of the peak

and mean channels for CD61 along with the t-values calculated. No statistically

significant differences are noted.

LE010                    LE010                    LE010

------- Quad Stats -------
File: LE010  Sample:   193
Date:  1/27/99  Gate G1= R1
Selected Preference: Arithmetic/Linear
Parameters: FL1-H(LOG),FL2-H(LOG)  Qua
Total=    10000  Gated=      211
Quad    Events   % Gated   % Total    Xm

1 UL       0     0.00      0.00      —
2 UR       0     0.00      0.00      —
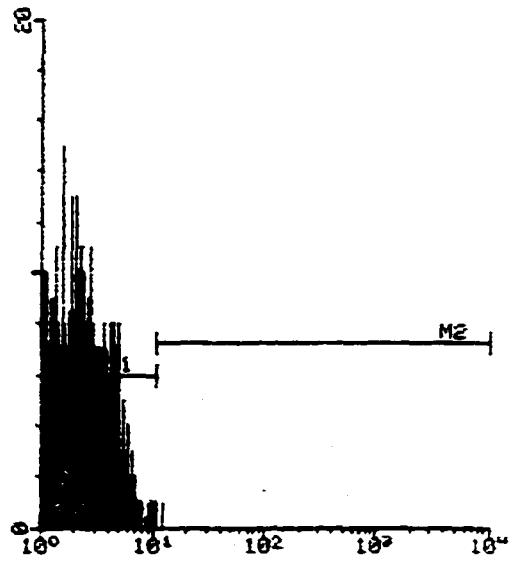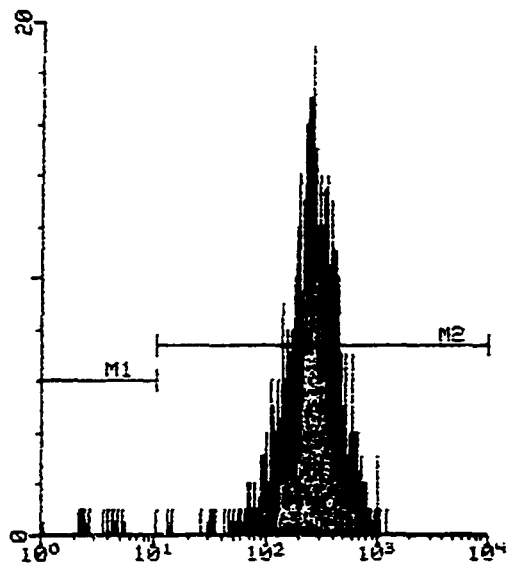3 LL      13     6.16      0.13      2
4 LR     198    93.84      1.98     370

LE010\FL1-H\CD42B          LE010\FL2-H\



LE010\FL1-H\CD42B                    LE010\FL2-H\

--- Arithmetic Histogram Statistics for LE010 ---     --- Arithmetic Histogram Statistics for LE010 ---
Selected Preferences: Arithmetic/Linear               Selected Preferences: Arithmetic/Linear
Parameter FL1-H CD42B    Gate G1= R1                   Parameter FL2-H    Gate G1= R1

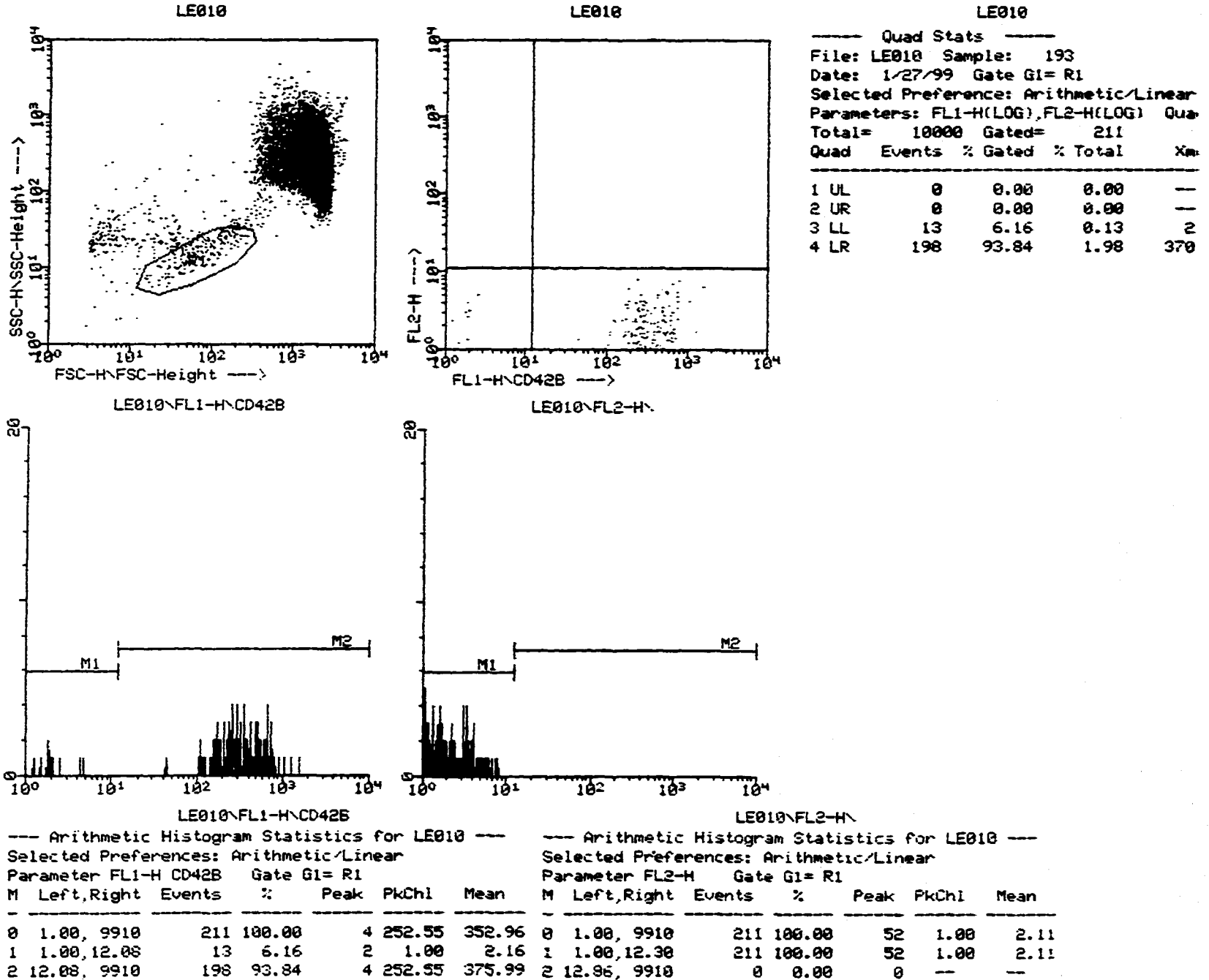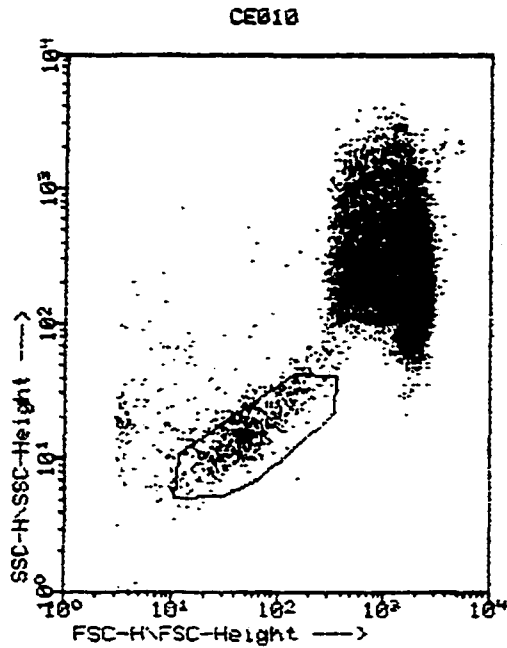| M | Left,Right | Events | % | Peak | PkChl | Mean | | M | Left,Right | Events | % | Peak | PkChl | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 1.00, 9910 | 211 | 100.00 | 4 | 252.55 | 352.96 | 0 | 1.00, 9910 | 211 | 100.00 | 52 | 1.00 | 2.11 |
| 1 | 1.00,12.08 | 13 | 6.16 | 2 | 1.00 | 2.16 | 1 | 1.00,12.30 | 211 | 100.00 | 52 | 1.00 | 2.11 |
| 2 | 12.08, 9910 | 198 | 93.84 | 4 | 252.55 | 375.99 | 2 | 12.96, 9910 | 0 | 0.00 | 0 | — | — |

Figure 17. Non-pregnant CD42b flow cytometric results

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DATE: 1-APR-99                                          TIME: 22:48:28

SELECTED PREFERENCES: Arithmetic/Linear



```
                                                      CE010
                                          ----- Quad Stats -----
                                          File: CE010  Sample:   032
                                          Date:  3/19/99  Gate G1= R1
                                          Selected Preference: Arithmetic/Linear
                                          Parameters: FL1-H(LOG),FL2-H(LOG)  Quad L
                                          Total=   10000  Gated=      459
                                          Quad    Events   % Gated  % Total    Xmean

                                          1 UL        0     0.00     0.00       —
                                          2 UR        0     0.00     0.00       --
                                          3 LL        4     0.87     0.04      2.12
                                          4 LR      455    99.13     4.55    294.73
```
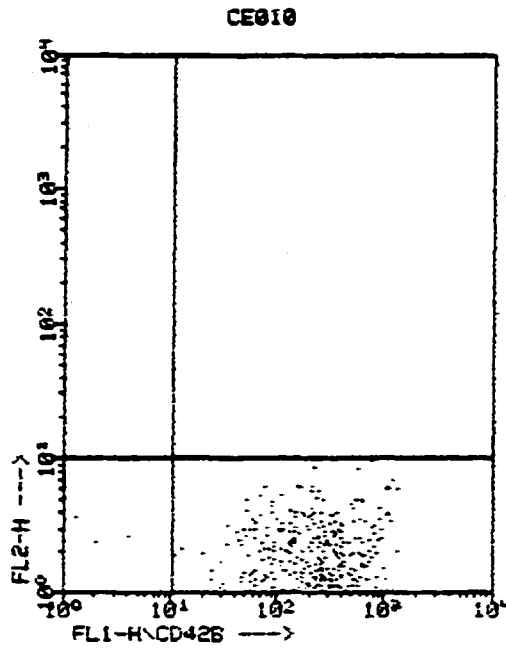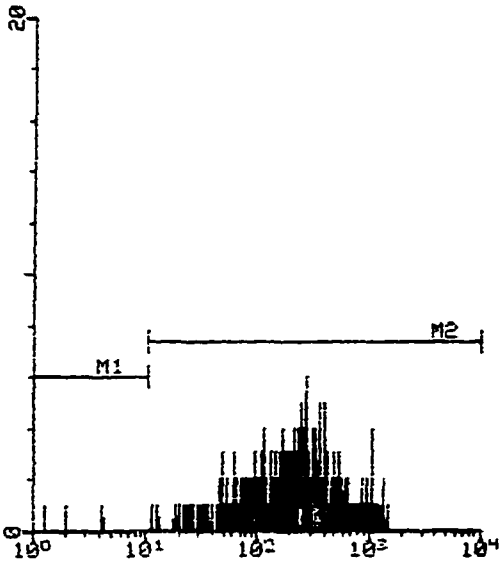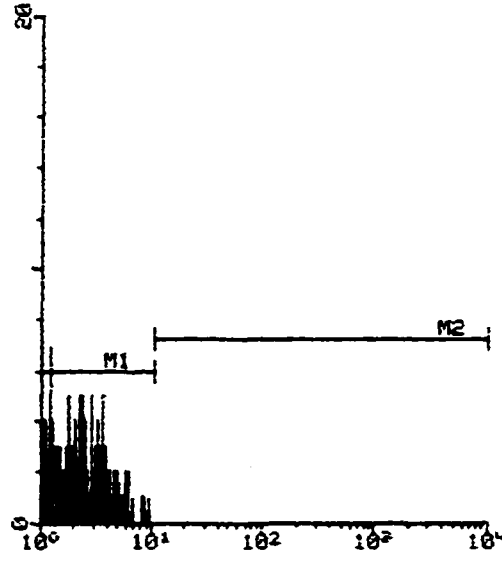
--- Arithmetic Histogram Statistics for CE010 ---
Selected Preferences: Arithmetic/Linear
Parameter FL1-H CD42B    Gate G1= R1

| M | Left,Right | Events | % | Peak | PkChl | Mean |
|---|---|---|---|---|---|---|
| 0 | 1.00, 9910 | 459 | 100.00 | 6 | 271.39 | 296.24 |
| 1 | 1.00,10.55 | 4 | 0.87 | 1 | 1.00 | 2.13 |
| 2 | 10.55, 9910 | 455 | 99.13 | 6 | 271.39 | 298.83 |

--- Arithmetic Histogram Statistics for CE010 ---
Selected Preferences: Arithmetic/Linear
Parameter FL2-H    Gate G1= R1

| M | Left,Right | Events | % | Peak | PkChl | Mean |
|---|---|---|---|---|---|---|
| 0 | 1.00, 9910 | 459 | 100.00 | 143 | 1.00 | 1.97 |
| 1 | 1.00,10.34 | 459 | 100.00 | 143 | 1.00 | 1.97 |
| 2 | 10.34, 9910 | 0 | 0.00 | 0 | -- | -- |

Figure 18. Pregnant CD42b flow cytometric results

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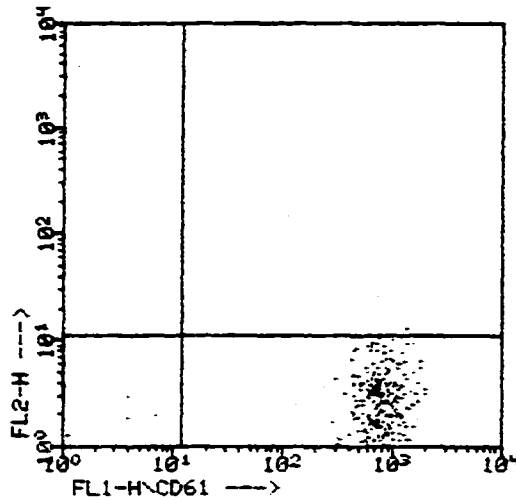DATE: 3-MAR-99                                    TIME: 11:28:40

SELECTED PREFERENCES: Arithmetic/Linear



LE011
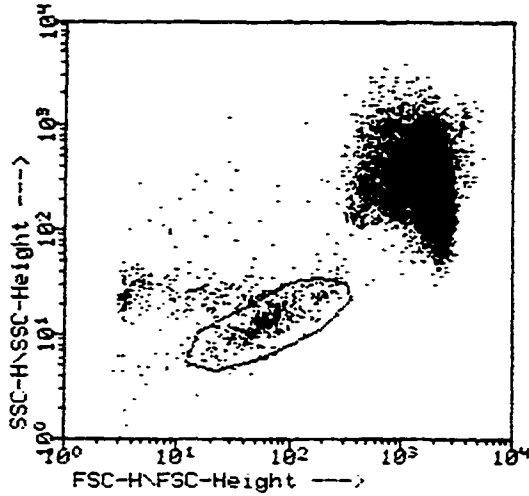
LE011

**LE011**

```
------ Quad Stats ------
File: LE011  Sample:   194
Date:  1/27/99  Gate G1= R1
Selected Preference: Arithmetic/Linear
Parameters: FL1-H(LOG),FL2-H(LOG)  Quad
Total=   10000  Gated=      370
Quad   Events  % Gated  % Total     Xm
```

| Quad | Events | % Gated | % Total | Xm |
|------|--------|---------|---------|------|
| 1 UL | 0 | 0.00 | 0.00 | — |
| 2 UR | 1 | 0.27 | 0.01 | 1333. |
| 3 LL | 10 | 2.70 | 0.10 | 3 |
| 4 LR | 359 | 97.03 | 3.59 | 808. |

LE011\FL1-H\CD61
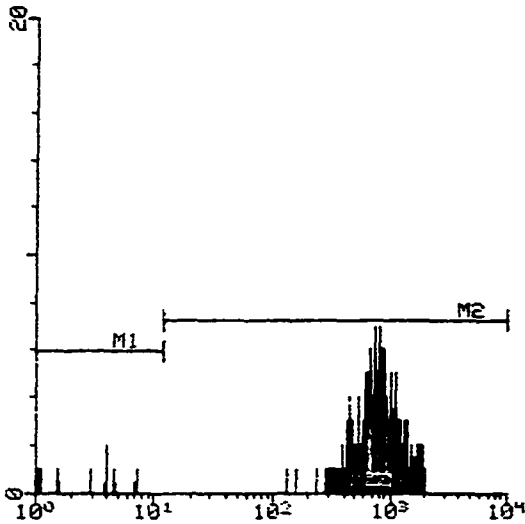
LE011\FL2-H\
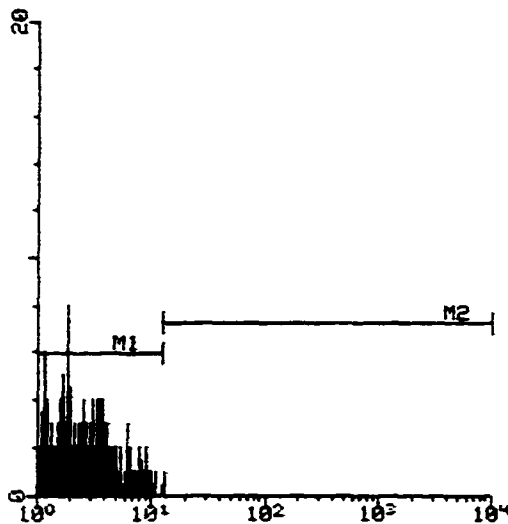
—— Arithmetic Histogram Statistics for LE011 ——
Selected Preferences: Arithmetic/Linear
Parameter FL1-H CD61    Gate G1= R1

| M | Left,Right | Events | % | Peak | PkChl | Mean |
|---|-----------|--------|-----|------|-------|------|
| 0 | 1.00, 9910 | 370 | 100.00 | 7 | 723.39 | 798.62 |
| 1 | 1.00,12.08 | 10 | 2.70 | 2 | 4.03 | 3.15 |
| 2 | 12.08, 9910 | 360 | 97.30 | 7 | 723.39 | 820.71 |

—— Arithmetic Histogram Statistics for LE011 ——
Selected Preferences: Arithmetic/Linear
Parameter FL2-H    Gate G1= R1

| M | Left,Right | Events | % | Peak | PkChl | Mean |
|---|-----------|--------|-----|------|-------|------|
| 0 | 1.00, 9910 | 370 | 100.00 | 76 | 1.00 | 2.70 |
| 1 | 1.00,12.30 | 369 | 99.73 | 76 | 1.00 | 2.67 |
| 2 | 12.86, 9910 | 1 | 0.27 | 1 | 12.98 | 12.98 |

Figure 19. Non-pregnant CD61 flow cytometric results

DATE: 29-MAR-99

TIME: 23:01:53

SELECTED PREFERENCES: Arithmetic/Linear
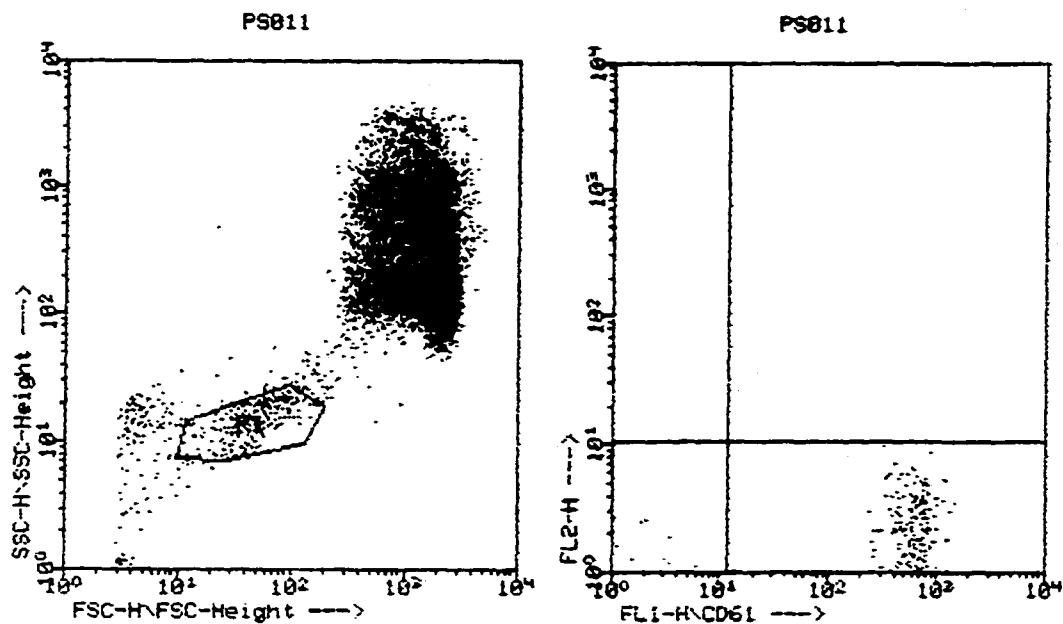


PS011

PS011

```
              PS011
      ---- Quad Stats ----
File: PS011  Sample:   060
Date:  3/25/99  Gate G1= R1
Selected Preference: Arithmetic/Linear
Parameters: FL1-H(LOG),FL2-H(LOG)   Qu=
Total=    10000  Gated=      250
Quad    Events  % Gated  % Total    Xm

1 UL       0     0.00     0.00      --
2 UR       0     0.00     0.00      --
3 LL      14     5.60     0.14       3
4 LR     236    94.40     2.36      62E
```



--- Arithmetic Histogram Statistics for PS011 ---
Selected Preferences: Arithmetic/Linear
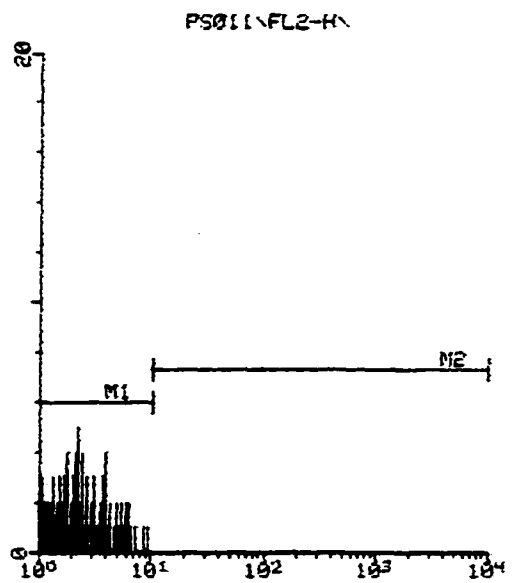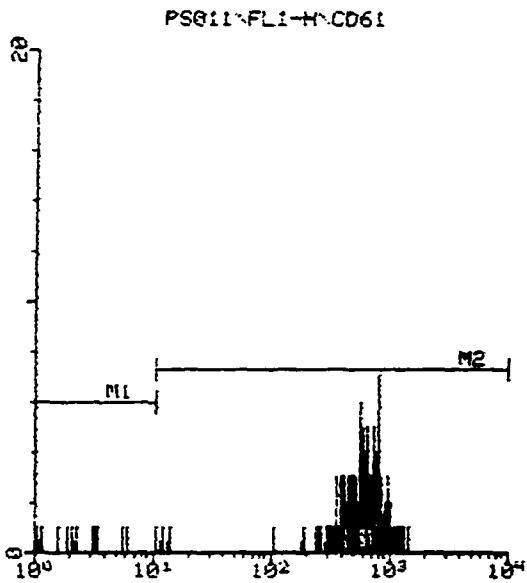Parameter FL1-H CD61    Gate G1= R1

| M | Left,Right | Events | % | Peak | PkChl | Mean |
|---|---|---|---|---|---|---|
| 0 | 1.00, 9910 | 250 | 100.00 | 7 | 820.47 | 601.08 |
| 1 | 1.00,10.84 | 13 | 5.20 | 1 | 1.00 | 3.36 |
| 2 | 10.84, 9910 | 237 | 94.90 | 7 | 820.47 | 633.86 |

--- Arithmetic Histogram Statistics for PS011 ---
Selected Preferences: Arithmetic/Linear
Parameter FL2-H    Gate G1= R1

| M | Left,Right | Events | % | Peak | PkChl | Mean |
|---|---|---|---|---|---|---|
| 0 | 1.00, 9910 | 250 | 100.00 | 65 | 1.00 | 2.24 |
| 1 | 1.00,10.65 | 250 | 100.00 | 65 | 1.00 | 2.24 |
| 2 | 10.65, 9910 | 0 | 0.00 | 0 | -- | -- |

Figure 20. Pregnant CD61 flow cytometric results

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**Table 9. CD61 Binding Results in nonpregnant and pregnant samples**

| | Nonpregnant (n=22) | Pregnant (n=18) | t-value | *S/NS |
|---|---|---|---|---|
| Peak Channel CD61 | 796.23+/- 150.0 | 811.44+/- 382.2 | -0.17 | NS |
| Mean Channel CD61 | 797.77+/- 80.6 | 861.06+/- 380.9 | -0.76 | NS |

\* Two-sided 99% confidence interval
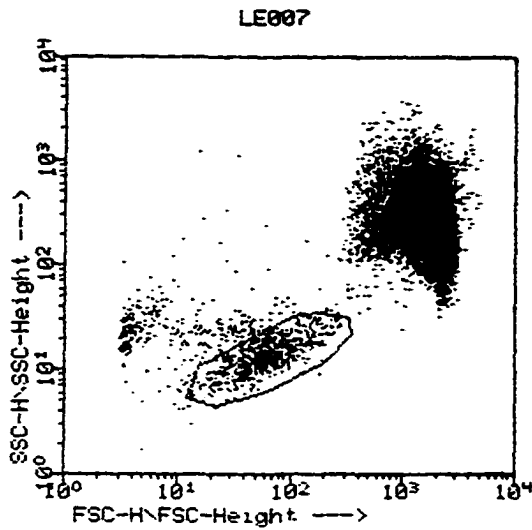
<u>CD62</u>

CD62 is the platelet adhesion molecule known as GMP-140. It is normally sequestered on the inner surface of the α-granules and only during degranulation would it be exposed on the platelets outer surface and accessible to the Ab. It is therefore an indicator of platelet activation. Figures 21 and 22 are examples of the results obtained from CD62 while table 10 summarizes the statistics for CD62. The average of the peak channels for the nonpregnant and pregnant groups were 16.50 and 21.12, respectively while the mean channel had averages of 42.70 and 75.53, respectively. Besides just recording peak and mean channels, which did not show any statistical difference (t-values of -1.09 and -1.43, respectively), the percent of platelets with the CD62 marker was also recorded. The percent of platelets with CD62 binding was 0.71% for nonpregnant females while the pregnant females had 20.19% binding. This was indicative that the percent of platelets with α- granule release was different between the two groups (t-value of -5.24).
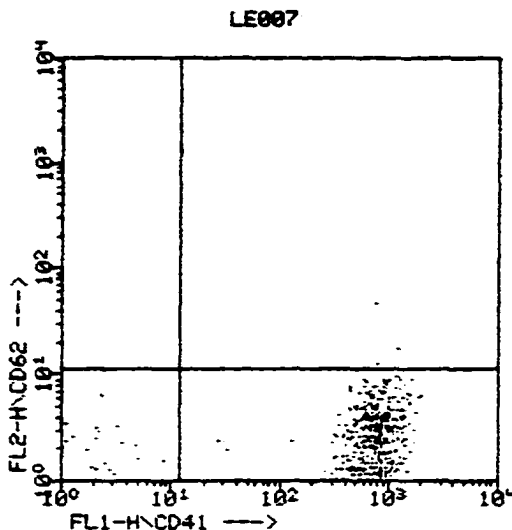
LYSYS II  Ver 1.1  2/6/90

DATE: 3-MAR-99                                      TIME: 11:17:25

SELECTED PREFERENCES: Arithmetic/Linear
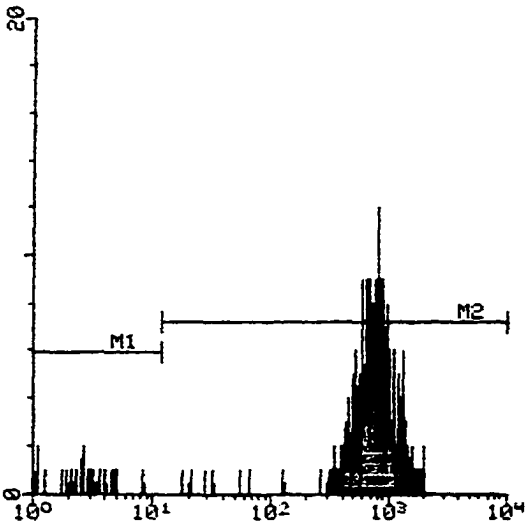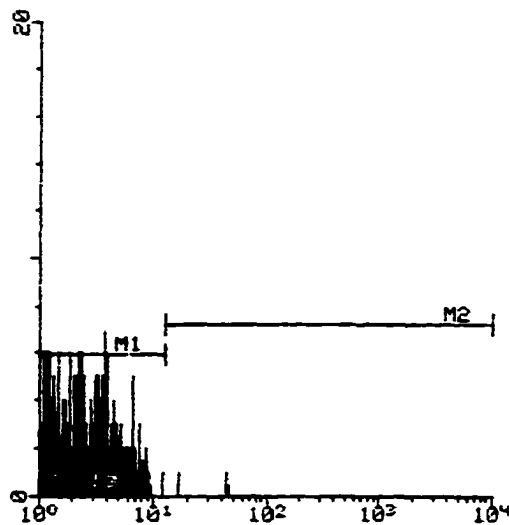


LE007

**Quad Stats**

File: LE007  Sample:   190
Date:  1/27/99  Gate G1= R1
Selected Preference: Arithmetic/Linear
Parameters: FL1-H(LOG),FL2-H(LOG)  Quad
Total=    10000  Gated=      600

| Quad | Events | % Gated | % Total | Xme |
|---|---|---|---|---|
| 1 UL | 0 | 0.00 | 0.00 | — |
| 2 UR | 3 | 0.50 | 0.03 | 913. |
| 3 LL | 29 | 4.83 | 0.29 | 2. |
| 4 LR | 568 | 94.67 | 5.68 | 775. |

LE007\FL1-H\CD41

--- Arithmetic Histogram Statistics for LE007 ---
Selected Preferences: Arithmetic/Linear
Parameter FL1-H CD41    Gate G1= R1

| M | Left,Right | Events | % | Peak | PkChl | Mean |
|---|---|---|---|---|---|---|
| 0 | 1.00, 9910 | 600 | 100.00 | 12 | 805.94 | 749.25 |
| 1 | 1.00,12.08 | 29 | 4.83 | 2 | 1.00 | 2.78 |
| 2 | 12.08, 9910 | 571 | 95.17 | 12 | 805.94 | 787.16 |

LE007\FL2-H\CD62

--- Arithmetic Histogram Statistics for LE007 ---
Selected Preferences: Arithmetic/Linear
Parameter FL2-H CD62    Gate G1= R1

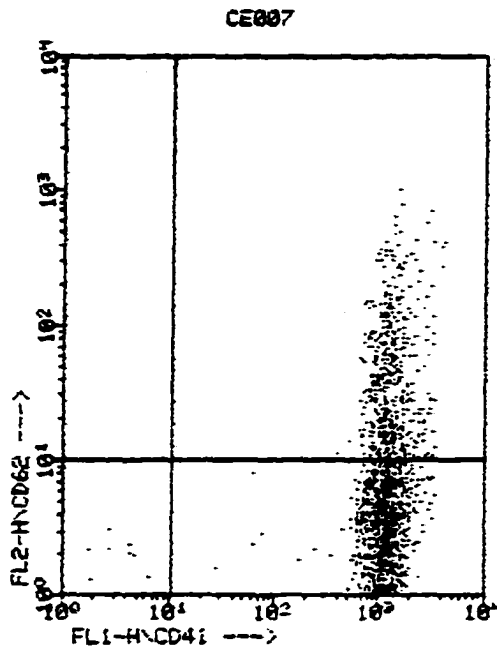| M | Left,Right | Events | % | Peak | PkChl | Mean |
|---|---|---|---|---|---|---|
| 0 | 1.00, 9910 | 600 | 100.00 | 131 | 1.00 | 2.71 |
| 1 | 1.00,12.30 | 598 | 99.67 | 131 | 1.00 | 2.62 |
| 2 | 12.86, 9910 | 2 | 0.33 | 1 | 16.70 | 30.80 |

Figure 21. Non-pregnant CD62 flow cytometric results

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SELECTED PREFERENCES: Arithmetic/Linear



Figure 22. Pregnant CD62 flow cytometric results

Table 10. CD62 Binding Results in nonpregnant and pregnant samples

| | Nonpregnant (n=22) | Pregnant (n=18) | t-value | *S/NS |
|---|---|---|---|---|
| % with CD62 marker | 0.71+/- 0.7 | 20.19+/- 17.5 | -5.24 | S |
| Peak Channel CD62 | 16.50+/- 15.4 | 21.12+/- 9.7 | -1.09 | NS |
| Mean Channel CD62 | 42.70+/- 83.6 | 75.53+/- 54.4 | -1.43 | NS |

* Two-sided 99% confidence interval

## PAC-1

PAC-1 is another platelet activation marker, which binds to activated GPIIb/ IIIa. Only after a process called inside-out signaling (see pages 22-23) does this marker present itself. Again, like CD62 it is important to consider the percentage of platelets with this marker besides the peak and mean channels of the ones that have it. The peak channel for the two groups was 673.41 and 1386.10 for the nonpregnant and pregnant groups while the mean channel had averages of 787.82 and 1890.50 respectively. The percent of platelets with PAC-1 binding was 0.48 and 0.33 for the nonpregnant and pregnant samples. Figures 23 and 24 are examples of the results of the pregnant and nonpregnant samples while table 11 summarizes the statistics. Here it is important to note the besides no statistical differences seen in peak and mean channels (t-values of -1.13 and -1.78, respectively), no statistical difference in the percent of platelets showing this activation marker was seen (t-value of 0.86).

DATE: 3-MAR-99                                         TIME: 12:02:13

SELECTED PREFERENCES: Arithmetic/Linear

JD008 (SSC-H\SSC-Height vs FSC-H\FSC-Height)
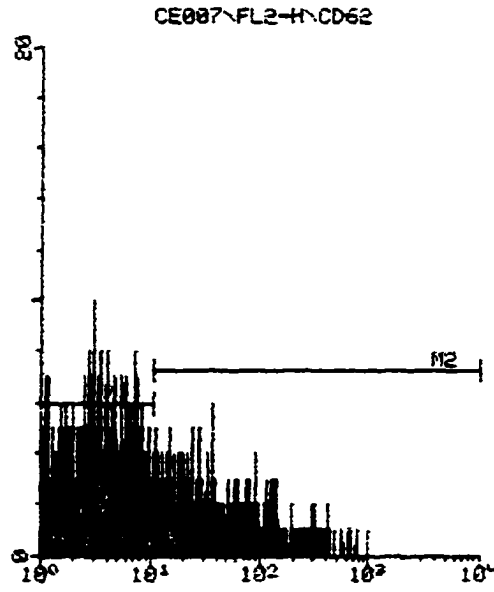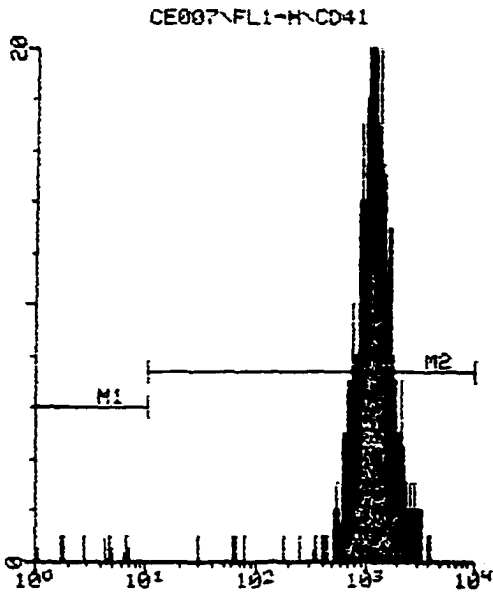
JD008 (FL2-H\CD41 vs FL1-H\PAC-1)

JD008

```
----- Quad Stats -----
File: JD008  Sample:   240
Date:  1/27/99  Gate G1= R1
Selected Preference: Arithmetic/Linear
Parameters: FL1-H(LOG),FL2-H(LOG)  Qua
Total=   10000  Gated=      590
Quad   Events  % Gated  % Total    Xm
```

| Quad | Events | % Gated | % Total | Xm |
|------|--------|---------|---------|-----|
| 1 UL | 451 | 76.44 | 4.51 | 1. |
| 2 UR | 0 | 0.00 | 0.00 | — |
| 3 LL | 138 | 23.39 | 1.38 | 2. |
| 4 LR | 1 | 0.17 | 0.01 | 523. |

JD008\FL1-H\PAC-1

JD008\FL2-H\CD41

--- Arithmetic Histogram Statistics for JD008 ---
Selected Preferences: Arithmetic/Linear
Parameter FL1-H PAC-1    Gate G1= R1

| M | Left,Right | Events | % | Peak | PkChl | Mean |
|---|-----------|--------|---|------|-------|------|
| 0 | 1.00, 9910 | 590 | 100.00 | 258 | 1.00 | 2.46 |
| 1 | 1.00,12.08 | 589 | 99.83 | 258 | 1.00 | 1.59 |
| 2 | 12.08, 9910 | 1 | 0.17 | 1 | 528.03 | 528.03 |

--- Arithmetic Histogram Statistics for JD008 ---
Selected Preferences: Arithmetic/Linear
Parameter FL2-H CD41    Gate G1= R1

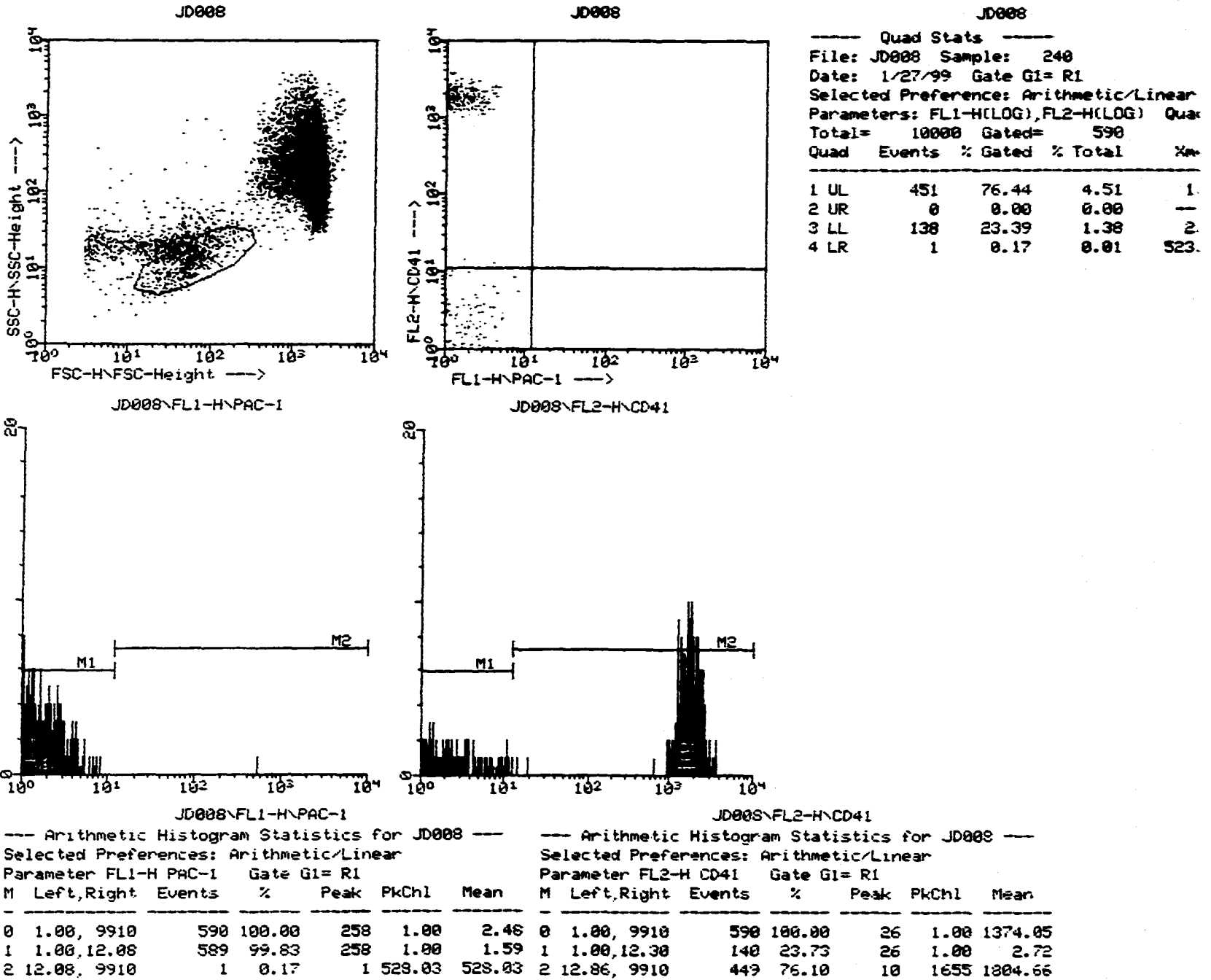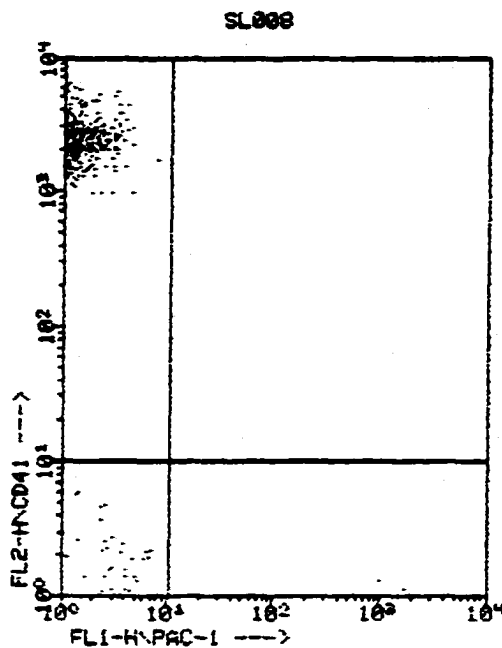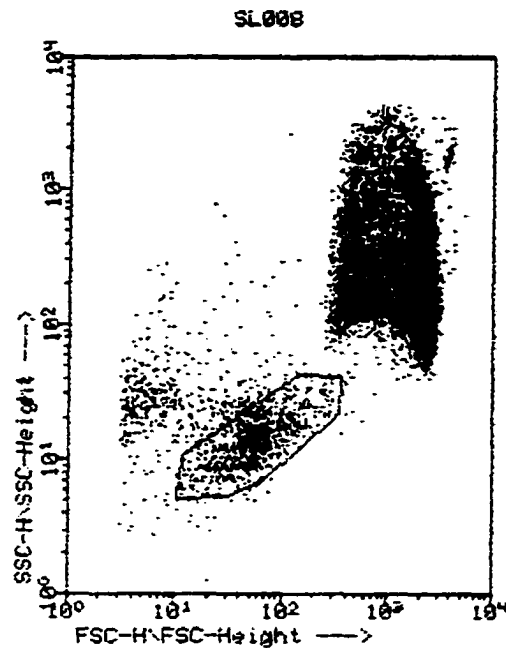| M | Left,Right | Events | % | Peak | PkChl | Mean |
|---|-----------|--------|---|------|-------|------|
| 0 | 1.00, 9910 | 590 | 100.00 | 26 | 1.00 | 1374.05 |
| 1 | 1.00,12.30 | 140 | 23.73 | 26 | 1.00 | 2.72 |
| 2 | 12.86, 9910 | 449 | 76.10 | 10 | 1655 | 1804.66 |

Figure 23. Non-pregnant PAC-1 flow cytometric results

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SELECTED PREFERENCES: Arithmetic/Linear



SL008

```
----- Quad Stats -----
File: SL008  Sample:   019
Date:  3/24/99  Gate G1= R1
Selected Preference: Arithmetic/Linear
Parameters: FL1-H(LOG),FL2-H(LOG)  Quad L
Total=   10000  Gated=     762
```

| Quad | Events | % Gated | % Total | Xmean |
|------|--------|---------|---------|-------|
| 1 UL | 714 | 93.70 | 7.14 | 1.3& |
| 2 UR | 0 | 0.00 | 0.00 | -- |
| 3 LL | 45 | 5.91 | 0.45 | 3.3& |
| 4 LR | 3 | 0.39 | 0.03 | 1161.8: |
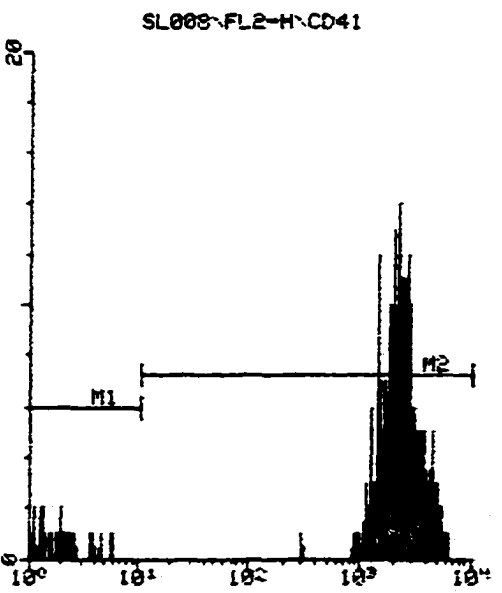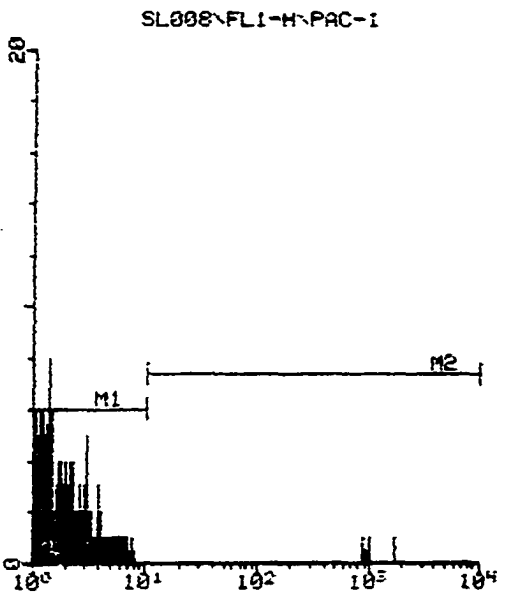


--- Arithmetic Histogram Statistics for SL008 ---
Selected Preferences: Arithmetic/Linear
Parameter FL1-H PAC-1      Gate G1= R1

| M | Left,Right | Events | % | Peak | PkChl | Mean |
|---|-----------|--------|---|------|-------|------|
| 0 | 1.00, 9910 | 762 | 100.00 | 411 | 1.00 | 6.14 |
| 1 | 1.00,10.55 | 759 | 99.61 | 411 | 1.00 | 1.45 |
| 2 | 10.55, 9910 | 3 | 0.39 | 1 | 899.65 | 1190.63 |

--- Arithmetic Histogram Statistics for SL008 ---
Selected Preferences: Arithmetic/Linear
Parameter FL2-H CD41      Gate G1= R1

| M | Left,Right | Events | % | Peak | PkChl | Mean |
|---|-----------|--------|---|------|-------|------|
| 0 | 1.00, 9910 | 762 | 100.00 | 14 | 2167 | 2318.42 |
| 1 | 1.00,10.84 | 48 | 6.30 | 10 | 1.00 | 1.96 |
| 2 | 10.84, 9910 | 714 | 93.70 | 14 | 2167 | 2474.15 |

Figure 24. Pregnant PAC-1 flow cytometric results

Table 11. PAC-1 Binding Results in nonpregnant and pregnant samples

| | Nonpregnant (n=22) | Pregnant (n=18) | t-value | *S/NS |
|---|---|---|---|---|
| % with PAC-1 marker | 0.48+/- 0.58 | 0.33+/- 0.47 | 0.86 | NS |
| Peak Channel PAC-1 | 673.41+/- 1696.9 | 1386.10+/- 2302.6 | -1.13 | NS |
| Mean Channel PAC-1 | 787.82+/- 1665.6 | 1890.50+/- 2251.0 | -1.78 | NS |

* Two-sided 99% confidence interval

# Chapter 5
## Discussion

## Overview

During normal pregnancy changes do occur on the platelet surface. In the results section it was shown that certain receptors do change in numbers. It is interesting that in all receptors that did statistically change in peak or mean channel values between nonpregnant and pregnant females, the platelet receptors were upregulated during pregnancy. This could potentially lead to abnormal platelet activation leading to aggregation. This supports the quote from the pre-eclampsia introduction, "during normal pregnancy there are various effects on the coagulation system that have been compared to a chronic state of compensated DIC" (Perry, 1992). More importantly, both hypotheses, stated in chapter 1, would be supported by the results presented in chapter 4:

1) During normal pregnancy, there are increases in platelet proteins, which are essential for platelet function.

->While not all of the platelet proteins did increase during pregnancy, two of them involved in platelet aggregation did, GPIIb and CD29, a collagen receptor.

2) During pregnancy there is an increase in circulating activated platelets.

->This hypothesis while it seems was proven needs to be looked at more closely. Since one activation marker did indicate an increase in circulating activated platelets the other, more accurate, marker did not indicate this. In the discussion on activation markers this topic will further be explored.

## Demographics

The ages were similar between the two groups, as were the platelet counts and MPV. This indicates that the groups were comparable. There was a difference on the issue of smoking. It is backward logic that a greater number of females would smoke during pregnancy than nonpregnancy (3 vs 0). This is where a limitation in the study arises. While the two groups of females are comparable (see above), the populations from which they were obtained were different. The pregnant females were obtained from a general OB/GYN practice in Grand Rapids while the nonpregnant females were all students in a graduate class taken by health professionals. This set of health professional students would be more informed and concerned with the risks of smoking than the general population. Since smoking affects the cardiovascular system it could have slightly skewed the data showing a larger increase in platelet changes than was actually there.

The difference in medications is also worth mentioning. Eleven of the nonpregnant females (50%) were on contraceptives while the only medications the pregnant females were on were prenatal vitamins. Obviously, only the nonpregnant females would be on contraceptives. It is not known, though, if contraceptives affect platelets and this may affect the results.

A third point to mention in the demographics data is prior pregnancies. These were not statistically different and this is important to note because preeclampsia is seen in a greater majority during the first pregnancies. Since both groups had similar

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numbers and the pregnancy group had an average of 1.8 prior pregnancies this can be seen as equal in both groups and therefore not be a significant difference.

## Platelet receptors

This study looked at six different platelet receptors. Two of them showed significant increases during pregnancy, CD29 and CD41. The purpose of this study was to see what changes occur during pregnancy and that is why both peak and mean channels were examined.

Both peak and mean channels were used in the analysis to identify differences in binding of the antibody to the platelet surface. Peak channel is the channel "median", which shows maximal binding of the specific Ab, while mean channel is the average channel of fluorescence taking into account all channels showing fluorescence in the positive region of the histogram. Both were used in identifying the potential differences in the two groups.

CD29 showed a significant (t-value of −5.34) increase in peak channel in the pregnant samples while the mean channel was within the normal range (t-value of −2.24). This translates into the "median" value of CD29, a general attachment protein to the ECM, increases during normal pregnancy. If there is an increase in a receptor that is responsible for general attachment this would make the platelet more "sticky" leading to the platelet to be activated more easily which supports both hypotheses.

The other platelet receptor that showed a significant increase between the nonpregnant and pregnant groups was CD41 or the GP IIb receptor. This is part of the receptor complex of GP IIb/ IIIa. CD41 binds to both the activated and unactivated GPIIb/ IIIa complex. An increase in the binding was observed in

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pregnancy. This increase may represent an upregulation in the proteins. Because GPIIb is involved in platelet aggregation through fibrinogen (Fb) binding, an upregulation may represent an increase in aggregation ability once activation has occurred. An increase in this receptor (supporting hypothesis #1) could lead to an increase in circulating activated platelets (hypothesis #2). The mean channels of all CD41s showed significant increases in the pregnancy group (-3.37, -4.77, & -8.52) while only the CD41 PE showed a significant increase in peak channel (-5.38). Overall, this supports the fact that CD41 very much increases during normal pregnancy.

An important corollary to this is that GP IIIa (CD61), the partner to GP IIb did not show any significant increases during pregnancy (t-values of –0.17 & -0.76). While these two are generally considered as one receptor, they are separate proteins and in the normal platelet they are noncovalently held together via $Ca^{2+}$. An increase in GP IIb with no corresponding increase in GP IIIa would yield extra GP IIb not being able to be coupled to GP IIIa. What effect, if any, this causes would need to be further investigated.

The other main receptor complex examined in this study was the complex of GP Ib. This study looked at two subunits of the larger complex CD 42a (GP IX) and CD 42b (GP Ibα). There were slight decreases in both of these during pregnancy, but neither was statistically significant so no change between the two groups can be assumed. Since the GP Ib complex is responsible for initial platelet adhesion via vWF, no change in primary platelet adhesion via GPIb/ GPIX can be assumed. vWF though, does increase during normal pregnancy, so an idea could be put forth that

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platelet adhesion could still increase by the greater amount of vWF saturating the GP Ib complex more readily leading to an increase in primary platelet adhesion.

The last platelet receptor examined in this study was CD 36, the platelet receptor for collagen V. No statistically significant changes were seen with this receptor (t-values of 0.328 & -2.65). Collagen V is not a very abundant form of collagen and therefore no increase in this receptor does not really say anything about platelet function or support or disprove either of the hypotheses.

## Platelet Activation Markers

Two platelet activation markers were examined in this study, GMP-140 (CD 62) and PAC-1 (activated GP IIb/ IIIa). While neither one of these showed significant differences between the two groups with regard to peak or mean channels, there was a difference when it came to percentage expressing these markers.

The nonpregnant group expressed the GMP-140 receptor on 0.7% of the platelets while over 20% of the pregnant sample expressed this activation marker. This is in contrast to the activated GP IIb/ IIIa marker, PAC-1, which showed no significant difference between the two groups (t-value of 0.856). This is where another limitation arises in the study. While all of the nonpregnant samples were fixed within one minute of collection, not all the pregnant samples could be fixed so quickly. This extra time, though not much more, could lead to platelet degranulation and therefore expression of GMP-140, CD62. On the other hand PAC-1 expression, which is activated GP IIb/ IIIa requires a stronger agonist for its expression to be seen on the platelet when compared to GMP-140. Pages 22-23 in chapter two describe this process of inside out signaling that occurs to yield the expression of this activation

marker. Thus, while it seems that the pregnancy sample did have an increased number of activated platelets further studies need to be done to make a concrete conclusion.

## Limitations

Certain limitations, besides the ones already mentioned above, were present in this study and could not be controlled. EDTA was used as the anticoagulant in the blood samples. It is a $Ca^{2+}$ chelator and therefore EDTA will bind the Ca2+. GPIIb/ IIIa is noncovalently held together via $Ca^{2+}$ and EDTA could therefore disrupt this relationship if it is tying up the $Ca^{2+}$. This would prevent proper PAC-1 expression from occurring and would therefore lead to the results seen with PAC-1 expression. Studies using different anticoagulants (non-$Ca^{2+}$ chelators) would need to be done to overcome this limitation.

Another limitation involves the populations from which each sample group was obtained. Again, as was mentioned above, one group was obtained from the general population while the other was from a graduate class of health professionals. There are confounding factors that can not be accounted for and therefore this needs to be mentioned as a limitation.

A third limitation was the time between when the samples were drawn and fixed. All nonpregnant samples were fixed immediately while some of the pregnant samples had a short amount of time go by before fixing was done. How much, if any, this affected platelet activation is not known and therefore needs to be mentioned here as a limitation.

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# APPENDIX A

## Proposal for Study

# Spectrum Health

May 10, 1999

Theresa Bacon-Baugley, Ph.D., R.N.
233 Padnos
Hall of Science, School of Health Science
Allendale, MI  49401-9403

Dear Dr. Bacon-Baguley:

At the May 4,1999, meeting of the Spectrum Health Research & Human Rights Committee your protocol entitled "Clinical Investigation on the Assessment of Platelet Structure and Function in Normal and Preeclamptic Patients" (#94-10), and the consent form, were reviewed and given annual reapproval.

The Research and Human Rights Committee and the F.D.A. requires you submit in writing, a progress report to the Committee by **April 1, 2000**, and you will need reapproval should your study be ongoing at that time.  Failure to do so could result in suspension of your study.  Please also be advised no additional personnel may be added to this study, nor may they have any access to study information unless the committee has been so advised and an Assurance Form has been signed.

Please be advised that any unexpected serious, adverse reactions must be promptly reported to the Research and Human Rights Committee within (5) five days and all changes made to the study after initiation require prior approval of the Human Rights Committee before changes are implemented.

If you have any questions, please phone me or Linda Pool at 391-1291\1299.

Sincerely,

Jeffrey S. Jones, M.D.
Chairman, Spectrum Health Research & Human Rights

JSJ/jfn

c:      Russell Jelsema, M.D.
        File

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# APPENDIX B

## Informed Consent Form

# INFORMED CONSENT INFORMATION FOR THE CLINICAL INVESTIGATION ON THE ASSESSMENT OF PLATELET STRUCTURE AND FUNCTION IN NORMAL AND PREECLAMPTIC PATIENTS

## INTRODUCTION

Platelets play a significant role in the prevention of bleeding. During blood vessel injury, platelets adhere to the site of injury and form a platelet plug. The platelet plug effectively forms a seal at the site of injury. A deficiency in the number of platelets or in the ability of platelets to form a platelet plug results in excessive bleeding even in the event of minor trauma. In a pregnancy complicated by preeclampsia there is a high incidence of thrombocytopenia, low platelet count. It has not been determined the exact nature of the thrombocytopenia. Therefore, you participation in the study may help in determining the cause of the thrombocytopenia as well as aid in the prediction of impending complications.

## PURPOSE AND RESULTS OF THE RESEARCH

The purpose of this study is to analyze both the function and structure of blood platelets from normal and preeclamptic pregnant females. The results of the platelets studies will be used to identify changes in preeclamptic females which could be used to predict subsequent complications and to aid in the future treatment in preeclamptic females.

## PROCEDURES

There will be approximately 50 participants in the study.
As a participant, you will have 6 ml of blood removed via venipuncture.

## POTENTIAL BENEFITS

The benefits of this study may include a contribution to the overall understanding on platelet structure and function in preeclamptic females which may result in changes in the treatment of platelet disorders during preeclampsia.

## POTENTIAL RISKS

The associated risks of venipuncture include a bruise at the site of puncture, inflammation of the vein and/or infection.

## STUDY PARTICIPATION AND MEDICAL RECORDS ACCESS

You have the right to refuse participation in the study if so desired at any time during the procedure without penalty or jeopardy of the quality or quantity of treatment you are now receiving or may receive in the future at Butterworth Hospital. In case of a problem or emergency, you can reach Dr. R. Jelesma at (616) 732-3681 or call Dr. Theresa Bacon-Baguley at (616) 895-3289.

The doctor and/or his representative at Butterworth Hospital may inspect your medical records for information purposes where appropriate and necessary. You are assured that your confidentiality will be preserved and that your name not be revealed in any publications or presentation resulting from this study without your expressed consent.

Questions regarding your rights as a participant can be answered by calling the Human Right's Committee representative, Linda Pool, at the following number: (616)774-1291/1299.

## FINANCIAL RESPONSIBILITY/STUDY RELATED PERSONAL INJURY

You will receive no payment for your participation in the study.

Costs associated with any injury will not be covered by the investigator or by Butterworth Hospital. Your signature on the consent form indicated that you have volunteered to participate in the study and have read the information provided.

## CONCLUSIONS

I have read this informed Consent and agree to participate in this research study. I will be provided with a copy of this consent form.


_____          _____
Signature of Patient                                       Date


_____          _____
Signature of Investigator                                  Date


_____          _____
Signature of Witness                                       Date

# APPENDIX C

## Demographic Sheet

# FLOW CYTOMETRIC ANALYSIS OF PLATELET STRUCTURE IN NORMAL AND HYPERTENSIVE PREGNANT FEMALES
## SUBJECT CHARACTERISTICS

Patient ID Number: _____

Patient Age: _____

Prior pregnancies: _____

Number of live births: _____

EDC(Pregnant subjects): _____

Subject Group

| | |
|---|---|
| _____ | Non-Pregnant |
| _____ | Normal Pregnant |
| _____ | PIH |
| _____ | Preeclampsia |
| _____ | Eclampsia |
| _____ | Other _____ |

| Medical History | No | Yes | Explain |
|---|---|---|---|
| Allergies | \_\_ | \_\_ | _____ |
| Bleeding disorders | \_\_ | \_\_ | _____ |
| Diabetes | \_\_ | \_\_ | _____ |
| Cardiovascular Disease | \_\_ | \_\_ | _____ |
| Smoker | \_\_ | \_\_ | _____ |

Other Significant Medical History _____

_____

Medications(Over the counter & Prescriptive):

_____

_____

Pregnancy Outcome

| | |
|---|---|
| _____ | Normal Vaginal Delivery |
| _____ | C-Section |
| _____ | Delivery complications (explain)_____ |

_____

Post-partum complications: _____