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Detection of Activated Platelets in Normal Pregnancy and Preeclampsia

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DETECTION OF ACTIVATED PLATELETS IN NORMAL PREGNANCY AND PREECLAMPSIA

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ABSTRACT

Normal pregnancy is a hypercoaguable state in and of itself. Evidence has shown that platelets may play a role in the pathogenesis and complications of preeclampsia. The objective of this study is to compare the presence of circulating activated platelets in normal pregnancy and preeclampsia. The subjects' blood was fixed, washed, incubated with antibodies (which detect platelets and activation of platelets), and evaluated using flow cytometry. Results of this study showed pregnant subjects (normal and preeclamptic) to have an increased percentage of activated platelets over non-pregnant subjects. Increased platelet activation may account for platelet consumption and bleeding complications in preeclamptic patients.

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Judy E. Herbolsheimer

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

PREFACE

Definition of Terms

Abrupto Placentae – Separation of the placenta implanted in normal position in a pregnancy of 20 weeks or more during labor before delivery of the fetus.

Aggregation – An accumulation of substances, objects, or individuals, as in the clumping of blood cells or the clustering of clients with the same disorder.

Angiotensin – A polypeptide in the blood that causes vasoconstriction, increased blood pressure, and the release of aldosterone from the adrenal cortex.

Catecholamine – A neurological chemical

Cerebral Hemorrhage – A hemorrhage from a blood vessel in the brain

Coagulation – The process of transforming a liquid into a solid, especially of the blood.

Convulsion – A hyperexcitation of neurons in the brain leading to a sudden, violent involuntary series of contractions of a group of muscles.

Cytokine - One of a large group of low molecular weight proteins secreted by various cell types and involved in cell-to-cell communication.

Diastolic Blood Pressure – The blood pressure at the instant of maximum cardiac relaxation.

Disseminated Intravascular Coagulation (DIC) – A grave coagulopathy resulting from the overstimulation of clotting and anticlotting processes in response to disease or injury.

Diuretic – A drug that promotes the formation and excretion of urine.

Eclampsia – The gravest form of pregnancy-induced hypertension. It is characterized by grand mal seizure, coma, hypertension, proteinuria, and edema.

Edema – The abnormal accumulation of fluid in interstitial spaces of tissue.

Endoplasmic Reticulum – An extensive network of membrane-enclosed tubles in the cytoplasm of cells. The structure functions the synthesis of proteins and lipids and in the transport of these metabolites within the cell.

Endothelin – Any of a group of vasoconstrictive peptides produced by endothelial cells

Endothelium – The layer of simple squamous epithelial cells that lines the heart, the blood and lymph vessels, and the serous cavities of the body.

Epigastric – Pertaining to the epigastrium, the area above the stomach

Fetal Hydrops (hydrops fetalis) – Massive edema in the fetus or newborn, usually in association with severe erythroblastosis fetalis.

Fibrin – A stringy insoluble protein produced by the action of thrombin on fibrinogen in the clotting process. Fibrin is responsible for the semisolid character of a blood clot.

Flow Cytometer – A Becton Dickson Flow Cytometer is used for this study. This machine is equipped with Cellquest software and is used to identify platelets and platelets tagged by antibody.

Gestation – in viviparous animal the period from the fertilization of the ovum until birth...in humans the average duration is 266 days.

Glomerular Filtration Rate – A kidney function test in which results can be determined from the amount of ultrafiltrate formed by plasma flowing thorough the glomeruli of the kidney. It may be calculated from insulin and creatinine clearance, serum creatinine and blood urea nitrogen. Normal values average 170 L/day for men and 150/day for women.

Glycoproteins – Any of the large group of conjugated proteins in which the nonprotein substance is a carbohydrate.

Gravida – A woman who is pregnant.

HELLP Syndrome – Acronym for a form of severe preeclampsia, a hypertensive complication of late pregnancy. The letters stand for *h*emolysis, *e*levated *l*iver function, and *l*ow *p*latelet level.

Hemolysis – The breakdown of red blood cells and the release of hemoglobin.

Hemorrhage – A loss of a large amount of blood in a short period, either externally or internally.

Hemostasis – The termination of bleeding by mechanical or chemical means or by the complex coagulation process of the body, which consists of vasoconstriction, platelet aggregation, and thrombin and fibrin synthesis.

Hepatic – Pertaining to the liver.

Hydatidiform Mole – An interuterine neoplastic mass of grapelike enlarged chorionic villi

Hyperreflexia – Increased reflex reactions

Hypertension – A common, often asymptomatic disorder characterized by elevated blood pressure persistently exceeding 140/90mm Hg.

Hypertensive encephalopathy – A set of symptoms, including headache, convulsions, and coma, associated with glomerulonephritis.

Hypoalbuminemia – A condition of abnormally low levels of albumin in the blood.

Ischemia – A decreased supply of oxygenated blood to a body organ or part.

Jaundice – A yellow discoloration of the skin, mucous membranes, and sclerae of the eyes, caused by greater than normal amounts of bilirubin in the blood.

Megakaryocytes – An extremely large bone marrow cell measuring between 35 and 160 μm in diameter and having a nucleus with many lobes. Magakaryocytes are essential for the production and proliferation of platelets in the marrow and are normally not present in the circulating blood.

Microangiophilic Hemolytic Anemia – A disorder in which narrowing or obstruction of small blood vessels results in distortion and fragmentation of erythrocytes, hemolysis, and anemia.

Mitochondria – An organelle within the cytoplasm that function in cellular metabolism and respiration. Mitochondria provide the principle source of cellular energy thorough oxidative phosphorylation and adenosine triphosphate synthesis.

Morbidity – An illness or abnormal condition or quality; the rate at which an illness or abnormality occurs

Mortality – The condition of being subject to death; the death rate.

Multifactoral Polygenetic Inheritance – The tendency to develop a physical appearance disease or condition that is a condition of many genetic and environmental factors, such as stature and blood pressure.

Norepinephrine – An adrenergic hormone that acts to increase blood pressure by vasoconstriction but does not affect cardiac output.

Nulliparity – A woman who has not given birth to a viable infant.

Papilledema – Swelling of the optic disc, visible on ophthalmoscopic examination of the fundus of the eye, caused by increase in intracranial pressure.

Parity – The classification of a woman by the number of live-born children and stillbirths she has delivered at more than 20 weeks gestation.

Pathogenesis – The source of cause of an illness or abnormal condition.

Pathophysiology – The study of the biologic and physical manifestations of disease as they correlate with the underlying abnormalities and physiologic disturbances. It explains the processes within the body that result in the signs and symptoms of a disease.

Perinatal – Pertaining to the time and process of giving birth or being born.

Platelet – The smallest cells in the blood. They are formed in the red bone marrow and some are stored in the spleen. Platelets are disk-shaped, contain no hemoglobin, and are essential for the coagulation of blood and in maintenance of hemostasis.

Preeclampsia – An abnormal condition of pregnancy characterized by the onset of acute hypertension after the twenty-fourth week of gestation. The classic triad of preeclampsia is hypertension, proteinuria, and edema. The cause the disease remains unknown. It occurs in 5-7% of pregnancies, most often in primigravidas. Termination of the pregnancy results in resolution of the signs and symptoms of the disease and in healing of the renal lesion. The most serious complication is eclampsia, which can result in maternal and fetal death. Formerly called Toxemia of Pregnancy.

Prostacycline – A prostaglandin that is a biologically active product of arachidonic acid metabolism in human vascular walls and a potent inhibitor of platelet aggregation. It inhibits the vasoconstrictor effect of angiotensin and stimulates renin release.

Proteinuria – The presence in the urine of abnormally large quantities of protein, usually albumin.

Pulmonary Edema – The accumulation of extravascular fluid in lung tissues and alveoli.

Renal – Pertaining to the kidney.

Serotonin – A naturally occurring derivative of tryptophan found in platelets and in cells of the brain and the intestine. Serotonin is released from platelets on damage to the blood vessel walls. It acts as a potent vasoconstrictor.

Spina Bifida – A congenital neural tube defect characterized by a developmental anomaly in the posterior vertebral arch.

Systolic Blood Pressure – The blood pressure measured during the period of ventricular contraction (systole).

Thrombin – An enzyme formed from prothrombin, calcium, and thromoplastin in plasma during the clotting process. Thrombin causes fibrinogen to change to fibrin, which is essential in the formation of a clot.

Thrombocytopenia – Reduction in the number of platelets. There may be a decreased production of platelets, decreased survival of platelets, and increases consumption of platelets.

Thromboxanes – Compounds synthesized by platelets and other cells that cause platelet aggregation and vasoconstriction.

Tonic/clonic seizure – An epileptic seizure characterized by a generalized involuntary muscular contraction and cessation of respiration followed by tonic and clonic spasms of the muscles.

Vasoactive – Tending to cause vasodilation or vasoconstriction

Vasoconstriction – A narrowing of the lumen of any blood vessel

Vasodilation – An increase in the diameter of a blood vessel.

Vasopressin – AKA Antidiuretic Hormone. A hormone that decreases the production of urine by increasing the reabsorption of water by the renal tubules.

Vasospasm – A spasm in a blood vessel

Definitions are taken from **Mosby's Medical, Nursing, and Allied Health Dictionary**. Fifth edition. 1998.

CHAPTER 1
INTRODUCTION

Preeclampsia, otherwise known as toxemia of pregnancy or pregnancy induced hypertension, is a problem in pregnant women consisting of three primary symptoms: hypertension (high blood pressure), edema (swelling), and proteinuria (protein in the urine). The cause and pathogenesis of preeclampsia is unknown. It usually develops after the twentieth week of gestation and the only curative treatment is delivery of the baby. When the fetus has not matured enough to deliver and live viably outside of the mother, preeclampsia is managed with bed rest, medications to lower the blood pressure, and medications to prevent eclamptic seizures while the baby matures (Fauci et al 1998, Cunningham et al 1997, Barron et al 2000, Witlin et al 1999, Witlin et al 2000).

Women who are younger than twenty years or older than thirty-five years during pregnancy are at a greater risk of developing preeclampsia (Cunningham et al 1997). Other risk factors include how many children they have had, preexisting hypertension, diabetes mellitus, renal disease, vascular disease, and a previous history or family history of preeclampsia. There are currently no tests available to predict preeclampsia during pregnancy (Fauci et al 1998, Barron et al 2000, Lenfant et al 1990, Burrow, Duffy 1999, Cunningham et al 1997, Redman 1992).

Preeclampsia affects 2 to 10% of all pregnancies in the United States and is responsible for 50 to 70% of hypertension in pregnancy. Preeclampsia is the most common cause of perinatal morbidity and mortality. It accounts for 35 to 300 deaths per 1000 births each year. It is also responsible for at least 76,000 deaths each year

world wide (Barron et al 2000, Konijnenberg A et al 1997 Feb, Cunningham et al 1997, Redman 1992). As demonstrated, preeclampsia is a serious complication in pregnancy.

Background

Twenty percent of women with preeclampsia develop bleeding problems during delivery that present serious risks to the mother and the infant. Platelets play a vital role in coagulation (the blood clotting mechanism), which stops profuse bleeding. Hematological changes occur during preeclampsia in both coagulation and platelet aggregation. These platelet changes are thought to be related to the bleeding problems experienced during delivery. It is believed that platelets may play an early pathogenic role in preeclampsia (Cunningham et al 1997).

Platelets play a crucial role in hemostasis by providing a surface where coagulation can take place and by aggregating together to form a clot. They are anuclear cells that are produced in the bone marrow by megakaryocytes. Upon stimulation, platelets release different granules and activate the coagulation system. The response is graded and depends on the extent of damage as to how much and which granules are released (Kumar et al 1997, Mc Cance KL, Huether SE, 1998, Silverthorn et al 1998).

For this study blood samples are drawn from the non-pregnant, normal pregnant, and preeclamptic groups of women and fixed to prevent further activation from that which had already occurred in vivo. The samples are taken to the flow cytometry lab where the platelets are separated from the rest of the cells and serum. After the platelets have been separated and washed, they are tagged with a fluorescent antibody to the normal proteins on the plasma cell surface. The markers are used to look for surface

proteins that identify the cell as a platelet as well as those that are present on the surface of activated platelets. The tagged platelets are then run through the flow cytometer, where the platelets are analyzed and the degree of fluorescence is recorded.

Flow cytometry quickly measures specific characteristics of a large number of individual cells. Before being run through the machine, the cell proteins are labeled with a fluorescent monoclonal antibody. Suspended cells can pass through the flow cytometer's focused laser beam at a rate of 1,000 to 10,000 per minute. The fluorescent antibodies are excited by a wavelength of light and a detector processes the light given off and the light scattered by the cells. These numbers and graphs show up on the computer monitor for the researchers to see instantly (Coon et al 1987, Leytin et al 2000, Shattil et al 1987).

The results obtained in the preeclamptic sample are compared to the results of previous studies performed in normal non-pregnant females and normal pregnant females. The goal of this study is to prove the hypothesis that the surface proteins on platelets of preeclamptic women are in a more activated state than in non-pregnant and normal pregnant women.

Problem Statement

Preeclampsia occurs in 2 to 10% of pregnancies and many of these lead to fetal and maternal morbidity and mortality (Cunningham et al 1997). The diagnosis is difficult at best and detection usually does not occur until the final trimester of pregnancy. There remains no definitive test to predict preeclampsia.

Twenty percent of women with preeclampsia experience bleeding problems

during their delivery. This study is investigating whether or not a test looking at the platelets for activation could be predictive of preeclampsia or could be predictive of bleeding problems at the time of delivery.

If platelets are activated early in pregnancy in women who have preeclampsia the platelets may be used up prior to delivery, leading to an insufficient amount of platelets remaining to allow clotting and hemostasis to occur. This could be the reason why some preeclamptic women have bleeding problems at delivery. If preeclampsia can be detected at an earlier stage by looking at the platelets for activation, the potential for preventing bleeding problems exists. If bleeding cannot be prevented, at the least the medical personnel can be better prepared at the time of delivery for the probability of bleeding to occur.

Purpose

The purpose of this study is to identify the surface proteins for activation on the platelet cells and to determine the amount of these surface proteins to discover what percentage of the platelets are activated in preeclamptic women. When these have been identified they will be compared to the percentage of activated surface markers in non-pregnant and normal pregnant women to see if there is an increase in activation of the platelets in preeclamptic women. Any differences noted could attribute to the bleeding problem experienced by 20% of women with preeclampsia.

The goal of this study is to prove the hypothesis that the surface proteins on the platelets of preeclamptic women are such that the platelet is circulating in a more activated state than in non-pregnant and normal pregnant women. This could prove as an

early detection test for preeclampsia. If the platelets are more activated the ultimate goal is to use this information to develop a test for determining those with preeclampsia who will develop bleeding problems.

Significance of the Problem

Preeclampsia, in and of itself, can be a serious problem leading to increased morbidity and mortality for the mother and fetus (Cunningham et al 1997). There is currently no test for early detection of preeclampsia, so the disorder is commonly not discovered until the third trimester, when the three main symptoms, hypertension, edema, and proteinuria, are commonly picked up. Alterations in blood vessels in the placenta are seen as the vascular supply of the placenta is being developed (Burrow, Duffy 1999, McCrae et al 1992). Changes in platelets, including activation of platelets, may occur as early as the second trimester when these vascular changes occur. A test for these changes, if they occur earlier in pregnancy, may lead to earlier detection of preeclampsia.

One in five women with pre-eclampsia develop bleeding problems during pregnancy but currently there is no way to determine which women with preeclampsia will develop these bleeding problems and which women will not. By looking at the activation of circulating platelets in non-pregnant, normal pregnant, and preeclamptic women and determining the level of activation a difference of activation is expected to be discovered. From this information a test can be developed to determine who will develop bleeding problems during delivery. By being able to predict who will have problems with bleeding, medical staff will be better prepared to control the bleeding that occurs at delivery or have the option of doing a c-section to control the bleeding. This will result in

less risk to the mother and the baby and decrease the morbidity and mortality of preeclampsia.

Hypothesis

From previous studies performed by GVSU students and Dr. Theresa Bacon-Baguley, it has been discovered that the activation of platelets is different in women who are pregnant than those of women who are not pregnant. This current research is looking at the differences in platelet activation in women with preeclampsia compared to non-pregnant and normal pregnant women. The goal of this study is to prove the hypothesis that the surface proteins on platelets of preeclamptic women are in a more activated state than in non-pregnant and normal pregnant women.

CHAPTER 2

REVIEW OF LITERATURE

Introduction

Preeclampsia, with a classic triad of hypertension, proteinuria, and edema, is a disease surrounded by mystery. Despite over 100 years of research by thousands of investigators, much about the disease is unknown. There is no concrete decision about the cause of preeclampsia and there is no "cure" for the disorder when it progresses other than delivery of the fetus (Anderson et al 1998).

The literature about preeclampsia is numerous. Throughout the years definitions have been added and taken away from the diagnosis. Many things have changed in the medical community's understanding of the disorder's diagnosis, risk factors, pathogenesis, and treatment. Even the name has changed from toxemia of pregnancy to preeclampsia (Anderson et al 1998). There is much more to be discovered about this disorder to know how to more quickly diagnose and more properly treat women with preeclampsia.

Preeclampsia

Preeclampsia is the most common complication of pregnancy today (Redman 1992). It relates to an increased risk of perinatal mortality and morbidity. This risk is increased for both the mother and the fetus (Cunningham et al. 1997). It is the most important cause of maternal mortality in the western world (Konijnenberg 1997 Feb).

Perfectly healthy women can die of preeclampsia, and that is why it is of such great concern (Redman 1992).

Preeclampsia (also known as toxemia syndrome (Fauci et al 1998, Anderson et al 1998)) usually occurs during the first pregnancy (Fauci et al 1998, Barron et al. 200, Lenfant et al.1990, Burrow, Duffy 1999, Cunningham et al. 1997, Redman 1992). This condition is unique to pregnancy and only occurs in that setting. It is a multisystem disorder that involves hypertension, proteinuria and generalized edema, with additional symptoms including coagulation abnormalities, liver function abnormalities (Barron et al 2000), sodium retention, and/or hyperreflexia (Fauci et al. 1998).

Preeclampsia is believed to originate in the placenta, an organ shared by the mother and the baby, which causes the effects to relate to both persons (Redman 1992). The pathogenesis and pathophysiology is not known and the theories of such will be discussed later.

Two to ten percent of all pregnancies result in preeclampsia (Barron et al 2000, Konijnenberg A et al. 1997 Feb, Burrow, Duffy 1999, Cunningham et al 1997, Redman 1992, Lenfant et al. 1990, Witlin et al. 1999). As many as 1 in 10 of all pregnancies and 1 in 5 of first time moms will get preeclampsia to some degree. One in 20 first time moms get a serious form of the disease and 1 in 100 first time moms will get a form so serious that it threatens the life of the mother or the baby (Redman 1992). The occurrence is seen to hit hardest the women on either end of the reproductive age spectrum and is more common in teenagers or those older than 35 years old (Cunningham et al. 1997).

While preeclampsia is primarily a disease of the first pregnancy, the risk of reoccurrence increases for the second and subsequent pregnancies if a woman had

preeclampsia during her first pregnancy (3.4% risk) when compared to women who had a normal first pregnancy (Burrow, Duffy 1999). A change in paternity between the first and subsequent pregnancies increases the risk up to the level of a first pregnancy all over again (Burrow, Duffy 1999, Li, Wi 2000).

Preeclampsia usually occurs at or after the 20th week of pregnancy (Barron et al 2000, Redman 1992, Lenfant et al. 1990). The foundations for getting preeclampsia are believed to be beginning to be set up in the first half of the pregnancy. However the symptoms do not occur until the last 3 months of pregnancy (Redman 1992). An early detection test, therefore, is a necessary tool and should be utilized to identify the women who may develop preeclampsia and try to treat them early before they progress to eclampsia. Early identification and treatment could prevent the progression to eclampsia.

Preeclampsia, if it is not controlled, can progress from a mild form to a serious form, and even to eclampsia itself. This progression can be slow, and not go beyond mild preeclampsia, or can be more rapid, progressing to severe disease in days to weeks. It can be fulminant also and progress from mild to severe to eclampsia in days or even hours. This is why it is very important to diagnose preeclampsia correctly and quickly. The goal of treatment is then to prevent eclampsia from occurring (Lenfant et al. 1990).

Eclampsia is the term for general tonic/clonic convulsions that develop in addition to the triad of hypertension, edema, and proteinuria in some women. The convulsions are induced by hypertension and/or aggravated by pregnancy. The woman can have 1 or 2 convulsions to even 100 or more. They can occur before (antepartum), during (intrapartum), or after (postpartum) labor. They are more common during the third trimester and the risk of developing eclamptic seizures in women with preeclampsia

increases as the woman gets closer to full term (Cunningham et al 1997). The peripartum seizures may be due to hypertensive encephalopathy (Barron et al 2000).

Preeclampsia can be pure (the disease alone) or superimposed on chronic hypertension. Either way the disease represents a great danger to the fetus and the mother (Lenfant et al. 1990). Approximately 50,000 women die each year from eclampsia worldwide. There is considerably less death in developed countries due to prenatal care. Even so, 18% of the 1450 maternal deaths in the United States from 1987 to 1990 were from complications of pregnancy-induced hypertension (Cunningham et al. 1997). Most maternal deaths attributed to preeclampsia occur in the setting of uncontrolled hypertension. Most are related to an ensuing intracerebral hemorrhage (Burrow, Duffy 1999).

With such a common and serious disease you would think that everyone knows about it and is on the lookout for it. This is not the case however. The general public knows much more about rare complications of pregnancy such as Down syndrome and spina bifida than they do about the more common problem of preeclampsia (Redman 1992). The public, and the medicinal community, need to be educated about this disease and more research must be done in this arena.

Diagnosis of Preeclampsia

The major signs and symptoms of preeclampsia are hypertension, proteinuria, and edema. Other signs and symptoms include hematoconcentration, hypoalbuminemia, hepatic function abnormalities, coagulation abnormalities, increased urate levels (Lenfant et al 1990), hyperreflexia, visual disturbances, thrombocytopenia (Fauci 1998), and

others. The diagnosis is often made based on the hypertension, edema, and proteinuria. The initial onset, however, is usually seen with the rapid development of edema and a rise in blood pressure (Fauci et al 1998).

Hypertension is the first thing that is looked at to diagnose an individual with preeclampsia. Hypertension in pregnancy can be diagnosed using the same methods as it is in non-pregnant individuals, using a spiromometer, but the definition of hypertension in a pregnant woman is different than a non-pregnant individual. There are also other issues involved in diagnosing the hypertension in pregnant women.

If the patient has been followed with normal prenatal care then hypertension can be diagnosed in one of two ways. If there is an increase of systolic blood pressure of 30 mm Hg or more in the late second trimester or third trimester compared to the average blood pressure of earlier pregnancy values hypertension can be diagnosed. The second way to diagnose hypertension is if the diastolic blood pressure has increased 15 mm Hg or more compared to the earlier values. If the patient's prior blood pressure is not known than readings of 140/90 mm Hg or above are considered hypertensive (Lenfant et al 1990). The earlier pregnancy values are used in the first method because normal blood pressure changes with pregnancy. Both systolic and diastolic blood pressure normally decrease 10 to 15 mm Hg during pregnancy, therefore a diastolic of >75 during the second trimester and > 85 during the third trimester are abnormal (Fauci et al. 1998). This means that a woman may be within the normal range (less than 140/90) and still have preeclampsia (Lenfant et al 1990).

Another problem in developing a diagnosis of hypertension in order to diagnose preeclampsia is that blood pressure measurements fluctuate throughout the day, and from

minute to minute, in these patients (Lenfant et al. 1990). If repeated measurements over a 4 to 6 hour time period show the blood pressure to be abnormal, the diagnosis can be made. It is important to note also that hypertension in these individuals is more severe at night (Fauci et al 1998).

The next step in making the diagnosis is testing for proteinuria. This value varies from woman to woman and from hour to hour in the same woman making this a difficult test as well. Abnormal values include proteinuria of at least 300mg/24hrs or 100mg or more in the last two specimens taken at least 6 hours apart (Cunningham et al. 1997).

Proteinuria is caused by problems in the renal system. In a normal pregnancy the glomerular filtration rate (GFR) of the kidneys increases by 30 to 50%. With preeclampsia the GFR is decreased instead of increased or may stay the same.

Glomerular filtration rate is tested for using serum creatinine and BUN laboratory tests. A serum creatinine of greater than 70μmol/L or a BUN of greater than 4.6 mmol/L are considered abnormal in pregnancy (Fauci et al 1998).

Proteinuria is a late sign of preeclampsia. It is minimal or lacking in the early and mild stages of the disease and shows up most in the severe forms. Proteinuria almost always develops later than hypertension, edema, and weight gain (Cunningham 1997).

Edema is the abnormal accumulation of fluid in the interstitial spaces of tissues (Anderson et al 1998). Some edema is a normal complication of pregnancy. However in preeclampsia the edema is seen as part of the triad of the disorder. Edema is diagnosed by looking for swelling and pitting in the legs, feet, and hands and by looking for abnormal weight gain.

Edema can be measured by looking at weight gain because fluid retention leads to weight gain. A sudden increase in weight may precede the development of preeclampsia. This may be the first visible sign of the impending disease. A weight gain of about 1 pound a week is normal in pregnancy. If the weight gain is greater than 2 pounds a week or 6 pounds in a month the diagnosis of preeclampsia must be considered (an the other diagnostic factors looked at). This weight gain is due to abnormal fluid retention in the preeclamptic patient. A fulminating disease course leading to eclampsia may have extreme fluid retention and a weight gain of 10 or more pounds in one week (Cunningham et al 1997).

Neurological symptoms that may be associated with preeclampsia include hyperreflexia, visual disturbances, and headache. If these neurological signs occur, and especially if they are persistent, concern should be raised for the development of convulsions (eclampsia) (Fauci et al. 1998). A headache is unusual in milder cases but increases in frequency in severe disease. The headaches are often frontal or may be occipital. *A severe headache almost always precedes the first eclamptic convulsions* so headaches in preeclamptic patients are very important (Cunningham et al. 1997).

Other signs and symptoms may be associated with the development of preeclampsia. One possibility is jaundice or abnormal liver functions. There can also be hematological problems such as thrombocytopenia, microangiophilic hemolytic anemia, HELLP syndrome (*H*emolysis, *E*levated *L*iver function tests, *L*ow *P*latelets), or disseminated intravascular coagulation associated with preeclampsia. Another problem that can occur with preeclampsia is fibrin deposits in the kidneys and liver (Fauci et al.

1998). Epigastric or right upper quadrant pain can also occur. This often a sign of severe preeclampsia and may be indicative of imminent convulsions (Cunningham et al 1997).

There is a test that can be used to help diagnose preeclampsia. It is the roll over test. It checks for an increased sensitivity to angiotensin II that is seen with preeclampsia. If there is an increase in diastolic blood pressure of 20 mmHg or more upon changing the patient's position from that of lying on the side to lying on the back the diagnosis can be confirmed (Fauci et al 1998).

Once the diagnosis of preeclampsia has been made by the discovery of these signs and symptoms, the severity can be assessed. Mild preeclampsia has a diastolic blood pressure of less than 100 mmHg and trace to 1+ proteinuria. Most other signs are absent or minimal (Cunningham et al 1997). Ominous signs in preeclampsia include blood pressure 160 mmHg or more systolic or 110 mmHg or more diastolic, proteinuria of 2.0gm or ore in 24 hours, increased serum creatinine, platelet count less than 100,000, evidence of microangiopathic hemolytic anemia, elevated liver enzymes, headache or visual disturbances, epigastric pain, retinal hemorrhage or exudate, papilledema, or pulmonary edema. These signs show multisystem involvement and serious disease (Lenfant et al 1990). Despite the staging of severity, all cases of preeclampsia (even mild) should be considered potentially dangerous because they can all lead to eclampsia (Burrow, Duffy 1999).

Cause/Pathophysiology of Preeclampsia

The exact cause of preeclampsia is unknown. Some possible explanations and theories have been explored however. These include an immunological basis, genetic

predisposition, dietary deficiencies, vasoactive compounds, and endothelial dysfunction (Cunningham et al. 1997).

Immunological theories for the development of preeclampsia hold that because preeclampsia is a condition of the first born maybe the disease due to the woman not having an effective immunization by a previous pregnancy. This would result in a woman not developing immunological tolerance to the fetus (Cunningham et al. 1997, Li, Wi 2000). This can be seen in a study with women who have changed paternal status between two children. If the woman changes her partner, the "protective status" of the first child is seen to be "lost". This is because the new father's immunological markers are different than the father of the first child and the fetus is seen as a different kind of foreign (Li, Wi 2000). This immunological theory is also supported since the first pregnancy, in which the mother has not been immunized by previous pregnancies, is seen to have a larger number of antigenic sites on the placenta compared to the amount of antibody (Cunningham et al 1997).

Genetic predisposition is seen as a possible cause of preeclampsia. The disorder does appear to have some familial tracing. It may be due to a single gene or multifactoral polygenetic inheritance (Cunningham et al 1997, Barron et al 2000).

There are possible dietary deficiencies implicated such as calcium and magnesium that may lead to preeclampsia. These, however, do not have a whole lot of proof to validate their role. However a study has shown that if you increase your calcium intake you decrease the risk of preeclampsia. This increased calcium does not adversely effect the fetus and may be recommended to patients (Cunningham et al 1997).

The major pathological feature of preeclampsia is increase in peripheral vascular resistance. Vascular resistance to blood flow leads to hypertension (Cunningham et al 1997). The pathogenesis of this is due to many factors. One of these is exaggerated vascular responsiveness to circulating angiotensin II and catacholamines (Lenfant et al 1990). It can also be due to increased production of endothelin 1 and serotonin from the activated platelets in the microvasculature, which can lead to hypertension (McCrae et al 1992). Another possible cause is increased vascular reactivity to pressors that is seen in preeclamptic patients. Normally pregnant women become refractory to vasopressors, but in preeclampsia they are not. In fact quite the opposite happens and they become even more reactive to them. These pressors can include norepinephrine, angiotensin II, vasopressin, etc. The exact mechanism of this is unknown (Cunningham et al 1997).

Endothelial damage can lead to the activation of endothelium. This promotes coagulation and makes the endothelium more sensitive to all of the vasopressor agents. This can be seen in the glomerular capillary endothelium in preeclamptic patients. Vasoconstriction leading to hypertension can also be due to vasospasm. These vasospasms can occur and damage the vessels. When this endothelial damage occurs the platelets come to be deposited in the subendothelium (Cunningham et all 1997).

One possible cause for the beginning of the pathogenesis of preeclampsia is that there is an alteration in the vasculature supply of blood to the placenta during the time when the arterial supply to the placenta is being developed. This may occur as early as the beginning of the second trimester. The manifestations of the ischemia due to this are not seen until the third trimester though. How these placental vascular abnormalities cause systemic disease is not known (Burrow, Duffy 1999, McCrae et al 1992). These

deficient arteries cause ischemia to the placenta the placenta releases diminished amounts of mediates or releases pathological factors. There is an increase in the amount of thromboxane and a decrease in the amount of prostacycline produced. This can lead to the development of hypertension, increased platelet activation, and reduced uteroplacental blood flow all seen in preeclampsia. (McCrae et al 1992).

Consequences of Preeclampsia

Both maternal and fetal consequences must be considered when thinking about preeclampsia. Preeclampsia originates in the placenta, an organ shared by the mother and the baby, which causes the effects of preeclampsia to relate to both persons (Redman 1992).

Preeclampsia can lead to two life threatening complications, HELLP syndrome and eclampsia. HELLP syndrome (*H*emolysis, *E*levated *L*iver function tests, *L*ow *P*latelets) is an emergency requiring prompt termination of the pregnancy (Lenfant et al 1990). HELLP is seen to be a variant of preeclampsia and not necessarily a unique disorder in and of itself. Thrombocytopenia is a prominent manifestation of HELLP.(McCrae et al 1992). HELLP is associated with a particularly adverse maternal and fetal prognosis (Burrow, Duffy 1999).

The second life threatening condition is eclampsia. This is a convulsive phase. It was at one time the major cause of cerebral bleeding and maternal death associated with preeclampsia (Lenfant et al 1990).

Things that can predispose a patient to the development of lethal complications include abrupto placentae, disseminated intravascular coagulation, cerebral hemorrhage,

hepatic failure, or acute renal failure (Lenfant et al 1990). These can be due to the pathophysiology or can be one of the other consequences of the disease.

Endocrine changes can occur during preeclampsia. In normal pregnancy renin, angiotensin II and aldosterone are all increased. In preeclamptic pregnancies they are not increased (Cunningham et al 1997).

The kidneys can also have changes in a preeclamptic patient. The renal perfusion and glomerular filtration rate are decreased in these women. The plasma uric acid concentration is increased. There is also proteinuria as has been discussed (Cunningham et al 1997).

Hematological changes occur during preeclampsia. There are changes in coagulation and platelet aggregation as well as thrombocytopenia. The thrombocytopenia can be induce acutely by preeclampsia or eclampsia. It reflects the severity of the disease. Overt thrombocytopenia with a platelet count of less than 100,000 is considered a very ominous sign and the infant should be delivered. Platelet aggregation is increased in preeclamptic women. This could be due to the immunological processes or due to platelet deposition at the site of endothelial damage (as discussed in pathophysiology). Platelets from preeclamptic women have surface alterations and are likely to be associated with IgG. Thrombocytopenia and preeclampsia are related to prolonged bleeding time (Cunningham et al. 1997).

The cardiovascular system is effected by preeclampsia. There are hemodynamic changes associated with preeclampsia, which include vascular resistance, cardiac output, left ventricular filling, heart rate, and many more. The blood volume is also seen to be effected. In a normal pregnancy the blood volume increase to 5000 mL but a

preeclamptic or eclamptic patients blood volume is seen to be only 3500mL. This means that they are sensitive to even the normal blood loss at delivery (Cunningham et al. 1997).

Consequences of preeclampsia that effect the fetus most directly are preterm delivery and intrauterine growth retardation. This combination can result in high perinatal mortality. The goal of treatment includes delivery of a healthy baby and the health restoration of the mother so these consequences must also be considered (Li, Wi 2000).

Treatment of Preeclampsia

There is nothing that can be done to "cure" preeclampsia short of delivering the fetus (Fauci et al 1998, Cunningham et al 1997, Barron et al 2000, Witlin et al 1999, Witlin et al. 2000). Prompt diagnosis, ongoing assessment of maternal and fetal health, and timely delivery are the key aspects to be addressed in managing a patient with preeclampsia (Barron et al. 2000).

There are really two management options. One is pregnancy prolongation (either expectant management or 48 hour delay for corticosteroid benefit) in an attempt to improve neonatal outcome. The other is immediate delivery (Witlin et al 2000).

Ending the pregnancy is always in the best interest of the mother, but may or may not be in the best interest of the fetus. If the baby is at greater than thirty-six weeks gestation it should be delivered because pulmonary maturity should have been achieved by this point. However, you must deliver regardless of age if there is evidence of advanced disease or impending eclampsia (Barron et al 2000). Other indications for

immediate delivery include non-reassuring fetal status, vaginal bleeding, eclampsia, uncontrolled severe hypertension, pulmonary edema, compromised renal function, persistent severe headaches or vision changes, or a platelet count less than 100,000/mm^3 (Witlin et al 1999).

One primary recommendation is bed rest in a quiet environment (Fauci et al 1998, Lenfant et al 1990). Bed rest is considered good because is maximizes the uteroplacental blood flow, helps reduce premature labor, lowers the blood pressure, and promotes diuresis (Lenfant et al 1990). It also helps to buy some time for the baby to mature.

Bed rest is often augmented with other treatments. These include control of the blood pressure(antihypertensives and decreased sodium intake) and neurological manifestations (magnesium sulfate) (Fauci et al 1998).

Blood pressure is controlled with antihypertensives to prolong the pregnancy until delivery can safely occur. Antihypertensives that can be used include beta-blockers, calcium channel blockers, vasodilators, and central sympathetic antagonists. Diuretics are avoided (Fauci et al 1998). ACE inhibitors are contraindicated in pregnancy because they cause fetal death (Cunningham et al 1997, Fauci et al 1998)

Medications are given prophylactically to prevent eclampsia from occurring. The rate of development of eclampsia for a woman with severe preeclampsia is 1% to 7% (Szal et al. 1999). The main treatment is with magnesium sulfate. It can be given prophylactically to prevent convulsions or as a treatment to stop the seizures when they occur. The question is when do you give this drug. For now the recommendations are to give it to all women with an established diagnosis of preeclampsia until further studies

are done (Barron et al. 2000). The risk of seizure development despite standard therapy is 0.3%(Szal et al 1999).

Prevention of Preeclampsia

No consistently effective treatment has been found to prevent preeclampsia, though many have been tried. Diuretics did not work. Salt restriction is not effective. Antihypertensives work to help decrease the blood pressure but do not effect the pathology of the preeclampsia itself since the primary pathology is not due to the high blood pressure. Calcium has been shown to help prevent the development of preeclampsia and does not hurt the fetus so it can be tried . Magnesium has been tried but does not help (Barron et al 2000).

Low dose aspirin seems to be the most promising preventative therapy attempted currently and is used in high-risk individuals. It inhibits platelets and thromboxane synthesis to inhibit platelet aggregation and vasoconstriction. It has been seen to reduce the incidence of preeclampsia by 9%. But it is not recommended for everyone...just high-risk individuals (Barron et al 2000).

Early Detection

Pregnant women are usually unaware of the two most important signs of preeclampsia, the hypertension and proteinuria. These are not things that people normally notice, because they do not produce noticeable symptoms. By the time the symptoms of headache, visual disturbance, epigastric pain, etc develop the preeclampsia is in the severe stage and delivery is imminent. Also disturbing is the fact that 10% of eclamptic

seizures occur before the overt proteinuria can be picked up by current methods, therefore before the patient is diagnosed with preeclampsia (Cunningham et al 1997). An early diagnostic or predictive test is therefore desperately needed for this disorder.

The first thing done for early detection is to look for risk factors. These include nulliparity, changing paternity in successive pregnancies, family history of preeclampsia or eclampsia, diabetes, multiple gestation, extremes of age, high body mass, hydatidiform mole, fetal hydrops, chronic hypertension (Barron et al 2000), renal disease, and other chronic vascular disease (Cunningham et al 1997). If they are predisposed, as seen in women with multiple risk factors, then they need to be observed very carefully. This includes checking for rapid weight gain and blood pressure changes every week during the last month of pregnancy and every two weeks for the two months preceding the last month of pregnancy. Education of the patient to report any signs and symptoms of headache, visual changes, epigastric pain, and edema are also very important (Cunningham et al 1997).

Risk factors and careful observation alone are not enough, many times, to predict early enough a woman's chance of becoming preeclamptic. Another test is needed. Numerous pathophysiologic events present in preeclamptic women weeks to months before the disorder is clinically recognized with the history, hypertension, and proteinuria (Barron et al 2000). Maybe one of these can become a test for the disease.

A platelet activity test out of Detroit (Wayne State) showed that abnormal platelet activity can be detected as early as the first trimester. Because of this study it may be possible to predict preeclampsia and explain why it is modifiable by antiplatelet agents (Redman 1992).

Platelets

Structure and Function

Platelets are not cells, but cell disk-shaped cell fragments. They are formed in the bone marrow from large cells called megakaryocytes. The outer edges of megakaryocytes extend into the lumen of the bone marrow blood sinuses and the extensions fragment into the disk-like platelets (McCance, Huether 1998, Silverthorn et al 1998).

Platelets are smaller than red blood cells. They have no color to them (Silverthorn et al 1998). They do not have a nucleus and therefore do not have any DNA. They are unable to divide to form new cells (Mc Cance, Huether 1998). Platelets do contain many intercellular organelles, however, including mitochondria, smooth endoplasmic reticulum, and granules. These granules are filled with the clotting proteins and cytokines the platelet needs to perform its job in clotting (Silverthorn et al 1998).

In an average person there are 140,000 to 340,000 platelets/mm^3 circulating in the blood. There are also an additional one third of the body's total amount of available platelets that are stored in the spleen most of the time (Mc Cance, Huether 1998). While the platelets are present in the circulating blood at all times, they are not activated unless there has been damage to the walls of the circulatory system. Platelets are involved in maintaining hemostasis, "the process of keeping blood within the blood vessels by repairing breaks without compromising the fluidity of the blood" (Silverthorn et al 1998). They are essential in coagulation and the control of bleeding when injuries occur (McCance, Huether 1998).

In their circulating, non-activated state a number of glycoproteins are present on the surface of the platelet. These glycoproteins allow chemicals and other substances to bind to the cell. The binding to some of these sites allows messages to be transported to the cell through the receptors made by the glycoproteins. Some of the platelet cell surface proteins include glycoprotein IIIa; glycoprotein IIb; glycoproteins IIa and Ic (Fibronectin Receptor); glycoproteins IIa and Ia (Collagen Receptor) glycoproteins Ib α, Ib β, IX, and V (Von Willebrand Factor Receptor); glycoproteins IIa and Ic (Laminin Receptor); and glycoprotein IIIb ou IV (Thrombospondin Receptor) (Immunotech International).

Platelets also contain two types of granules. The first type of granule is the alpha granule. This granule has the adhesion molecule P-selectin on its membrane surface and contains many of the molecules used in coagulation and the coagulation cascade. These include fibrinogen, fibronectin, factors V and VIII, platelet factor 4, platelet derived growth factor, and transforming growth factor β. The second type of granule is the dense body or delta granule. This granule contains adenine nucleotides (ADP and APT for engery), ionized calcium, histamine, serotonin, and epinephrine (Kumar et al 1997).

When there is a vascular injury the initial hemostasis occurs by blocking the hole with a platelet plug (Silverthorn et al 1998). When vascular injury occurs the endothelial extracellular matrix is exposed. This leads to exposure of collagen, proteoglycans, fibronetin, and other adhesive glycoproteins. When the platelet comes in contact with these substances it becomes activated and undergoes three main reactions: adhesion and shape change, secretion of granule contents, and aggregation. The platelets adhere to the damaged vessel's endothelial extracellular matrix with interaction with Von Willebrand's factor. The released granules are used both to stimulate and to be used in the coagulation

cascade and in mediating aggregation. In aggregation the platelets adhere to one another forming a platelet plug using vasoconstrictor thromboxane A_2 (TXA_2), which the platelet secretes when it is activated, and ADP, which is released from the delta granule. Also at this time the platelet is activated. This activation leads to the expression of a phospholipid comples that becomes a binding site for calcium and coagulation factors needed in the intrinsic clotting pathway. The coagulation cascade, activated by the platelets and the injured cells, produces thrombin, which binds to and reinforces the platelet plug (Kumar et al 1997).

When platelets are activated their surface proteins change also. The glycoproteins present on an activated platelet include sites for the binding of GMP 140 and glycoprotein 53. The other glycoproteins on the surface of the platelet form receptors are bound to their respective molecules in activation (Immunotech International).

Platelets and Preeclampsia

Platelets are associated with vasospasms, occlusive vascular lesions, in numerous organ systems including the placenta. They are also known to activate the coagulation system. Evidence for the involvement of circulating platelets in preeclampsia is in the decreased platelet count, change in platelet aggregability, and high levels of platelet specific protein B-thromboglobulin that have been noted in preeclamptic patients. Explanations for increased platelet activation are an intrinsic change in platelet responsiveness or increased consumption/turnover in the microvasculature secondary to hypertensive vasospasm (Socol et al 1985). Whatever the cause this could be our chance at a predictive test.

Platelets play a substantial role in the pathogenesis of preeclampsia. Platelets may be activated by damaged endothelium in systemic or placental circulation during preeclampsia. Despite where the damaged endothelial extracellular matrix is exposed the result is the same, activation of platelets.

Activated platelets have different exposure of glycoproteins and glycoproteins complexes on their surfaces compared with non activated platelets. Flow cytometry can detect the activated platelets in circulation. This has been done by Janes and Goodall. They discovered a significant increase in the platelet surface expression of CD 63 in preeclamptic patients (Konijnenberg et al 1997 Feb).

Thrombocytopenia and Preeclampsia

Thrombocytopenia is a reduction in the number of platelets. It is the most common cause of bleeding disorders (Anderson et al 1998). "Between 15 and 50% of patients with preeclampsia develop thrombocytopenia at some point in the course of their illness making preeclampsia a common cause of significant thrombocytopenia during the third trimester of pregnancy" (McCrae et al.1992).

The thrombocytopenia can lead to bleeding problems in delivery. Major bleeding is rare, but minor bleeding is common as well as post-operative oozing after cesarean section. This bleeding may result from the effects of a coagulopathy involved with the preeclampsia. Treatment is replacing the lost platelets with donor platelets, but it may not help if the preeclampsia is causing platelet destruction (McCrae et al. 1992).

Flow Cytometry

The FACScan flow cytometer is a multiparameter instrument with up to three fluorescence and two scatter measurements for each cell (Coon et al 1987). The fluorescence is measured based on the number of antibodies with fluorescent markers that are attached to the antigens on the cell surface (Leytin et al 2000). The forward and side scatter measurements determine the type of cell, separating platelets, erythrocytes, and white blood cells based on size (Shattil et al1987). Many of the adjustments, to settings of the optical alignment and laser power, are under software control making it an easy instrument to work with. (Coon et al 1987)

Flow cytometry is commonly performed in a flow laboratory. The flow cytometer is compatible with IBM computers. This allows the data to be transferred to any IBM compatible computer and then analyzed off site as well as on site (Coon et al 1987). The software used in this study is the Cellquest software system.

Detection of Platelets and Platelet Activation

The first step to flow cytometric analysis of platelets is the differentiation of platelets from other cells in the whole blood sample. The platelets need to be differentiated from erythrocytes and white blood cells. This can be done by the flow cytometer using the light scatter profile. On the forward and side scatter profiles the platelets can be clearly separated from the larger erythrocytes and white blood cells. This scatter is not affected by the presence of platelet agonists. For platelet analysis a gate is set around the scatter area thought to be platelets. Then this area, containing mostly

platelets with approximately 5% erythrocytes, can be assessed for the following surface glycoproteins (Shattil et al 1987).

Platelets and platelet activation are detected with the help of monoclonal antibodies to the glycoproteins on the cell surface. Monoclonal antibodies have been discovered which recognize cell surface differentiation antigens. These monoclonal antibodies were studied to determine what antigens they were reactive to. Then they were grouped according to the reactivities on specific target cells. Cluster analysis was used to define groups of antibodies that were statistically similar and these clusters were termed clusters of differentiation (CD) (Coon et al 1987).

CD 41 is a monoclonal antibody that binds to glycoprotein IIb on the surface of a resting platelet. CD 61 is a monoclonal antibody that binds to glycoprotein IIIa, which is also present on the surface of a resting platelet. The presence of these glycoproteins, identified by the monoclonal antibody markers confirms that these cells are indeed the platelets that we wish to analyze (Immunotech International)

CD62 is a monoclonal antibody that binds to GMP-140, a protein from the alpha granule of the platelet that is expressed on the surface of the platelet after activation (Leytin et al 2000). Since the alpha granule only releases it's contents after activation of the platelet this is a good marker to look for when attempting to identify activated platelets.

PAC-1 is an IgM antibody that binds to the activated form of the glycoprotein IIb/IIIa complex. This complex is only present after activation of the platelet (Shattil et al 1987). Therefore it is an excellent marker to use when looking for activated platelets.

In order to determine platelet activation two parameters are looked at when using CD 62, these are the percentage of CD62 positive platelets in the total platelet population and the mean fluorescence of the CD62 positive cells, which is expressed as mean channel fluorescence. The percentage of CD62 positive platelets tells us the proportion of activated cells in the total platelet population. This gives us a quantitative idea of how many activated cells are present in the total platelet population. The mean fluorescence gives us an idea of the quality of the activation of the platelets. It is based on density of the CD 62 molecules present in the sample (Leytin et al 2000). This tells us how strongly the platelets are reacting to the antibody.

Similar Studies

Two studies have been performed in the Netherlands that are similar to this study being performed here.

In February of 1997 Konijnenberg et al released a study that investigated, via flow cytometry, to what extent platelets circulate in an activated state during normal pregnancy. They then looked to see what extent platelets circulate in an activated state during a preeclamptic pregnancy. It was seen (comparing 10 preeclamptic with 10 normotensive pregnant women in the third trimester) that there is enhanced platelet aggragation in some normal pregnant women (4) and in all of the preeclamptic women (10). This was discovered using numerous platelet markers. It was also found in this study that platelet count and mean platelet volume did not differ significantly, but the activated platelets were significantly higher in preeclamptic patients. An obvious

downfall of this study is the small numbers, but as a pilot study almost to ours it is very useful.

Another study, done by Konijnenberg et al in August of 1997 took this a step further. They checked the blood of 244 patients at the first, second, and third trimester and after delivery for the surface antigens antiCD62P, antiCD63, and antiCD31. It was discovered that anti CD63 was the only one that worked to predict preeclampsia and that it could do so at 13 weeks gestation. Therefore increased first trimester CD63 is an independent risk factor for development of preeclampsia. This increased activation of the platelet was seen on average 21 weeks (with a range of 3 to 27) before the onset of the clinical signs and symptoms of preeclampsia would be seen. This opens a new perspective for early identification and clinical intervention in these people. This study was intended to show the use of combining both the platelet activation test with initial blood pressure measurements as a tool to identify those at high risk for developing preeclampsia and to get intervention to those that need it. It was suggested to possibly use aspirin therapy on these individuals.

Implications of This Study

The implications of this study can be obviously seen. Preeclampsia is a serious disorder with serious consequences to the mother and the fetus. Eclampsia can be prevented and preeclampsia can be treated (to a point) if it is picked up early enough. Currently there are no early tests available to help find the women that are at high risk for this disease. If platelet markers turn out to be truly predictive women at high risk of developing preeclampsia can be identified earlier in the development of the disorder and

monitored more closely. The protective factors that have been discovered and are currently in use (aspirin and calcium) can be used in these women to try to prevent the development of preeclampsia.

At the same time, there is currently no way of predicting which women with preeclampsia will progress on to bleeding problems during delivery. If platelet activation is identified early in a pregnancy with a high number of the cells being activated, and therefore commonly used up prior to delivery, there could be a way to predict bleeding complications and possibly treat these women with platelet transfusions to prevent the bleeding problems. At the very lease the medical staff would be better prepared for the chance of extreme bleeding at the time of delivery.

CHAPTER 3
MATERIALS AND METHODS

Study Design

This study is a cross sectional study looking at the platelet surface glycoproteins for activation in preeclamptic pregnant women. The sample population was obtained through West Michigan OB/GYN and Spectrum Health. Consent for this study was obtained from Spectrum Health Research and Human Rights Comittee by way of approval of the research proposal (Appendix A).

The independent variable in this study is the preeclamptic females. The dependent variables are the platelet number, mean volume, and the activation status of the platelets, measured by flow cytometric analysis of the platelet surface glycoproteins.

Study Site and Subjects

The sample population consisted of non pregnant, normal pregnant, and preeclamptic pregnant females. Preeclamptic pregnant females were defined as women with pregnancy induced hypertension. The population used for this study was obtained from Dr. Russel Jelesma, M.D. at West Michigan OB/GYN. These women were chosen because pregnancy induced hypertension puts a woman at risk of developing eclampsia.

Demographic information on each of the subjects was obtained by Dr. Theresa Bacon-Baguley (thesis chair) via the patient's records. Inclusion criteria for placement in the study group of preeclamptic females were women with pregnancy induced

hypertension. Exclusion criteria for this group were females who had preexisting hypertension.

Equipment and Instruments

The laboratory analysis was performed at the Spectrum Health Flow Cytometry laboratory. A Sysmex K-1000 (Coulter Co.) was used to perform the complete blood count. A Becton –Dickson FACSort flow cytometer (Becton-Dickson Immunocytometery Systems, San Jose, CA) equipped with Cellquest software was used for the analysis of the platelets.

Antibodies specific for platelets were used to mark the cells as platelets. These antibodies wee specific for the GPIIb binding site on the platelets. In this study the antibodies used for this purpose were CD 41 (FITC, F7088, DAKO, Denmark) and CD 41 (PE, R7058, DAKO, Denmark). Specific antibodies were used for the identification of activated platelets. These bound to the GMP-140 site. The antibody used in this study to identify activated platelets was CD62 (PE, 348107). These antibodies were tagged to be read by the flow cytometer with the presence of two colored labels, FL1-FITC (green) and FL2-PE (red).

Procedure

Platelet (Blood) collection

After the selection process described above was completed, two 5ml EDTA tubes of blood were collected from each subject in the sample population using venipuncture with a 21-gauge needle or larger. The first 5 ml EDTA tube of blood, when available,

was used to run a complete blood count (CBC). It was then discarded in case of thrombin formation. Then 100μl of blood from the second 5 ml EDTA tube was fixed with 1 ml of 1% formaldehyde solution (at 2-8°C) to prevent non specific activation of the platelets. This blood was then used for flow cytometric analysis. The blood was fixed within 30 minutes of collection to prevent activation.

Platelet Size and Mean Platelet Volume

In order to obtain a platelet count and mean platelet volume a complete blood count was performed using a Sysmex K-1000. As stated previously, the first 5 ml EDTA tube of blood collected, when available, was used for this procedure. This blood was not prepared in any special fashion.

Preparation of Platelets for Flow Cytometric Analysis

The 100 µl of blood that was fixed with the 1 ml of 1% formaldehyde was incubated for thirty minutes to ensure that fixing occurred. Next, the blood was centrifuged at 2500 RPM for 5 minutes (at 20-25° C). The supernatant was then aspirated and the pelleted blood was resuspended in 2 ml of PBS with 0.1% of sodium azide and vortexed. Centrifuging at 2500 RPM for 5 minutes (at 20-25° C) was repeated and the supernatant aspirated. The pellet was then resuspended in 1 ml PBS with fetal calf serum and 0.1% sodium azide. The blood was then ready to be incubated with the monoclonal antibodies.

Each antibody is directly conjugated to one of two fluorescent fluorochrome labels called Flourescein Isothiocynate (FITC) or R-Phycoerythrin (PE). When bound to

the cell, these labels emit colored fluorescence and can be detected by the flow cytometer. Following the above fixing and washing, 50 µl of the resuspended blood was incubated with 20 µl of mouse monoclonal antibodies used for controls or commercially available antibodies specific for:

- GP IIb (CD 41, FITC, F7088, DAKO, Denmark),
- GP IIb (CD 41, PE, R7058, 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).

The blood and antibodies were vortexed, and incubated in the dark for 15-20 minutes. Following this period, 2 ml of PBS with 0.1% sodium azide was added to wash and the solution vortexed. This was now centrifuged at 2500 RPM for 5 minutes (at 20-25° C) and the supernatant aspirated. Finally, the pellet was resuspended in 1 ml PBS with 0.1% sodium azide and was now ready for flow cytometric analysis.

Flow Cytometric Analysis

A Becton-Dickson FACSort flow cytometer (Becton-Dickson Immunocytometry Systems, San Jose, CA) equipped with Cellquest software was used for the analysis of the platelets. Calbrite beads (Becton-Dickson) established fluorescent compensation. Platelets were distinguished from erythrocytes and white blood cells on the basis of their forward scatter and side scatter profiles. The flow cytometer provides information on relative size of a cell (forward scatter of laser light-FSC) and the granularity and

complexity of a cell (side scatter of the laser light – SSC). These are compared to known values for platelets and the area of platelets is identified. The flow cytometer also provides information on the fluorescence intensity of the two colored labels used (FL1-FITC and FL2-PE). The cells passed through the laser beam at a rate of 1000 cells per second. Using the forward scatter and side scatter, electronic gates were established to include only platelets and exclude debris. The software was set in acquisition mode and the following parameters set to obtain optimal data:

- FSC and SSC were collected on a log scale.
- FACSort detectors were set to: FSC (E00), SSC (322), FL1 (749), FL2 (690).

Compensation was set at 1.8% for FL1, 14.9% for FL2, and 0% for the other detectors, which were not in use. Compensation threshold was set according to the FSC-Height 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 histograms of the amounts of fluorescence emitted per cell. Every sample run was saved and analyzed later.

Data Analysis

After each sample had been run through the flow cytometer, the data obtained was printed out and analyzed. An example of an analysis is provided in Figure 1. The diagram in the upper left hand corner shows a dot plot on a log vs. log scale of forward scatter vs. side scatter of the total cells collected (10,000). A gate around just the platelets was then created based on how the cells scattered and the known sizes and granularity of platelets.

Next to the FSC vs. SSC dot plot is another plot of FL1 (Fluorescence 1) for FITC vs. FL2 (Fluorescence 2) for PE. This data was plotted on a log vs. log scale and quadrants were set at 10' on both the FL1 and FL2. The quadrants were set to separate Ab bound platelets in the data analysis. Below the two dot plots are histograms of each of the FL1 and FL2. The statistics for each histogram give the peak and mean channels for each antibody (the fluorescence intensity). Since each Ab binds a specific platelet glycoprotein, the peak and mean values of Ab bound represents the peak and mean values of the platelet glycoprotein expressed. The values obtained via flow cytometry are for CD 41- FITC (IIb), CD 41- PE (IIb), CD 62- PE (GMP-140), and PAC-1- FITC (activated IIb/IIIa).

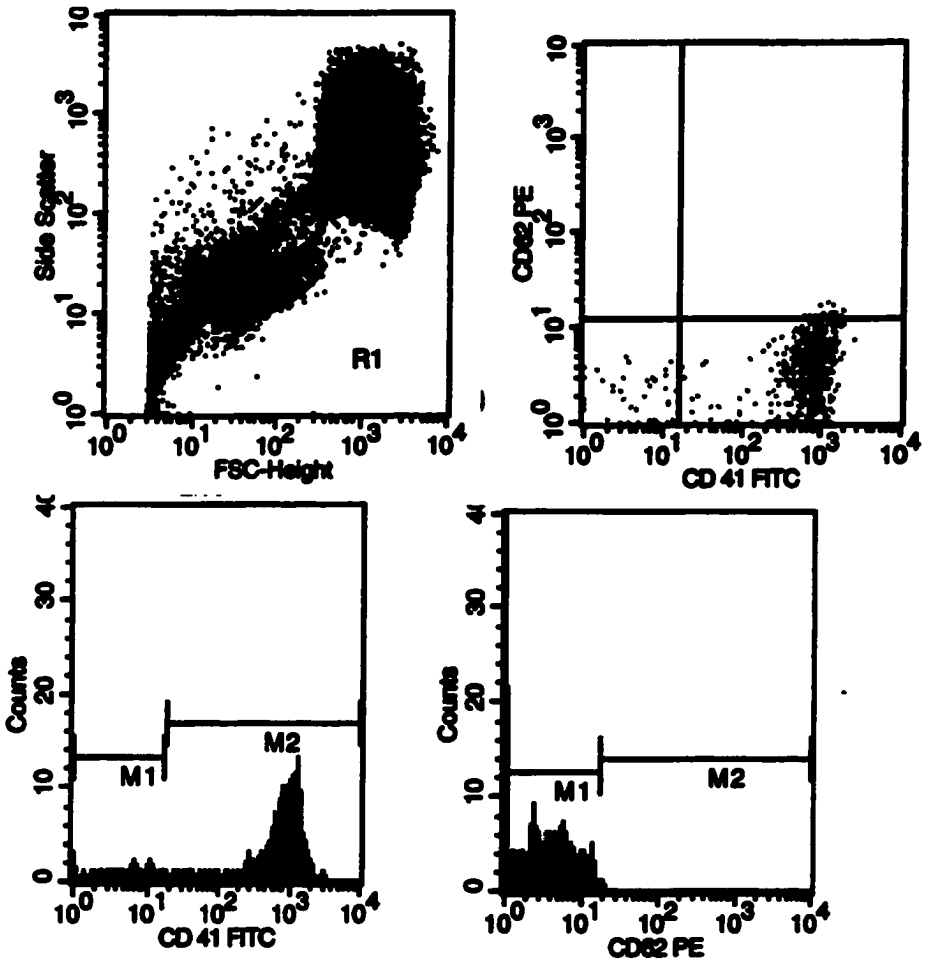


Figure 1: Example of Flow Cytometry Results. Upper left diagram shows all of the cells with the area thought to be platelets (according to size) gated (circled). These are the cells analyzed in the rest of the diagrams. The upper right diagram has CD 41(platelet marker) on the horizontal axis and CD 62 (activation marker) on the vertical axis. Cells to the right are platelets. Cells higher have more activation marker on them. The bottom two graphs show spikes in fluorescence for CD 41 (left) and CD 62 (right).

CHAPTER 4
RESULTS/DATA ANALYSIS

Technique of Data Analysis

Data was analyzed using the computer program SPSS to run statistical analysis. The raw data for age, prior pregnancies, number of live births, gestational age, platelet count, mean platelet volume, CD 41 peak, CD 41 mean, CD 41 with CD 62 peak, CD 41 with CD 62 mean, CD 62%, CD 62 peak, CD62 mean, PAC1%, PAC1 peak and PAC1 mean were all entered into the program for analysis.

An independent t-test was run on the values comparing the non pregnant to the normal pregnant, the non-pregnant to the preeclamptic, and the normal pregnant to the preeclamptic. The values were looked at independent of each other so that individual changes could be seen in the different groups.

The mean for each variable was assessed to look at the numbers as a whole. The standard deviation was also given for each variable for each group to assess the variablility within the group as well as between the groups.

The values were assessed initially for equal variances using the Levene's Test for Equality of Variances. If the significance of the variances was greater than 0.05, equal variance was assumed. When this was the case the initial set of values, in the equal variances assumed row, were used for results and analysis. If the significance of the variances was less than 0.05, equal variance could not be assumed and the values in the second row, equal variances not assumed, were used for results and analysis.

Next the t-test for equality of means was looked at. The variables were examined to see if they were statistically significantly different. Since the program runs the significance two tailed each of these numbers were divided by 2 in order to get the one sided p-values to look at the statistical significance. Variables with p-values less than 0.05 are not equal and are thus statistically significant results.

Characteristics of Subjects

A total of 50 subjects were analyzed for this study. Twenty- two of them were non-pregnant females used as controls, eighteen were normal pregnant females, and six were preeclamptic patients. Four of the subjects were excluded due to other circumstances including spontaneous abortion, hysterectomy in control population, etc.

The average age of the non-pregnant females was 27.7. The average age of the normal pregnant females was 29.4. The average age of the preeclamptic females was 29.5. None of the age differences between groups showed any statistically significance (Table 1). This was evident by a p value >0.1 for all three comparisons. There was also no statistical significance between the number of prior pregnancies or number of live births between the three groups (Table 1).

Table 1: Characteristics of Subjects

	Non-Pregnant (n=22)	Normal Pregnant (n=18)	Preeclamptic (n=6)
Mean Age	27.6818	29.4444	29.5000
Mean Number of Prior Pregnancies	1.1364	1.7778	1.5000
Mean Number of Live Births	0.8182	1.2778	0.8333

Results of Platelet Counts and Platelet Volume

Platelet counts between the three groups were not statistically significant. The mean platelet count for non-pregnant females was 226,000 +/- 57.0. The mean platelet count for normal pregnant females was 235,000 +/- 45.7. The mean platelet count for the preeclamptic females was 254,833 +/- 54.7 (Table 2). This was not statistically significant with $p>0.1$ in comparisons between all of the groups (Table 3). This means that the groups had comparable total number of platelets. No one group had significantly more or less number of platelets.

Table 2: Platelet Counts

	Non Pregnant (n=22)	Normal Pregnant (n=18)	Preeclamptic (n=6)
Mean Platelet Count (Standard Deviation)	226,0000 (+/- 56.9987)	235,000 (+/-) 45.7088	254,000 (+/-) 54.6897

Table 3: Significance of Platelet Counts

	Non Pregnant vs. Normal Pregnant	Non Pregnant vs. Preeclamptic	Normal Pregnant vs. Preeclamptic
P-valne	0.2955	0.1395	0.1945

The mean platelet volumes (MPV) were compared between the groups. There were no statistically significant differences seen in the mean platelet volumes between these groups. The mean platelet volume for the non-pregnant group was 9.66 (+/- 0.77). The mean platelet volume for the normal pregnant group was 9.70 (+/- 0.73). The mean platelet volume for the preeclamptic group was 9.62 (+/- 0.78) (Table 4). This means that the size of the platelets was comparable between the groups and there were no major differences in platelet size (Table 5).

Table 4: Mean Platelet Volume

	Non Pregnant (n=22)	Normal Pregnant (n=18)	Preeclamptic (n=6)
Mean Platelet Volume (Standard Deviation)	9.6591 (+/- 0.7725)	9.7000 (+/- 0.7340)	9.6167 (+/- 0.7808)

Table 5: Significance of Mean Platelet Volume

	Non Pregnant vs. Normal Pregnant	Non Pregnant vs. Preeclamptic	Normal Pregnant vs. Preeclamptic
P-value	0.433	0.453	0.4075

Results of CD 41

The peak and mean of CD 41 is analyzed next. This is the marker for platelets. The flow cytometric results for a non-pregnant patient are seen in Figure 2. The platelets with CD 41 present are to the right on the horizontal axis (CD41). These results are recorded by the flow cytometer as number values for peak and mean fluorescence, which were then analyzed and compared.

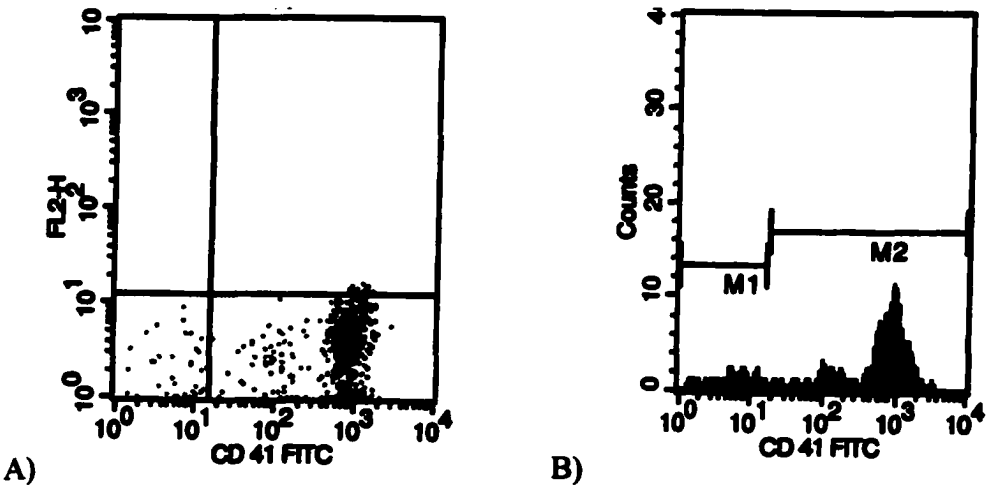


Figure 2: Flow cytometry results of CD 41 in Non-Pregnant (Control) Subjects. The first diagram (A) shows the forward scatter. Cells that are platelets are moved to the right along the vertical axis as they show more concentration of fluorescence. The second diagram (B) shows the peaking of cells with increased fluorescence.

The peak values of CD 41 for non-pregnant subjects were 781.1 (+/- 134.4), for normal pregnant subjects were 1300.9 (+/- 1642.2), and for preeclamptic subjects were 761.2 (+/- 206.5). The mean values for CD 41 in non-pregnant patients were 787.1 (+/- 64.4), in normal pregnant patients were 994.3 (+/- 279.8), and in preeclamptic patients were 762.2 (+/- 211.5)(table 6).

Table 6: Mean and Peak of CD 41

	Non Pregnant (n=22)	**Normal Pregnant (n=18)**	**Preeclamptic (n=6)**
Mean CD 41 (Standard Deviation)	787.0909 (+/- 64.4367)	994.3333 (+/- 279.7661)	762.1667 (+/- 211.4989)
Peak CD 41 (Standard Deviation)	781.1364 (+/- 134.4461)	1300.9444 (+/- 1642.2194)	761.1667 (+/- 206.4920)

The differences between non-pregnant and normal pregnant as well as normal pregnant and preeclamptic in the mean amount of CD 41 seen were statistically significant (Table 7). However there was no statistical significance in the values for

preeclamptic compared to non-pregnant individuals. This shows that there are more platelets showing fluorescence in normal pregnancy than in non-pregnant and preeclamptic. The preeclamptic pregnant patients have mean CD 41 values that decrease from the normal pregnancy state to lower than the non-pregnant state (Figure 2).

Table 7: Significance of Mean and Peak CD 41

	Non Pregnant vs. Normal Pregnant	Non Pregnant vs. Preeclamptic	Normal Pregnant vs. Preeclamptic
Mean CD 41 P-value	0.003	0.393	0.0385
Peak CD 41 P-value	0.099	0.415	0.2185

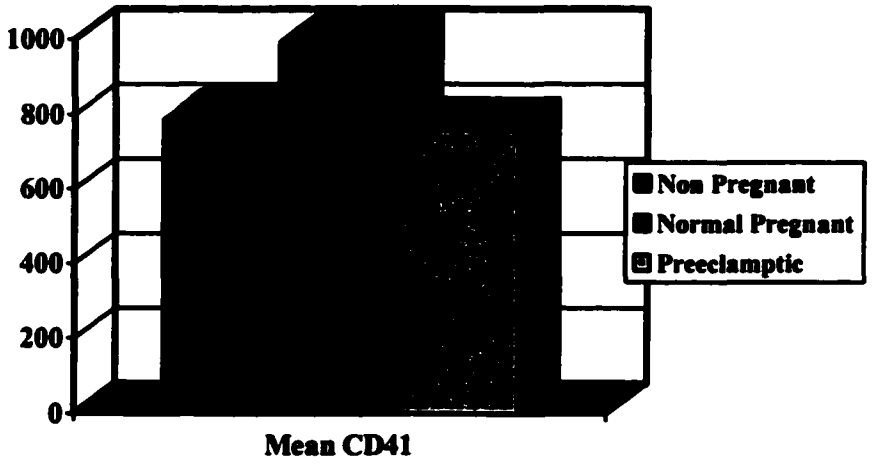
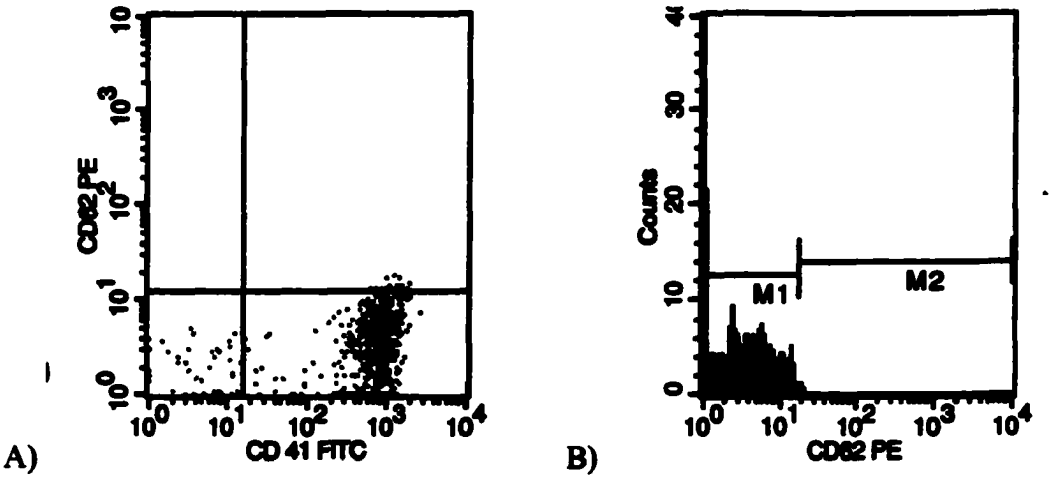


Figure 3: The mean fluorescence of platelets containing CD 41 is significantly different between non-pregnant and normal pregnant and between normal pregnant and preeclamptic. However there is no significant difference between the non-pregnant and preeclamptic groups.

Results of CD 62

In analysis of the CD 62 marker it is seen that the presence of circulating activated platelet membrane proteins was different between the various groups. This means that there is difference in activation between these groups.

Figure 4 shows the flow cytometry readouts for the non-pregnant subjects. The platelets are present (peak to the right on the horizontal axis (CD 41) of A) but not activated (not high on the vertical axis (CD 62) of A. Also not showing much activation on the horizontal axis (CD 62) of B.



A) B)

Figure 4: The flow cytometry CD 62 for non-pregnant subjects. (A) Platelets are identified with CD 41. The cells that have CD 41 bound to them are to the right on the horizontal axis. Activation is identified on the vertical axis. (B) The peaks of activation are highlighted. There is not much activation.

Figure 5 shows the flow cytometry information from the normal pregnant subjects. This sample shows that the majority of the cells are platelets (to the right on the horizontal axis, CD41) and many of those platelet cells are activated (high on the vertical axis, CD 62). This shows pregnancy to be a hypercoaguable state with activated platelets looking to release their contents, bind to each other, and form a platelet aggregate.

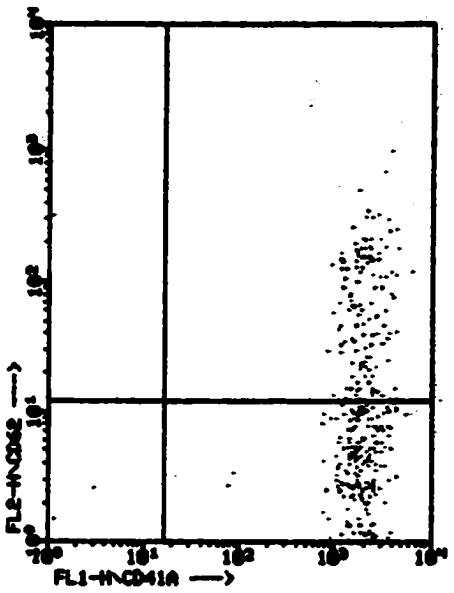


Figure 5: Flow cytometry CD 62 information for normal pregnant women. The cells are noted to be mostly platelets (to the right on the horizontal (CD41) axis. Many of the cells are activated as is seen by their vertical rise on the vertical axis (CD 62).

Preeclamptic subjects are shown in figure 6. The platelets show an interesting finding in that there are two spikes of platelets seen as well as an area that is the same size as the platelets (fell within the gated area to be analyzed) that did bind the CD 41, which marked the cells as platelets.

Another interesting finding in the preeclamptic group is the variation in the subjects. These two samples both show the two spikes but one shows a large number and percentage of the cells to be activated, while the other patient has very few activated platelet cells.

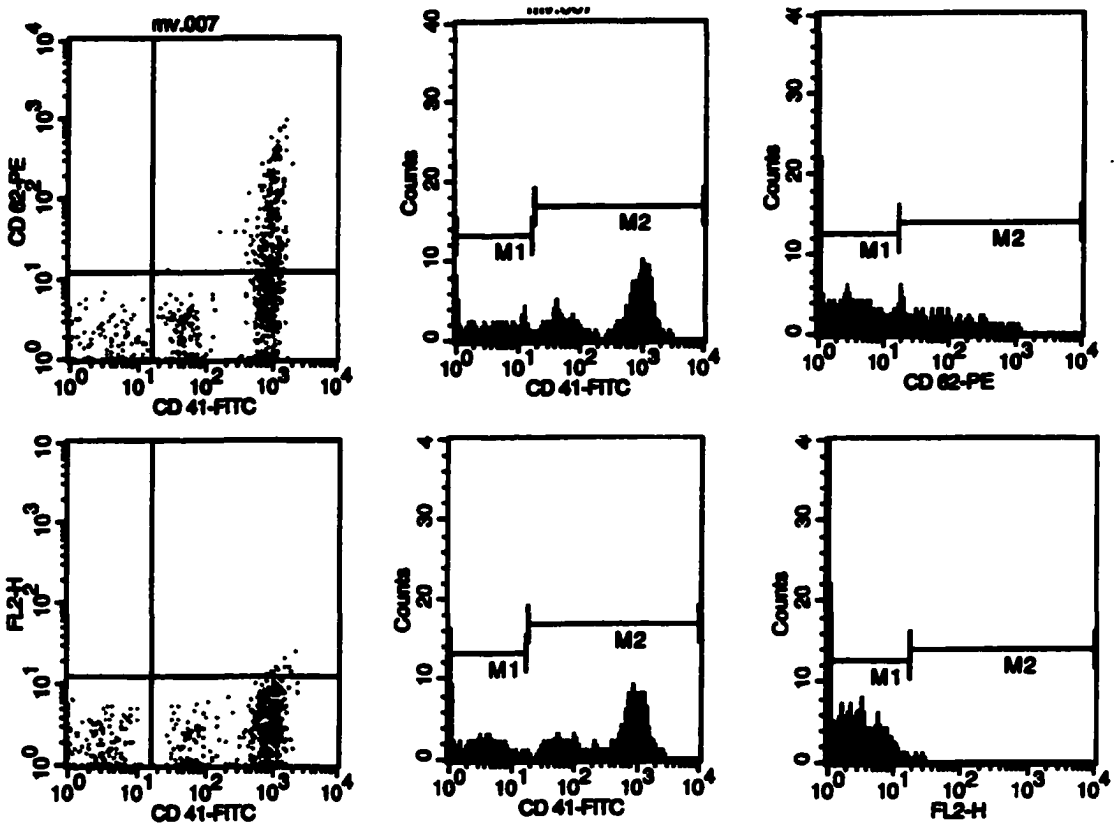


Figure 6: Flow cytometry data from preeclamptic subjects. These readouts are from two different subjects in the preeclamptic group. Both subjects have an interesting finding of two spikes of platelets along the horizontal (CD41) axis as well as cells that fell in the gated area to be analyzed but do not appear to be platelets (do not have CD 41 on them). Also the two patients have different amounts of activated platelets with the top patient having quite a few activated platelets (higher on vertical axis, CD 62) while the bottom patient has a smaller number and smaller percent of the total cells activated.

The mean amount of CD 62 present on the platelets of the non-pregnant population was 40.2 (+/- 72.8). The mean CD 62 present in normal pregnancy was 75.5 (+/- 54.4). The preeclamptic group was similar to the normal pregnancy group with a mean CD 62 of 70.2 (+/- 21.2). The peak amount of CD 62 was 16.6 (+/- 15.4) for the non-pregnant subjects, 21.1 (+/- 9.7) for the normal pregnant subjects and 37.0 (+/- 26.8) for the preeclamptic subjects (Table 8).

Table 8: Mean and Peak of CD 62

	Non Pregnant (n=22)	Normal Pregnant (n=18)	Preeclamptic (n=6)
Mean CD 62 (Standard Deviation)	40.2000 (+/- 72.8333)	75.5294 (+/- 54.4221)	70.1667 (+/- 21.2360)
Peak CD 62 (Standard Deviation)	16.6000 (+/- 15.3705)	21.1176 (+/- 9.7396)	37.0000 (+/- 26.8403)

No statistically significant differences are seen in either the mean CD 62 presence or in the peak of CD 62 found amongst any of the groups. However the p-values are very close to statistically significant. The p-value between non-pregnant and normal pregnant for mean CD 62 is 0.0545, nearly statistically significant. At the same time the p-value between non-pregnant and preeclamptic for peak CD 62 is 0.063, also close to being statistically significant (Table 9).

Table 9: Significance of Mean and Peak CD 62

	Non Pregnant vs. Normal Pregnant	Non Pregnant vs. Preeclamptic	Normal Pregnant vs. Preeclamptic
Mean CD 62 P-valne	0.0545	0.168	0.4095
Peak CD 62 P-value	0.1515	0.063	0.1055

Looking at the numbers and graphing them out this near statistical significance is even more evident. Figure 7 shows bar graphs for mean CD 62 and peak CD 62 values between the non-pregnant, normal pregnant, and preeclamptic groups. The peak CD62 values show a difference between the non-pregnant and the preeclamptic as well as between the normal pregnant and the preeclamptic groups. This is not a statistically significant difference. The mean CD 62 values appear to show a difference between the

non-pregnant and both the normal pregnant and preeclamptic groups. Again, this was not statistically significant

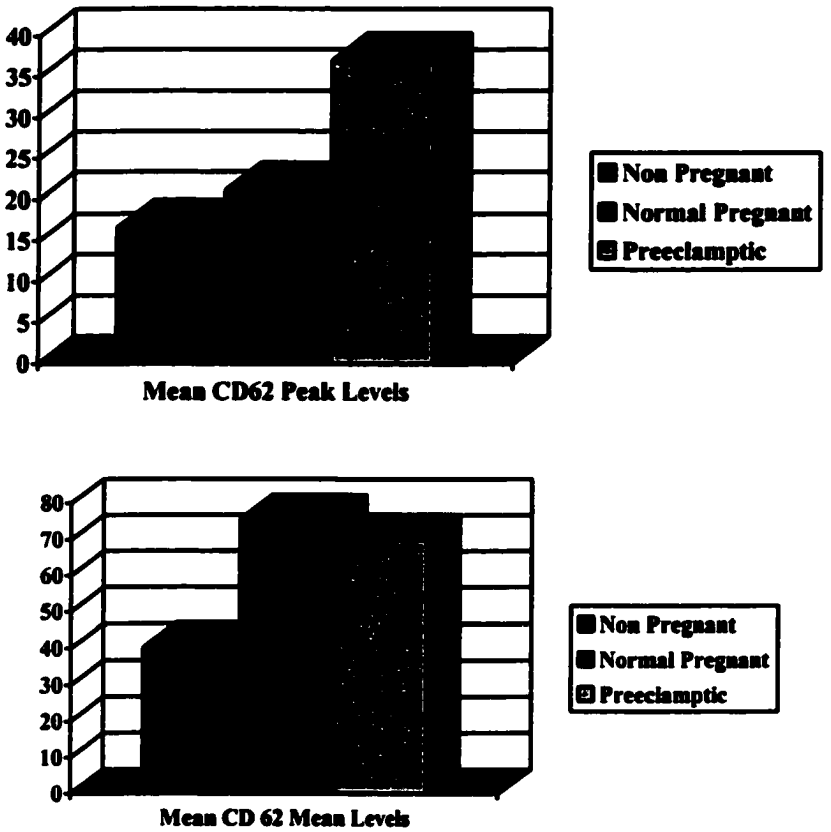


Figure 7: The peak CD62 values are shown in the top bar graph. A difference is seen between the non-pregnant and the preeclamptic as well as between the normal pregnant and the preeclamptic groups. This is not a statistically significant difference. The mean CD 62 values for the three groups are shown in the bottom graph. A difference is seen between the non-pregnant and both the normal pregnant and preeclamptic groups. Again, this was not statistically significant.

Another aspect of the CD62 marker that was analyzed was the percent of platelets that were activated. This looked at the total number of platelets present and the percent of those platelets that were activated. The mean percent of activated platelets (marked by the presence of CD62) in the non-pregnant subjects was 0.71% (+/- .67). In the normal pregnant women the mean percent of platelets that were activated rose to 20.28% (+/-

17.59). The preeclamptic subjects had a mean percent of activated platelets 23.17% (+/- 10.68) (Table 10).

Table 10: Percent of Platelets Expressing CD 62

	Non Pregnant (n=22)	Normal Pregnant (n=18)	Preeclamptic (n=6)
Percent of Platelets Expressing CD 62 (Standard Deviation)	0.7064% (+/- 0.6715)	20.28% (+/- 17.5921)	23.1667% (+/- 10.6849)

There is a statistically significant difference between the percent of cells expressing CD 62 in non-pregnant and preeclamptic women (p-value 0.000). There is also statistical significance between the non-pregnant women and the preeclamptic subjects (p-value 0.002). There is not statistically significance between the normal pregnant and preeclamptic subjects (p-value 0.355) (Table 11). However in looking at the raw data 5 out of the 6 preeclamptic women had greater than 25% of their platelets activated which is significant when looking at the raw numbers of the other groups. The difference in means is seen when the values are graphed out (Figure 8).

Table 11: Significance of Percent of Platelets Expressing CD 62

	Non Pregnant vs. Normal Pregnant	Non Pregnant vs. Preeclamptic	Normal Pregnant vs. Preeclamptic
Percent CD 62 P-value	0.000	0.004	0.355

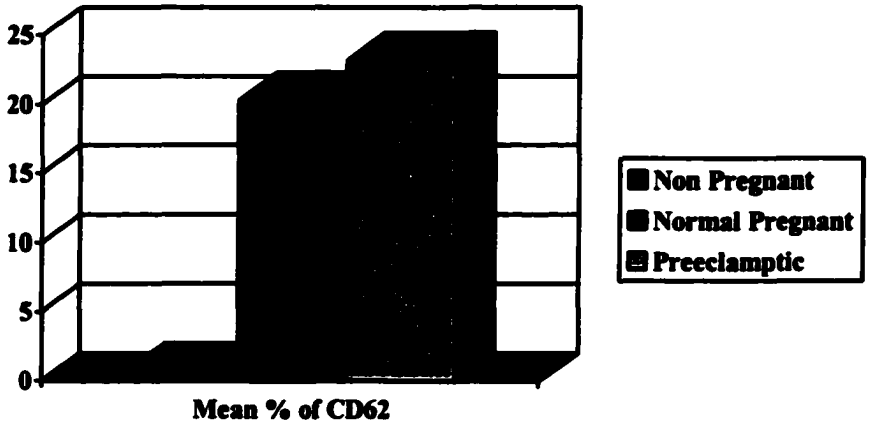


Figure 8: The percent of platelets in each group with the CD 62 marker showing activation. Not many of the platelets present in the non-pregnant group are activated. However, 20% of the platelets in the normal pregnant sample are activated and 23% of the platelets in the preeclamptic group are activated.

Results of PAC 1

When looking at the PAC-1 values it is seen that none of the groups are statistically significantly different in the expression of PAC-1. The percent of platelets expressing the PAC-1 site for non-pregnant subjects was 0.47% (+/- 0.58). The percent of normal pregnant women in the study with the PAC-1 binding site was 0.33% (+/- 0.47). The percent PAC-1 on preeclamptic patients was 24.33% (+/- 32.65) (Table 12).

Table 12: Percent of Platelets Expressing PAC-1

	Non Pregnant (n=22)	Normal Pregnant (n=18)	Preeclamptic (n=6)
Percent of Platelets Expressing PAC-1 (Standard Deviation)	0.4745 (+/- 0.5841)	0.3300 (+/- 0.4658)	24.3333 (+/- 32.6548)

The peak and mean PAC-1 values also do not show any statistically significant differences between groups. The non-pregnant mean PAC-1 is 787.8 (+/- 1665.6) and the peak PAC-1 is 673.4 (+/- 169.9). The normal pregnant mean PAC-1 is 1890.5 (+/-

2251.0) and peak PAC-1 is 1386.1 (+/- 2302.6). The preeclamptic mean PAC-1 is 137.0 (+/- 75.0) and the peak PAC-1 is 22.0 (+/- 5.2) (Table 13). The p-values show that there is no statistical significance between the groups in their PAC-1 expression (Table 14).

Table 13: Mean and Peak of PAC-1

	Non Pregnant (n=22)	Normal Pregnant (n=18)	Preeclamptic (n=6)
Mean PAC-1 (Standard Deviation)	787.8235 (+/- 1665.5745)	1890.5000 (+/- 2250.9941)	137.000 (+/- 75.0267)
Peak PAC-1 (Standard Deviation)	673.4118 (+/-169.8528)	1386.1000 (+/- 2302.5516)	22.0000 (+/- 5.1962)

Table 14: Significance of Mean and Peak PAC-1

	Non Pregnant vs. Normal Pregnant	Non Pregnant vs. Preeclamptic	Normal Pregnant vs. Preeclamptic
Mean PAC-1 P-value	0.157	0.517	0.218
Peak PAC-1 P-value	0.365	0.524	0.341

CHAPTER 5
DISCUSSION AND IMPLICATION

Discussion of Findings

This study has attempted to show an increase in platelet activation in preeclamptic women that can be used as an early detection test for women who will develop preeclampsia. It also attempted to show that women with increased activation will have an increased percentage of activated platelets. The activation of platelets was determined by the presence of CD62 and PAC-1.

In looking at the data it is important to remember that the preeclamptic women are pregnant, just as the normal pregnant women are pregnant. However there are differences even between the two groups showing that something is definitely going on within the circulation and in the platelets themselves.

The study discovered that the platelet count and mean platelet volume does not change from non-pregnant women to pregnant women, with or without preeclampsia. It also does not change between normal pregnancy and preeclamptic pregnancies. This supports the previous research discovered by Konijnenberg et al in February and August of 1997.

In looking at CD 41, a resting platelet marker, it is seen that normal pregnant women had more CD 41 present than both the non-pregnant and the preeclamptic groups. This may be due to the normal hypercoaguable state of pregnancy. However the decrease of platelet presence in preeclamptic subjects may be due to the platelets being used up by activation, leading to the bleeding problems some women with preeclampsia develop.

The decreased binding of antibody CD41 could also possibly be explained by the increased activation of platelets in preeclampsia. When platelets are activated the glycoprotein IIb/IIIa binds calcium, undergoes a conformational change, and subsequently binds fibrinogen. It could be hypothesized from the results of this study that the decrease in CD 41 presence in preeclamptic subjects is due to binding of fibrinogen secondary to activation. Since fibrinogen is using up the glycoprotein that CD 41 normally binds to, there is no place for CD 41 to bind on these activated cells. This hypothesis could be proven by studies looking at fibrinogen binding to the platelet.

When platelet activation was looked at, using CD 62 – a marker for platelet activation, it was noted that the peak and mean presence of CD 62 was not statistically significant amongst any of the groups. However the difference in mean CD 62, looking at the actual quantity of cells activated, between non-pregnant and normal pregnant is a p-value of 0.0545, which is nearly statistically significant. This tells us that the quantity of the activation protein on the platelets activated is nearly, but not quite, statistically significant between non pregnant and normal pregnant women. The possible reasons for this will be discussed in the limitations section below. However, it is also noted that there is no statistical significance between the non-pregnant and the preeclamptic pregnant women in the mean amount of cells activated (p-value 0.168). This is an interesting finding because these preeclamptic women are pregnant and should have similar values to the normal pregnant making them nearly statistically significant as well. This is not the case.

The p-value looking at peak CD 62, the amount of fluorescence (or activity) in the activated cells, between non-pregnant and preeclamptic is 0.063, which is also nearly

statistically significant. This value looks at how much the cells are activated rather than how many cells are activated. Looking at all of the data tells us that the normal pregnant women have slightly more activation than the non-pregnant women. The preeclamptic women have an even larger amount of activation from the pregnant women and because of that they have quite a bit more activation than the non-pregnant who were less than the normal pregnant. This value is not quite statistically significant, however.

The percent of platelets expressing CD 62, however, was statistically significant. There was a statistically significant difference between non-pregnant and preeclamptic pregnant women with a p-value of 0.000. This tells us that the percent of platelets that are activated, compared to the total number of platelets present, is increased in women with preeclampsia. However, there was not a statistically significant difference between preeclamptic and normal pregnant subjects. This supports the hypothesis that platelets are more activated in preeclamptic women.

Applications

The results of this study can be used to continue to look for a better test to predict preeclampsia and to predict bleeding problems in women with preeclampsia. Also, women who are currently found to have an increased percentage or level of activation of their platelets who are not diagnosed as preeclamptic could be watched more closely for the development of preeclampsia. This study is a step in the right direction in looking for better ways to predict and prevent the serious disorder of preeclampsia.

Limitations

The largest limitation of this study was the small sample size of preeclamptic patients. With only 6 patients to compare to the 22 and 18 of the non-pregnant and preeclamptic groups respectively the comparisons were difficult to make and the statistics were more difficult to compare. Also, with having only 6 preeclamptic patients, the one outlier (whose values were quite different from the rest of the group) cause a large variation.

Another limitation is the small sample size total. With only 46 total patients it is difficult to generalize the findings to the population. The small size of all of the groups lead to the means of the data having a large standard deviation due to outliers.

The reason that the values for CD 62 peak and mean were not statistically significant brings us back to the actual data from the statistical analysis. This shows us there are some individuals who are outliers, whose data is not consistent with the results of the rest of the individuals in the group. Had these outliers not been included in the study the statistical significance of these groups would have been great. Also, had the sample size for the groups been larger, the outliers would have averaged in better because there would also be outliers in the other direction along the bell curve.

In the normal pregnant group, when looking at the mean CD 62 analysis, there is one subject with 227 mean CD 62. This may throw the rest of the group, most of which are under 100, off track. In the preeclamptic mean CD 62 group one individual has a mean CD 62 of 110 while the other 5 are in the range of 47-71. Also in the non-pregnant group there was one individual with a CD 62 mean of 388 while the rest of the non-pregnant individuals were below 100, with most being less than 25.

At the same time, when looking at the CD 62 peak group of preeclamptic subjects, 4 of the 6 have peak values less than 25, but two have values of 64 and 78, which are not consistent with the rest of the group. The non-pregnant individuals all have peak CD 62 that is less than 20 except for two, one which is 22 and not that far off the rest of the group, and one that is 81, a far cry from the consistency of the rest of the group. The normal pregnant peak values are very much in line with each other.

Looking at the raw data for CD62 percent the non-pregnant group is consistently less than 2 with the exception of one individual. The normal pregnancy group ranges from 0.32% and 59.15%, with most of the individuals in the teens to 30's. The preeclamptic group however appears to have an obvious outlier. While 4 of the 6 individuals have CD 62 percentage values greater than 25% and one of the 6 has a value of 23% (almost to the 25%), there is one individual with only 3% of her platelets activated. Had this outlier been excluded, or had there been more individuals in the study to bring the mean up to above 25% seen in the majority of the subjects, this would have made the percent of activated platelets more significant.

In comparing the limitations of this study to the limitations of other studies in this arena of medicine it is noted that the limitations are similar. While the sample population in this study is small, the sample population of Knijnenberg et al in 1997 was 20 (10 preeclamptic and 10 normotensive pregnant women). This is a common limitation when working with human subjects.

Suggestions for Future Research

Further research could repeat this study and continue it for a longer period of time to allow more subjects to be studied. A larger subject population would allow for many

of the limitations of the study to be overcome and could look for a trend that is more like the general population. Outliers would not be as big of a problem in a larger study because they would be equaled out by the outliers in the opposite direction. A longer period of time would allow more women to be studied.

One area of research that could be looked at is in the realm of a predictive test. A large sample of pregnant women could be followed with blood draws every 4-6 weeks in the 2nd trimester and every 2-4 weeks in the 3rd trimester to look at the activation of platelets. These women could be followed closely for the development of preeclampsia. The platelet activation could then be compared to the development of preeclampsia to see if there is any predictive value in the test.

Further research could also assess whether or not preeclampsia can be predicted in the early second trimester in a similar way. Women who have their blood drawn early, mid, and late second trimester could be followed for the development of preeclampsia. If preeclampsia can be detected earlier research could continue to look for a way to prevent preeclampsia and eclampsia from occurring. Also these women who are at higher risk could be treated with current or future treatment methods.

Another aspect that further research could look at is the aspect of prediction of bleeding. Women in the study would need to be followed with blood draws before twenty weeks, at twenty weeks, after twenty weeks, just before delivery, and after delivery. The platelets could be looked at for activation at these various times to see if the activation increases during the pregnancy. Also these women could be followed for bleeding complications in delivery and the activations of platelets could be compared to the actual occurance of bleeding complications in pregnancy. In this way it could be seen whether

or not activation of platelets at a certain time, or above a certain percent of the platelets, could be predictive of bleeding problems.

Conclusions

With preeclampsia being such a serious disease, leading to 76,000 deaths each year world-wide and affecting two to ten percent of all pregnancies in the United States (Barron et al 2000, Konijnenberg A et al 1997, Cunningham 1997, Redman 1992) something must be done to lead earlier diagnosis, better treatments, and prevention of complications such as bleeding.

This study, similar to other studies, showed an increased activation in pregnancy and preeclampsia. It continued to show pregnancy as a hypercoaguable state. It also demonstrated a difference in the platelet markers and activation markers between normal pregnancy and preeclampsia. This information needs to be looked into further to discover the pathogenesis behind why the platelet changes from normal pregnancy to preeclampsia.

In conclusion, this study is an excellent stepping-stone for other, longer, more complete and comprehensive studies. More studies need to be done to look for a predictive test for preeclampsia and for the bleeding problems commonly associated with it.

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APPENDIX A

Spectrum Health

Butterworth Campus
100 MICHIGAN STREET NE GRAND RAPIDS MI 49503-2560
616 391 1774 FAX 391 2745 *www.spectrum-health.org*

March 6, 2002

Theresa Baguley,RN, PhD
Grand Rapids Medical Education and Research Center for Health Professions
1000 Monroe NW
Grand Rapids, MI 49503

Dear Theresa,

At the March 5, 2002 meeting of the Spectrum Health Research & Human Rights Committee your project "Clinical Investigation on the Assessment of Platelet Structure and Function in Normal and Preeclamptic Patients" (Spectrum Health #1994-010) was 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 **February 1,2003** and you will need reapproval should your study be ongoing at that time.

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,

[redacted]

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

JSJ/tjv

c:
File

APPENDIX B

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, your participation 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 result of the platelet 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 240 participants in the study. As a participant, you will have 6 ml of blood removed via venipuncture. You may be asked to return 6-12 weeks after the delivery of your baby for baseline analysis

Potential Benefits
The benefits of this study 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. In accordance with Michigan Law, an HIV and Hepatitis test may be performed on you without written consent if a healthcare worker/researcher is exposed to the blood. If the results of an HIV or Hepatitis test indicate that you are HIV or Hepatitis infected, you will be informed of these results and provided with the appropriate counseling.

_____
Patient Initials

Study Participation and Medical Records Access
You have the right to refuse participation in the study (thereby refusing to sign this consent form) if so desired, without any fear of prejudice to additional treatment. In addition, you may refuse to continue on this study at any time after the start of therapy without fear of prejudice to additional treatment. In case of a problem or emergency, you can reach Dr. R. Jelesma at (616) 774-7036 or call Dr. Theresa Bacon-Baguley at (616) 233-6505.

The doctor and/or his representative at Spectrum Health may inspect your medical records for information purposes where appropriate and necessary via mail, FAX or in person. Your confidentiality will be protected to the extent permitted by law. Your name will not be revealed in any publications or presentation resulting from this study without you expressed written consent.

Questions regarding your rights as a participant can be answered by calling the Spectrum Health Research & Human Rights representative, Linda Pool, at the following number: (616) 391-1291/1299.

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 Spectrum Health Downtown Campus. Your signature on the consent form indicates 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 by signing this form. By signing this form, I have not waived any of the legal rights I otherwise would have as a patient. I will be provided with a signed copy of this consent form and my signature indicates that I have volunteered to participate in this study having read the information provided.

Signature of Patient	Date
Signature of Investigator	Date
Signature of Witness	Date

APPENDIX C

SUBJECT NUMBER_____
DATE BLOOD DRAWN_____
DATE BLOOD ANALYZED_____

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: _____