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# Comparison of Leukocyte Classes Most Likely to Cause Vascular Dysfunction in Preeclampsia

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COMPARISON OF LEUKOCYTE CLASSES MOST LIKELY TO CAUSE  
VASCULAR DYSFUNCTION IN PREECLAMPSIA

A thesis submitted in partial fulfillment of the requirements for the degree of Master of  
Science at Virginia Commonwealth University

by

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## Table of Contents

	Page
Acknowledgements.....	ii
List of Tables .....	v
List of Figures .....	vi
List of Abbreviations .....	viii
List of Unit Measurements.....	ix
 Chapter	
1 General Introduction .....	1
A. Preeclampsia.....	1
B. Maternal Vascular Cell Dysfunction .....	1
i. Oxidative Stress.....	2
ii. Neutrophils.....	3
iii. Lymphocytes & Monocytes/Macrophages .....	4
C. Summary .....	7
D. Purpose of Study.....	7
E. Hypothesis.....	7
F. Significance of Study .....	8
2 Materials and Methods.....	9
A. Study Subjects .....	9

B. Collection of Fat .....	10
C. Protocol for Formalin-fixed Tissue .....	11
D. Data Analysis.....	13
E. Statistical Analysis.....	13
3 Results.....	15
A. Subjects.....	15
B. Immunohistochemical Staining .....	16
4 General Discussion .....	40
Literature Cited .....	46
Vita.....	51

List of Tables

Page

Table 1: Clinical Descriptive Statistics for Patient Groups.. .....19

## List of Figures

	Page
Figure 1: A visual hypothesis of leukocyte activation and infiltration causing endothelial and vascular cell dysfunction.....	18
Figure 2: Percent vessel staining of resistance sized vessels for monocytes, lymphocytes and neutrophils in normal non-pregnant women. ....	20
Figure 3: Percent vessel staining of resistance sized vessels for monocytes, lymphocytes and neutrophils in normal pregnant women.....	21
Figure 4: Percent vessel staining of resistance sized vessels for monocytes, lymphocytes and neutrophils in preeclamptic women .....	22
Figure 5: Number of leukocytes per vessel for normal non-pregnant patients.....	23
Figure 6: Number of leukocytes per vessel for normal pregnant patients. ....	24
Figure 7: Number of leukocytes per vessel for preeclamptic patients.....	25
Figure 8: Comparison of CD66b percent vessel staining for NNP, NP and PE .....	26
Figure 9: CD66b positive control staining example .....	27
Figure 10: Visual representation of CD66b staining for NNP, NP and PE patients.....	28
Figure 11: Comparison of CD99 percent vessel staining for NNP, NP, and PE .....	29
Figure 12: CD99 positive control staining example .....	30
Figure 13: Visual representation of CD99 staining from NNP, NP, and PE patients.....	31
Figure 14: Comparison of CD14 staining per field of view among groups.....	32



Figure 15: CD14 positive control staining example. ....	33
Figure 16: Visual representation of CD14 staining from NNP, NP and PE patients.....	34
Figure 17: Comparison of CD99 visual score for NNP, NP and PE .....	35
Figure 18: Visual scoring examples.....	36
Figure 19: Comparison of CD66b visual score for NNP, NP and PE .....	37
Figure 20: Comparison of CD66b optical densities for NNP, NP and PE .....	38
Figure 21: Visual score and density measurement correlation for CD66b staining .....	39

## List of Abbreviations

A	Adipocytes
dH <sub>2</sub> O	Deionized Water
EC	Endothelial Cells
NNP	Normal Non-Pregnant Women
NP	Normal Pregnant Women
PBS	Phosphate Buffered Saline
PE	Preeclamptic Women
ROS	Reactive Oxygen Species
TNF $\alpha$	Tumor Necrosis Factor Alpha
TX	Thromboxane
VL	Vessel Lumen
VSM	Vascular Smooth Muscle

## List of Unit Measurements

°C	Degrees Celsius
g	Gram
mg	Milligram
mmHg	Millimeters of Mercury
mM	Millimoles
μm	Micrometers
μM	Micromoles
μl	Microliters
ml	Milliliters

## Abstract

### COMPARISON OF LEUKOCYTE CLASSES MOST LIKELY TO CAUSE VASCULAR DYSFUNCTION IN PREECLAMPSIA

By Kristen Anne Cadden, M.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of  
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Virginia Commonwealth University, 2006

Major Director: Scott W. Walsh, Ph.D.  
Departments of Obstetrics and Gynecology, and Physiology

Leukocytes are activated in women with preeclampsia, but the class of leukocyte most likely to cause vascular dysfunction is not known. We hypothesized that neutrophils may be the class of leukocyte most involved in causing this dysfunction because neutrophils are the most abundant of the leukocytes and their numbers increase during pregnancy. In this study we compared vascular infiltration of neutrophils (CD66b) with monocytes/macrophages (CD14) and lymphocytes (CD99) in normal non-pregnant women (NNP), normal pregnant women (NP), and preeclamptic women (PE). There was no

significant difference in the infiltration of lymphocytes into the maternal vasculature in PE as compared to NNP or NP. There was significantly more neutrophil infiltration into the systemic vasculature in PE women than in NP and NNP women. Monocytes/macrophages were present in tissue but not in vessels. We speculate that neutrophils are the class of leukocyte that causes the majority of vascular cell dysfunction in preeclampsia.

# **General Introduction**

## **A. Preeclampsia**

Preeclampsia is a pregnancy induced hypertensive disorder that is among one of the leading causes of maternal and fetal death<sup>1</sup>, as well as premature<sup>2</sup> and small for gestational age births<sup>3</sup>. The clinical definition of preeclampsia is an elevated blood pressure of 140/90 mmHg or above after 20 weeks gestation along with proteinuria. Preeclampsia is also characterized by poor blood flow to the fetal-placental unit<sup>4</sup>. There are many hypotheses as to the causes of preeclampsia, some of which pertain to the activation of maternal inflammatory response mechanisms.

## **B. Maternal Vascular and Endothelial Cell Dysfunction**

Normal pregnancy is characterized by activation of the immune system as a protection mechanism against infection<sup>5</sup>, of which the endothelium plays a key role. The endothelial tissue is critical for maintaining vascular tone, preventing coagulation, and for influencing vascular smooth muscle response to various inflammatory agents<sup>4</sup>. Preeclampsia, however, goes beyond this protective mechanism and causes harmful immune system activation that can lead to vascular and endothelial damage. Maternal

vasculature is critical in controlling blood pressure, and has been linked to problems in preeclampsia. Sakai et al found preeclampsia to be correlated with increased peripheral resistance and decreased venous distensibility<sup>6</sup>. This study reinforces the theory that preeclampsia is associated with vascular damage.

Evidence also suggests an endothelial cell functional disturbance in preeclampsia<sup>7</sup>. Krauss et al found that midgestation prenatal measurements of specific adhesion molecules, such as ICAM and VCAM (intercellular adhesion molecule and vascular cell adhesion molecule, respectively), could predict pregnancy complications including preeclampsia. This study revealed a maternal inflammatory response to pregnancy and an altered expression of endothelial cell molecules in these women<sup>8</sup>. ICAM and VCAM were found in several studies to be increased in preeclampsia and are believed to be an indication of increased endothelial cell activation<sup>8,9</sup>.

#### i. Oxidative Stress

Maternal endothelial cell dysfunction in preeclampsia has also been linked with an increase in oxidative stress and lipid peroxidation<sup>10,11</sup>. Walsh described this association as an imbalance between an increase in thromboxane levels and a decrease in prostacyclin production, which in conjunction with decreased antioxidants and increased oxidative stress can play a major role in preeclampsia. Thromboxane is also known to be a strong vasoconstrictor, which in the placenta can cause adverse effects and greatly decrease uteroplacental blood flow. Oxidative stress can also cause an increase in thromboxane

synthesis, and a decrease in prostacyclin synthesis. Since prostacyclin is a strong vasodilator, this imbalance leads to a decrease in uteroplacental blood flow. The prostacyclin and thromboxane imbalance could also be responsible for the high blood pressure in preeclamptic patients. In normal pregnancy prostacyclin dominates and in preeclampsia thromboxane dominates.<sup>10</sup>

Evidence also suggests that oxidative stress and lipid peroxides are increased in preeclamptic women. Highly reactive oxygen free radicals interact with polyunsaturated fatty acids to create lipid peroxides whose double bonds react with the oxygen free radicals. Antioxidants counteract both free radicals and the lipid peroxidation process. Normal pregnant women have increased lipid peroxidation, but also increased antioxidants to counteract them. Preeclamptic women, however, have an increase in lipid peroxidation but a decrease in antioxidants creating an imbalance, which may cause the symptoms of preeclampsia. One cause of oxidative stress in preeclampsia may be circulating maternal leukocytes. Neutrophils, for example, generate the superoxide anion causing oxidative stress. They also produce cytokines that increase the inflammatory response.<sup>10</sup>

## ii. Neutrophils

Neutrophils not only are a key component of the host inflammatory response, but they are also the most abundant class of leukocyte. Research has found their activation levels to be elevated in preeclamptic patients, and evidence suggests that neutrophils are one class of leukocyte that plays a role in endothelial cell dysfunction when activated<sup>12</sup>.



Cell dysfunction occurs when the neutrophils attach to the endothelial cell lining and cause lipid peroxidation<sup>10</sup>.

Wang et al found an increase in neutrophil adhesion to human umbilical cord venous endothelial cells obtained from preeclamptic versus normal pregnant women<sup>9</sup>. These investigators also found an increase in cell surface adhesion molecules in the endothelial cells obtained from preeclamptic women. Up-regulation of cell surface adhesion molecules was believed to be the cause for neutrophils to adhere more in preeclamptic cells. Another study found that neutrophils were important for communicating between the placenta and maternal vascular endothelium in preeclamptic women<sup>13</sup>. Increased amounts of neutrophils in preeclamptic women were reported to be due to delayed neutrophil apoptosis in preeclampsia as compared to normal pregnancy. Normal pregnancy showed some apoptotic delay, which is consistent with evidence that normal pregnancy causes some inflammation. The excessive delay, however, in preeclampsia is believed to result in too many activated neutrophils remaining in the circulation, which then causes endothelial activation and dysfunction.

### iii. Lymphocytes and Monocytes/Macrophages

Lymphocytes are another class of leukocytes that are believed to be involved in preeclampsia. Barden et al reported an increase in reactive oxygen species found in lymphocytes in preeclamptic women<sup>11</sup>. Mor et al also reported that lymphocytes are low during normal pregnancy, hinting at a more innate immune response involving

macrophages and neutrophils<sup>14</sup>. Sacks et al used whole blood intracellular reactive oxygen species and surface markers of activation to find evidence for circulating leukocyte activation in preeclampsia. This study found that preeclamptic women had more leukocyte reactive oxygen species activation than normal pregnant women, who subsequently had more activation than normal non-pregnant women. This study suggests that the inflammatory changes and reactive oxygen species found elevated in preeclamptic women are merely an exaggeration of those seen in the normal pregnant women. The intracellular reactive oxygen species measured in this study were found elevated in lymphocytes, as well as granulocytes and monocytes. Monocytes and granulocytes also had increased expression of surface markers activated in normal pregnant and preeclamptic women.<sup>15</sup>

Gervasi et al found that preterm labor, commonly found in preeclamptic births, was associated with maternal granulocyte and monocyte phenotypic and metabolic changes. This study's findings agree with the belief that the innate maternal immune system plays a role in preterm labor, and therefore possibly preeclampsia<sup>16</sup>. Gervasi et al then tested peripheral venous blood and examined the phenotypic and metabolic activity of granulocytes and monocytes<sup>17</sup>. They found an increase in reactive oxygen species, and concluded that preeclampsia is characteristic of phenotypic and metabolic changes in both monocytes and granulocytes. Most studies found that granulocytes and monocytes are activated and producing reactive oxygen species, but lymphocytes can also create them, and therefore could also potentially be responsible for the endothelial dysfunction found in preeclampsia<sup>18</sup>.

Normal pregnancy and preeclampsia are both associated with increased platelet, monocyte and lymphocyte activation which are more extensive in preeclamptic than in normal pregnant patients<sup>19</sup>. This study indicated that pregnancy changes the adherence of platelets to leukocytes by causing increases in the expression of platelet-leukocyte adhesion molecule, P-selectin glycoprotein ligand-1 (PSGL-1). These investigators also found that monocytes were more active in platelet attachment than lymphocytes and, therefore, contribute more to the inflammation reaction in preeclamptic blood<sup>19</sup>.

Mor et al found that macrophages were one of the leukocyte classes also contributing to preeclampsia<sup>14</sup>. One of the functions of macrophages is to rid the body of dead apoptotic cells. This process is critical during pregnancy, especially during preeclampsia, because trophoblast cells slough off the placenta. Macrophages were found to be a source of increased cytokine production in preeclampsia causing a greater pro-inflammatory response<sup>14</sup>. Preeclamptic patients were reported to have an increased level of placental macrophage colony stimulating factor (M-CSF) as compared to normal pregnant patients<sup>20</sup>. Hayashi et al found that an increase in placental M-CSF caused an increase in the number of macrophages, and therefore another potential interaction between the maternal and placental tissues, which could result in vascular and uterine blood flow problems<sup>21</sup>.

Mellembakken et al compared adhesion molecules on neutrophils and monocytes to determine leukocyte activation from venous blood from both the uterus and the forearm of preeclamptic and normal pregnant women. These investigators reported a higher amount of activated monocytes and neutrophils in uterine blood samples than in forearm samples

from preeclamptic women, implicating that leukocyte activation in preeclampsia occurs within the uteroplacental circulation<sup>22</sup>.

### **C. Summary**

In summary, evidence shows a factor cytotoxic to endothelial cells is present in preeclamptic pregnancies<sup>23</sup>. This harmful factor leading to cellular dysfunction in preeclampsia is believed to be associated with activated leukocytes. Leukocyte production of toxic substances, such as ROS and TNF $\alpha$ , are believed to cause oxidative stress and inflammation in endothelial and vascular smooth muscle cells resulting in vascular dysfunction in preeclampsia.

### **D. Purpose of Study**

In this study we compared vascular infiltration of neutrophils with vascular infiltration of monocytes/macrophages and lymphocytes in normal non-pregnant women, normal pregnant women, and preeclamptic women.

### **E. Hypothesis**

We hypothesized that neutrophils are the class of leukocytes mainly involved in infiltrating the vasculature in preeclampsia.

## **F. Significance of Study**

The significance of this study is that by discovering the potential cause of vascular and endothelial damage in preeclampsia, we might discover a way to counteract and prevent the onset of this condition and consequently decrease maternal and fetal mortality rates.

## **Materials and Methods**

### **A. Study Subjects**

Subcutaneous fat biopsies were collected from patients at MCV Hospital at Virginia Commonwealth University Medical Center. The fat biopsies were obtained during cesarean section surgeries for the preeclamptic patients (PE, n = 7) and for the normal pregnant patients (NP, n = 7). Criteria for preeclamptic patients included a blood pressure at or above 140/90 mmHg after 20 weeks gestation in a previously normotensive woman along with a proteinuria level above 300 mg/L or 500 mg/24h. Criteria for the normal pregnant patients included a blood pressure less than 140/90 mmHg, no proteinuria, and no other complications. Fat biopsies from normal non-pregnant patients (NNP, n = 7) were obtained during abdominal surgeries for conditions such as uterine fibroids. The criteria for the normal non-pregnant patients required that they had a blood pressure less than 140/90 mmHg, and that they had no inflammatory conditions. All subjects from each condition were non-smokers. The Office of Research Subjects Protection at Virginia Commonwealth University approved this study, and all patients were consented prior to surgery.

## **B. Collection of Fat**

The subcutaneous fat biopsies taken during surgery were placed in 10% neutral buffered formalin in the operating room. The biopsies were then cut into smaller pieces in the lab and placed in Tissue Tek Cassettes labeled with the patient's initials, date of surgery, and patient condition (PE, NP, NNP). The cassettes were placed in formalin and onto a rotating shaker for five days before being removed from the formalin. The cassettes were opened so that the tissue could be seen and felt to ensure proper fixation. The cassette was then re-closed, rinsed in deionized water (dH<sub>2</sub>O), and placed in a phosphate buffer of pH 7.5. An automated tissue processor was used overnight on the cassettes to prepare the tissue for paraffin embedding. The next morning the paraffin prepared tissue samples were removed from the processor and then from the cassettes to be placed in metal trays. A plastic embedding ring (labeled with the patient initials, date of surgery, and condition) was then placed on top of the metal tray while it was being filled with paraffin. The tray was then placed on ice to cool. Once cooled, the tissue block was removed from the metal tray, and after sitting overnight, the tissue sections were sliced into 8  $\mu$ m sections using a microtome. The tissue sections were sliced into two piece ribbons and put in a 42°C dH<sub>2</sub>O bath before they were placed onto glass slides (Superfrost Plus, Fisher Scientific, Pittsburg, PA) labeled with patient initials, date of surgery and condition. Two tissue sections were placed on each slide and warmed on a slide warmer at 37°C, and then placed in a 37°C oven overnight for firm adherence of the tissue sections to the slides.

### C. Protocol for Formalin-fixed Tissue

Immunohistochemical staining of tissue sections began with three separate passages through HistoClear (National Diagnostics, Atlanta, GA) for five minutes each to remove the paraffin, and then through a hydrating alcohol series for two minutes in each of the following concentrations: 100%, 100%, 95%, 95%, 85%, 50%. The slides were then placed in 100 mM phosphate buffer for ten minutes before being incubated in 0.3% hydrogen peroxide in methanol for 30 minutes to quench endogenous tissue peroxidase. The slides were then placed back in the 100 mM phosphate buffer for five minutes and then into dH<sub>2</sub>O for six minutes. Antigen retrieval was performed on the tissue sections beginning with a five-minute, low-set pressure cooker incubation in 10 mM citrate buffer. After letting the slides cool, they were rinsed in 0.05% enzyme grade tween (FisherBiotech, NJ) in phosphate buffered saline (PBS). The CD14 slides were then incubated for ten minutes with a trypsin solution consisting of one drop 0.5% trypsin to three drops of trypsin liquid diluent (Zymed/Invitrogen, South San Francisco, CA) to enhance antigen retrieval. The CD99, CD66b, and control slides were kept in the 100 mM phosphate buffer while the CD14 slides were being treated with trypsin. After trypsin incubation of the CD14 slides was complete, the slides were rinsed with 0.05% tween PBS. Slides were then incubated for 30 minutes with 100  $\mu$ l of primary antibody. Primary antibodies were: 1) CD14, a mouse IgG anti-human antibody and monocyte/macrophage specific antigen in a 1:50 titer (Zymed/Invitrogen, South San Francisco, CA), 2) CD99, a mouse IgG anti-human antibody and lymphocyte specific antigen in a 1:400 titer (Serotec,



Oxford, UK), 3) CD66b, a mouse IgM anti-human monoclonal antibody and neutrophil specific antigen in a 1:50 titer (BD PharMingen, San Diego, CA), 4) IgM, a negative control rabbit monoclonal isotype for CD66b pre-diluted in phosphate buffered saline (Zymed/Invitrogen, South San Francisco, CA), and 5) IgG, a negative control mouse monoclonal isotype for CD14 and CD99 pre-diluted in phosphate buffered saline (Zymed/Invitrogen, South San Francisco, CA). All slides were then rinsed with 0.05% tween PBS three times at two minutes each before 100  $\mu$ l of horseradish peroxidase polymer conjugate (ready to use SuperPicture kit, Zymed/Invitrogen, South San Francisco, CA) was applied to each section and incubated for ten minutes. The slides were rinsed with 0.05% tween PBS three times at two minutes each before 100  $\mu$ l diaminobenzidine substrate (DAB; SuperPicture kit, Zymed/Invitrogen, South San Francisco, CA) was applied to each section and incubated for five minutes. After this step, the slides were rinsed with 0.05% tween PBS three times at two minutes each. Slides were then immersed in hematoxylin, for counterstaining purposes, for ten seconds and then immediately rinsed with two changes of dH<sub>2</sub>O. The slides were then dipped five times in 0.05% acetic acid in acetone, and placed back into each concentration (50%, 85%, 95%, 95%, 100%, 100%) in the alcohol series for two minutes each to dehydrate the tissue. Two separate clearances through histoclear were performed for three minutes each, and then a coverslip was applied to the slides using Vectamount (Vector Laboratories, Burlingame, CA), dried overnight, and analyzed.

#### D. Data Analysis

The fat biopsies immunohistochemically stained for CD66b, CD99, and CD14 were all analyzed using a microscope (Olympus BH2, Japan) at 400x magnification with a digital camera attached (Olympus QColor5, Canada). The tissue was evaluated for staining in resistance sized vessels and the number of leukocytes stained in each vessel was recorded. Stained vessels were given a visual score ranging from zero to four, with zero corresponding to no staining and four corresponding to intense staining. Staining intensity was also assessed by optical density using analytical software (IP Lab, Scanalytics, Inc., Fairfax, VA). The percentage of vessel staining was calculated by the following equation:

$$\frac{\# \text{ Vessels Stained}}{\# \text{ Vessels}} \times 100 = \% \text{ Vessels Stained}$$

The number of leukocytes per vessel was determined using the following equation:

$$\frac{\text{Total \# Leukocytes Counted per Patient}}{\text{Total \# Vessels}} = \# \text{ Leukocytes per Vessel}$$

#### E. Statistical Analysis

Data were analyzed using Prism 4.0 statistical analysis software (GraphPad Software, Inc, San Diego, CA). Data within each patient group were analyzed using unpaired, two tailed t-tests because CD14 showed no vessel staining. Data across patient groups for each antibody were compared using parametric one-way ANOVA with Newman-Keuls Multiple Comparison post hoc test to determine which means were

significantly different. All data were presented as mean  $\pm$  SE, and a probability of  $P < 0.05$  was considered statistically significant.

## Results

### A. Subjects

Clinical data collected from each subject included the mother's age, pre-pregnancy BMI and blood pressure. The preeclamptic and normal pregnant subjects had the following additional data collected: urinary protein, parity, gestational age and infant birth weight. Patient demographics are displayed in Table 1. Normal non-pregnant patients were significantly older than normal pregnant or preeclamptic patients ( $37.9 \pm 2.8$  vs.  $22.6 \pm 0.7$  and  $24.6 \pm 1.7$  years, respectively,  $P < 0.001$ ). Preeclamptic patients had a significantly higher mean systolic blood pressure than normal pregnant and normal non-pregnant patients ( $172.9 \pm 4.7$  vs.  $114.0 \pm 4.7$  and  $125.4 \pm 5.9$  mmHg, respectively,  $P < 0.001$ ). Preeclamptic patients also had a significantly higher diastolic blood pressure than normal pregnant and normal non-pregnant patients ( $106.2 \pm 3.2$  vs.  $67.3 \pm 2.8$  and  $76.7 \pm 6.3$  mmHg, respectively,  $P < 0.001$ ). Preeclamptic patients delivered at a significantly lower gestational age than normal pregnant patients ( $32.9 \pm 1.1$  vs.  $39.5 \pm 0.3$  weeks,  $P < 0.001$ ) and their babies had a significantly lower birth weight than normal pregnant patients ( $1804 \pm 297.2$  vs.  $3256 \pm 142.3$  grams,  $P < 0.01$ ).

## B. IHC Staining Results

Fat biopsies were evaluated based on the percent resistance sized vessels (10-200  $\mu\text{m}$ ) stained, number of leukocytes per vessel, visual score, and intensity of staining.

NNP had no CD14 vessel staining and no difference between CD99 and CD66b % vessel staining ( $17.0 \pm 5.8$  vs.  $21.3 \pm 4.9\%$ , respectively,  $P > 0.05$ )(Figure 1). NP also had no CD14 vessel staining and no difference between CD99 and CD66b % vessel staining ( $29.8 \pm 7.4$  vs.  $42.6 \pm 8.5\%$ , respectively,  $P > 0.05$ )(Figure 2). PE also had no CD14 vessel staining, but had significantly higher % vessel staining for CD66b than CD99 ( $70.0 \pm 5.8$  vs.  $33.3 \pm 10.8\%$ , respectively,  $P < 0.05$ )(Figure 3).

Figure 4 shows that there was no difference in the average number of leukocytes stained per vessel for NNP between CD99 and CD66b ( $0.6 \pm 0.2$  vs.  $0.7 \pm 0.2$ , respectively,  $P > 0.05$ ). Figure 5 shows no difference in the number of leukocytes stained per vessel for NP between CD99 and CD66b ( $1.1 \pm 0.3$  vs.  $1.8 \pm 0.6$ , respectively,  $P > 0.05$ ). PE, however, had significantly more leukocytes stained per vessel for CD66b than CD99 ( $3.5 \pm 0.9$  vs.  $1.1 \pm 0.3$ , respectively,  $P < 0.05$ )(Figure 6).

Figure 7 shows significantly more CD66b vessel staining for PE than for NP ( $P < 0.01$ ) and NNP ( $P < 0.001$ ). There was also significantly more CD66b staining for NP than for NNP ( $P < 0.05$ ). There was no significant difference, however, among groups for CD99 % vessel staining (Figure 8). Monocytes/macrophages (CD14) were not present in vessels, but were found infiltrated into fat tissue. The number of monocytes/macrophages

per field of view in each tissue biopsy was not statistically different between groups (Figure 9).

CD99 visual scoring showed no differences among groups, as illustrated in Figure 10 (NNP:  $0.2 \pm 0.08$ , NP:  $0.4 \pm 0.1$ , PE:  $0.6 \pm 0.2$ ,  $P > 0.05$ ). CD66b had significantly higher visual scoring results for PE than for NP ( $1.8 \pm 0.3$  vs.  $0.9 \pm 0.2$ , respectively,  $P < 0.01$ ) and NNP ( $0.3 \pm 0.08$ ,  $P < 0.001$ )(Figure 11). There was no difference, however, between NP and NNP groups for CD66b visual scoring. CD66b also had significantly higher optical density for PE than for NP with the average background optical density control being 150.0 ( $179.1 \pm 4.9$  vs.  $165.0 \pm 4.0$ , respectively,  $P < 0.05$ ) and NNP ( $154.1 \pm 3.7$ ,  $P < 0.01$ ), but there was no difference between NP and NNP (Figure 12).

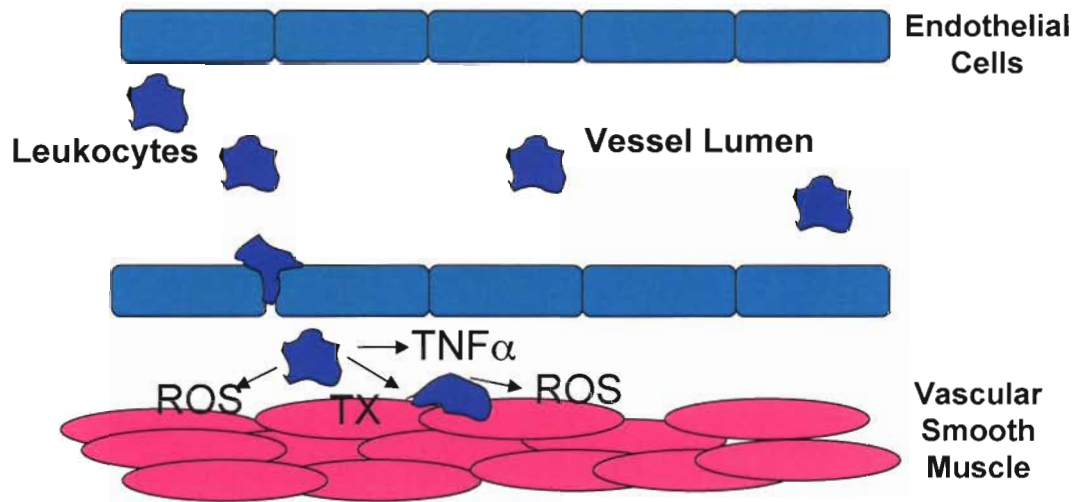


Figure 1. A visual hypothesis of leukocyte activation and infiltration causing endothelial and vascular cell dysfunction.

Activated leukocytes in the maternal circulation infiltrate the endothelial cells and release reactive oxygen species, TNF $\alpha$ , and thromboxane (TX), which cause oxidative stress and vascular smooth muscle dysfunction.

Table 1. Clinical Descriptive Statistics for Patient Groups.

	NNP (n = 7)	NP (n = 7)	PE (n = 7)
Maternal Age, years	37.9±2.8***	22.6±0.7	24.6±1.7
Pre-pregnancy BMI	25.8±0.9	29.7±2.6	29.0±3.1
Systolic blood pressure, mmHg	125.4±5.9	114.0±4.7	172.9±4.7***
Diastolic blood pressure, mmHg	76.7±6.3	67.3±2.8	106.2±3.2***
Proteinuria, mg/24 h	NA	ND	690.0±247.9(n=3)
Protein Urinary Dipstick	NA	ND	3.0±0.4(n=4)
Parity	NA	1.4±0.4	0.6±0.3
Gestational age, weeks	NA	39.5±0.3	32.9±1.1***
Infant birth weight, grams	NA	3256±142.3	1804±297.2**

Values are mean ± SE.

NNP indicates normal non-pregnant; NP indicates normal pregnant; PE indicates preeclamptic; NA indicates not applicable; ND indicates not determined.

\*\*P < 0.01 compared to NP

\*\*\*P < 0.001 compared to other two groups



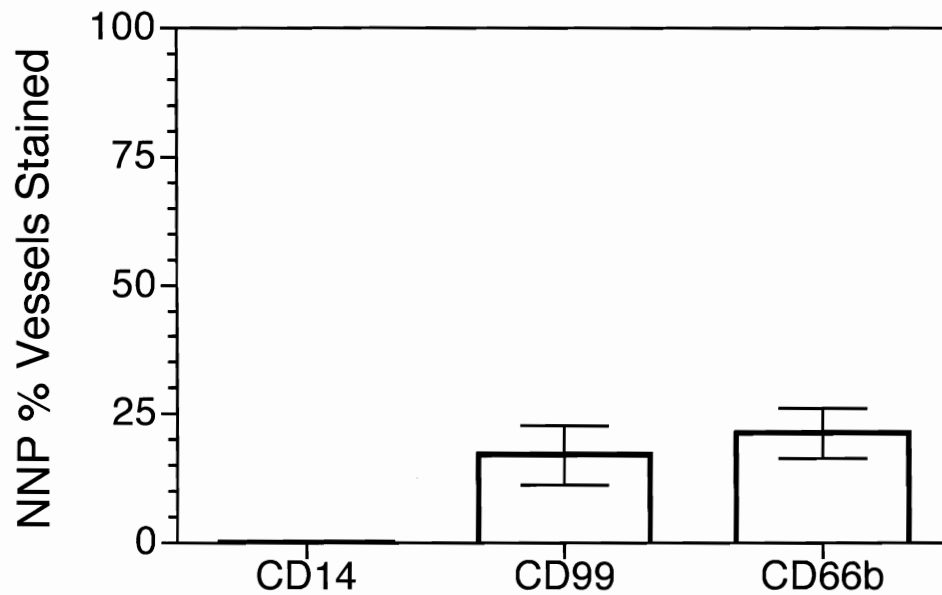


Figure 2. Percent vessel staining of resistance sized vessels for monocytes, lymphocytes and neutrophils in normal non-pregnant women.

There was no CD14 vessel staining, CD99 had a mean of 17% vessel staining and CD66b had a mean of 21% vessel staining. There was no statistical difference between CD99 and CD66b staining ( $P > 0.05$ ). Data represent mean  $\pm$  SE.

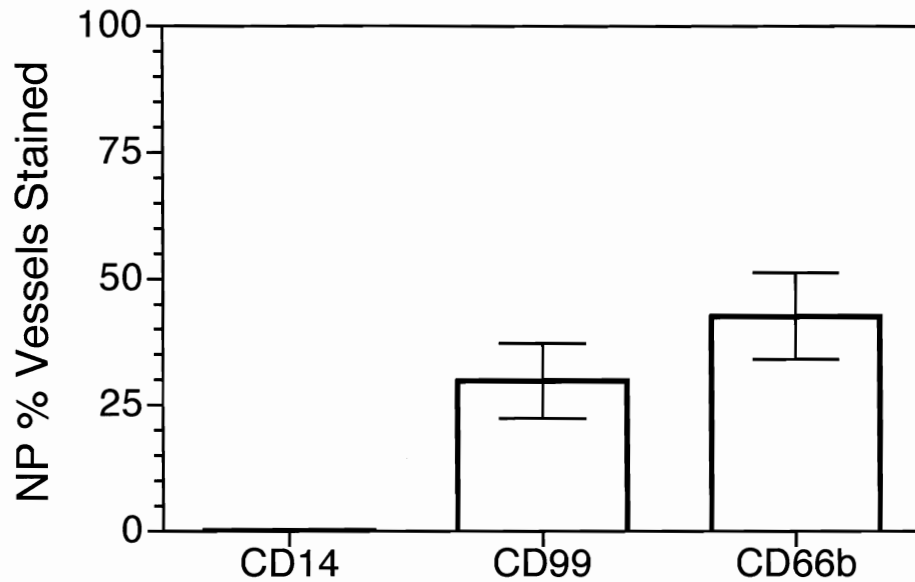


Figure 3. Percent vessel staining of resistance sized vessels for monocytes, lymphocytes and neutrophils in normal pregnant women.

There was no CD14 vessel staining, CD99 had a mean of 30% vessel staining and CD66b had a mean of 43% vessel staining. There was no statistical difference between CD99 and CD66b vessel staining ( $P > 0.05$ ). Data represent mean  $\pm$  SE.

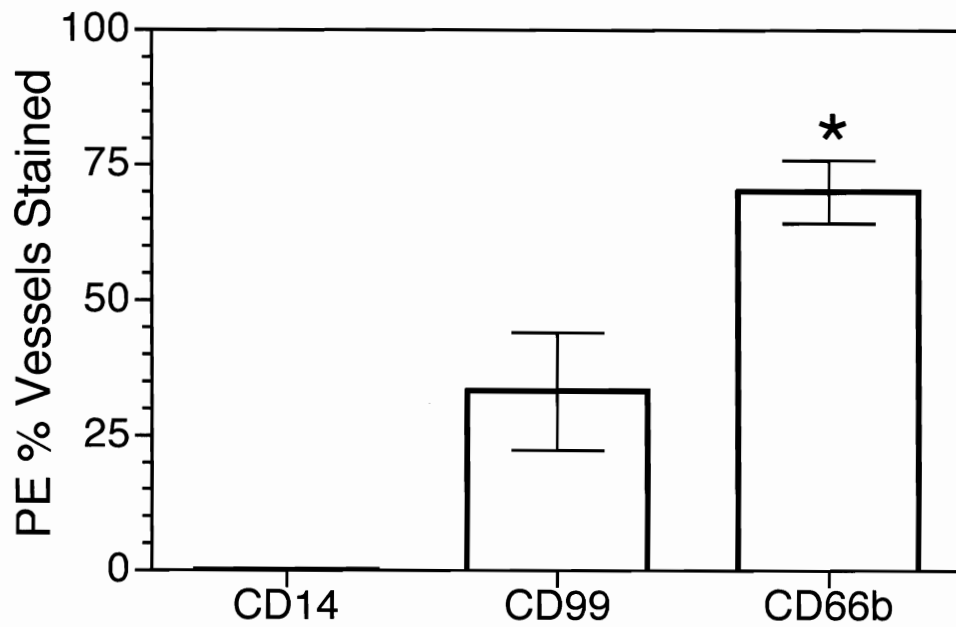


Figure 4. Percent vessel staining of resistance sized vessels for monocytes, lymphocytes and neutrophils in preeclamptic women.

There was no CD14 vessel staining, CD99 had a mean of 33% vessel staining and CD66b had a mean of 70% vessel staining. CD66b vessel staining was significantly higher than CD99 vessel staining ( $P < 0.05$ ). Data represent mean  $\pm$  SE.

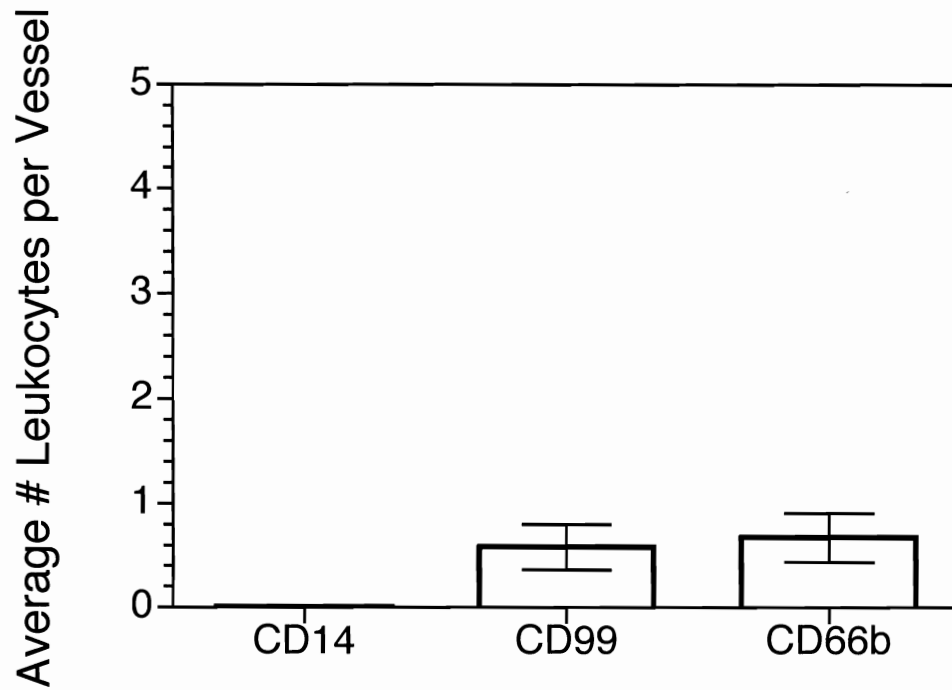


Figure 5. Number of leukocytes per vessel for normal non-pregnant patients.

There was no CD14 staining found within the vessels, CD99 had a mean number of 0.6 lymphocytes per vessel and CD66b had a mean of 0.7 neutrophils per vessel. There was no statistical difference between CD99 and CD66b ( $P > 0.05$ ). Data represent mean  $\pm$  SE.

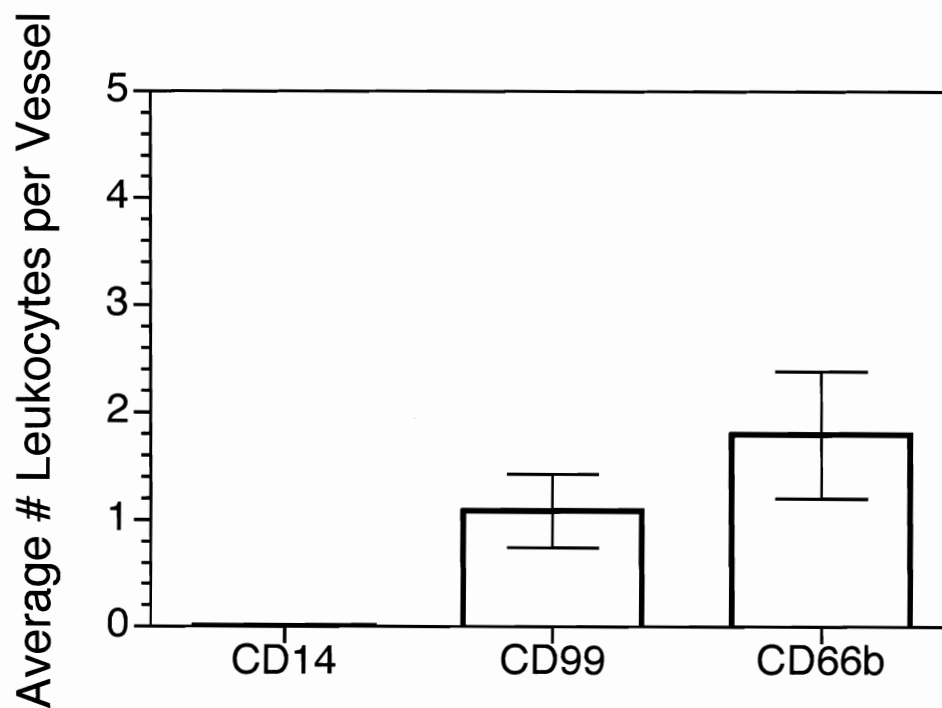


Figure 6. Number of leukocytes per vessel for normal pregnant patients.

There was no CD14 staining found within the vessels, CD99 had a mean number of 1.1 lymphocytes per vessel and CD66b had a mean of 1.8 neutrophils per vessel. There was no statistical difference between CD99 and CD66b ( $P > 0.05$ ). Data represent mean  $\pm$  SE.

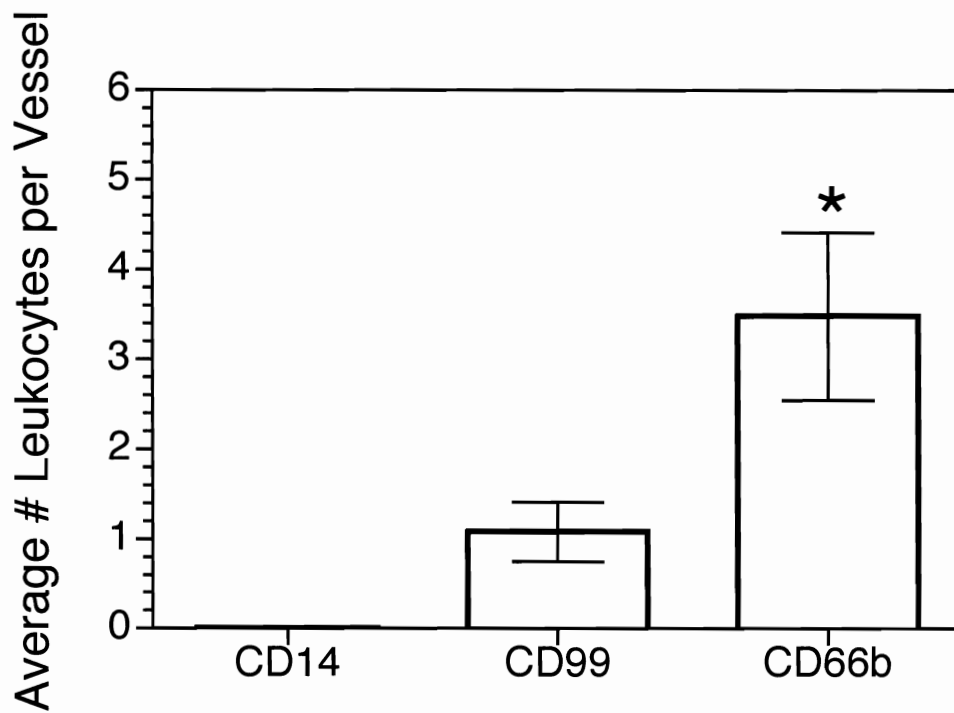


Figure 7. Number of leukocytes per vessel for preeclamptic patients.

There was no CD14 staining found within the vessels, CD99 had a mean number of 1.1 lymphocytes per vessel and CD66b had a mean of 3.5 neutrophils per vessel. There were significantly more neutrophils than lymphocytes stained per vessel in preeclamptic patients ( $P < 0.05$ ). Data represent mean  $\pm$  SE.

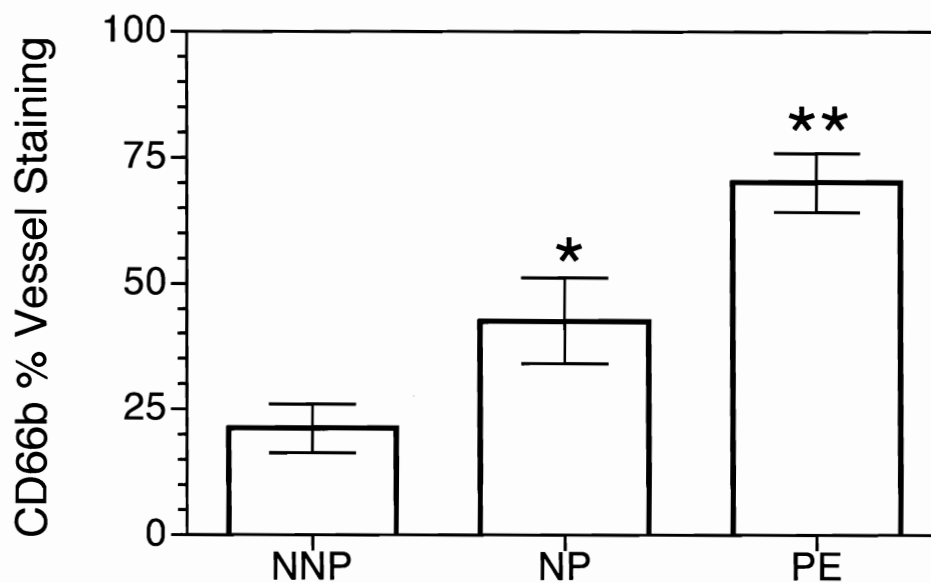


Figure 8. Comparison of CD66b percent vessel staining for NNP, NP and PE.

There was significantly more CD66b % vessel staining for PE than for both NP ( $P < 0.01$ ) and NNP ( $P < 0.001$ ), and there was significantly more CD66b % vessel staining for NP than for NNP ( $P < 0.05$ ). Data represent mean  $\pm$  SE.

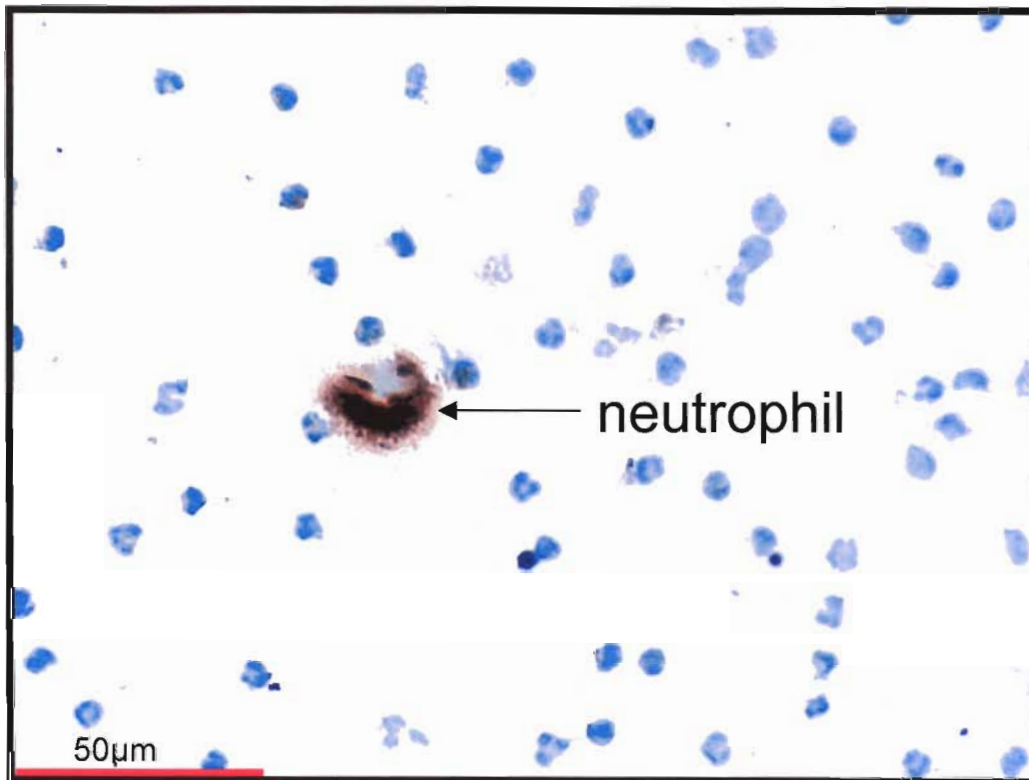


Figure 9. CD66b positive control staining example.

Granulocyte blood smear revealed positive brown staining for CD66b.



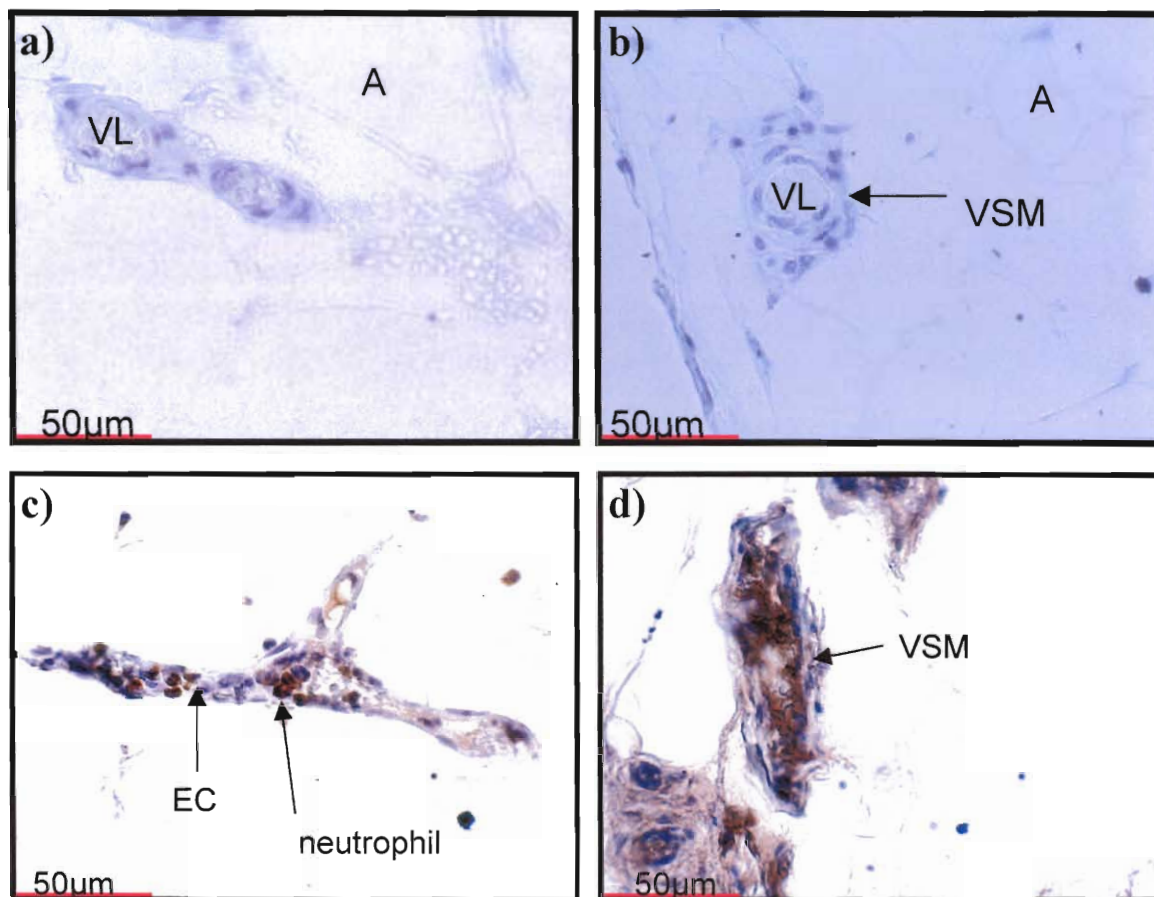


Figure 10. Visual representation of CD66b staining for NNP, NP, and PE patients.

a) IgM negative control, b) NNP with no staining visible, c) NP with minimal staining, d) PE shows intense neutrophil CD66b staining throughout the vessel, with most concentrated on the endothelium and vascular smooth muscle. Magnification x400.

(A = adipocyte, EC = endothelial cells, VL = vessel lumen, and VSM = vascular smooth muscle)

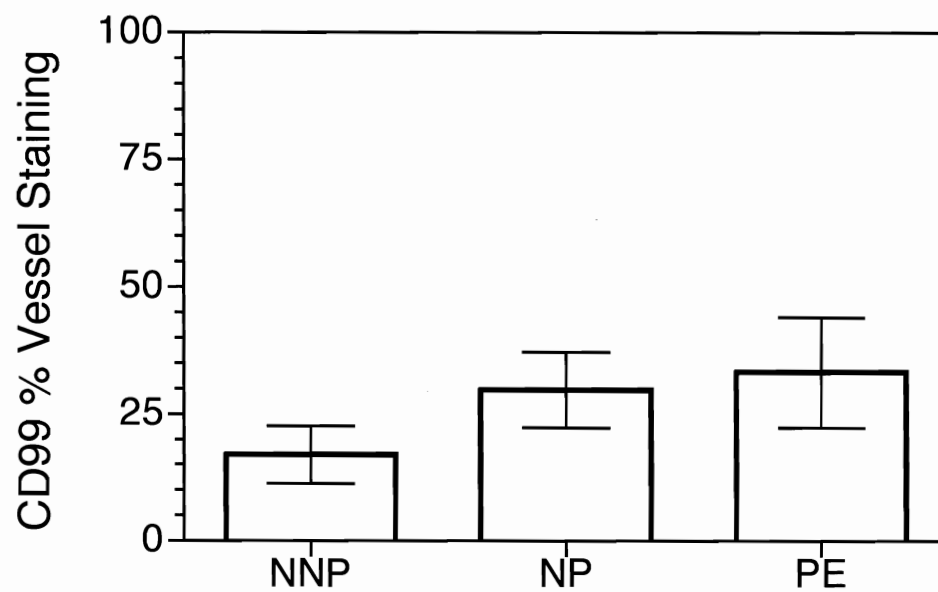


Figure 11. Comparison of CD99 percent vessel staining for NNP, NP and PE.

There were no statistically significant differences among groups for CD99 % vessel staining ( $P > 0.05$ ). Data represent mean  $\pm$  SE.

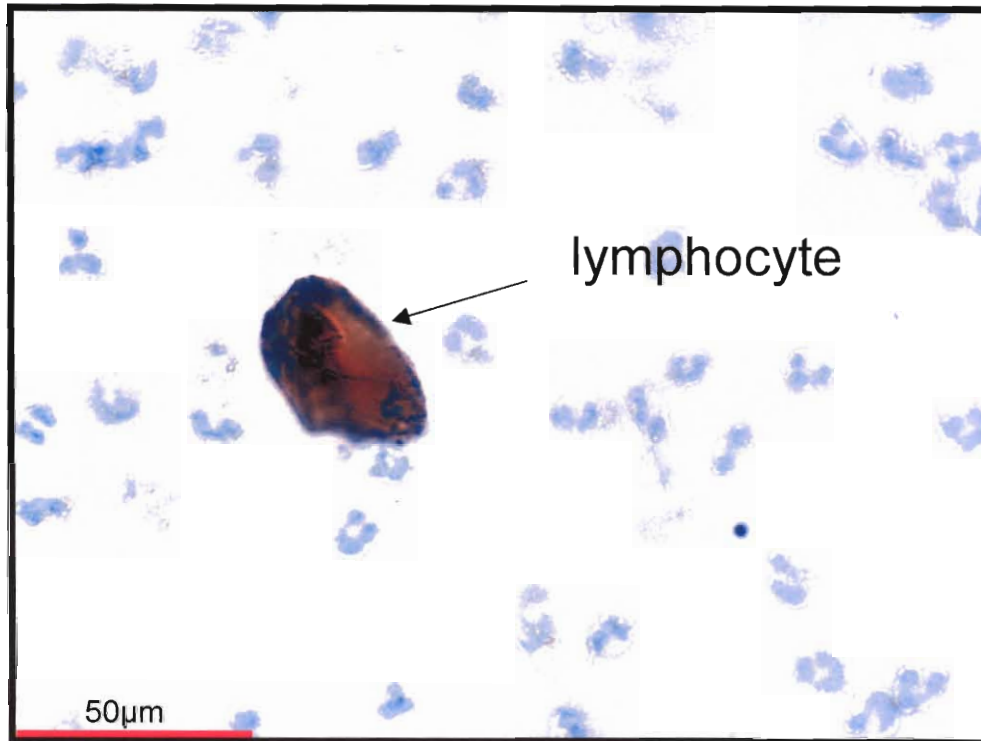


Figure 12. CD99 positive control staining example.

Lymphocyte blood smear showed positive brown staining for CD99.

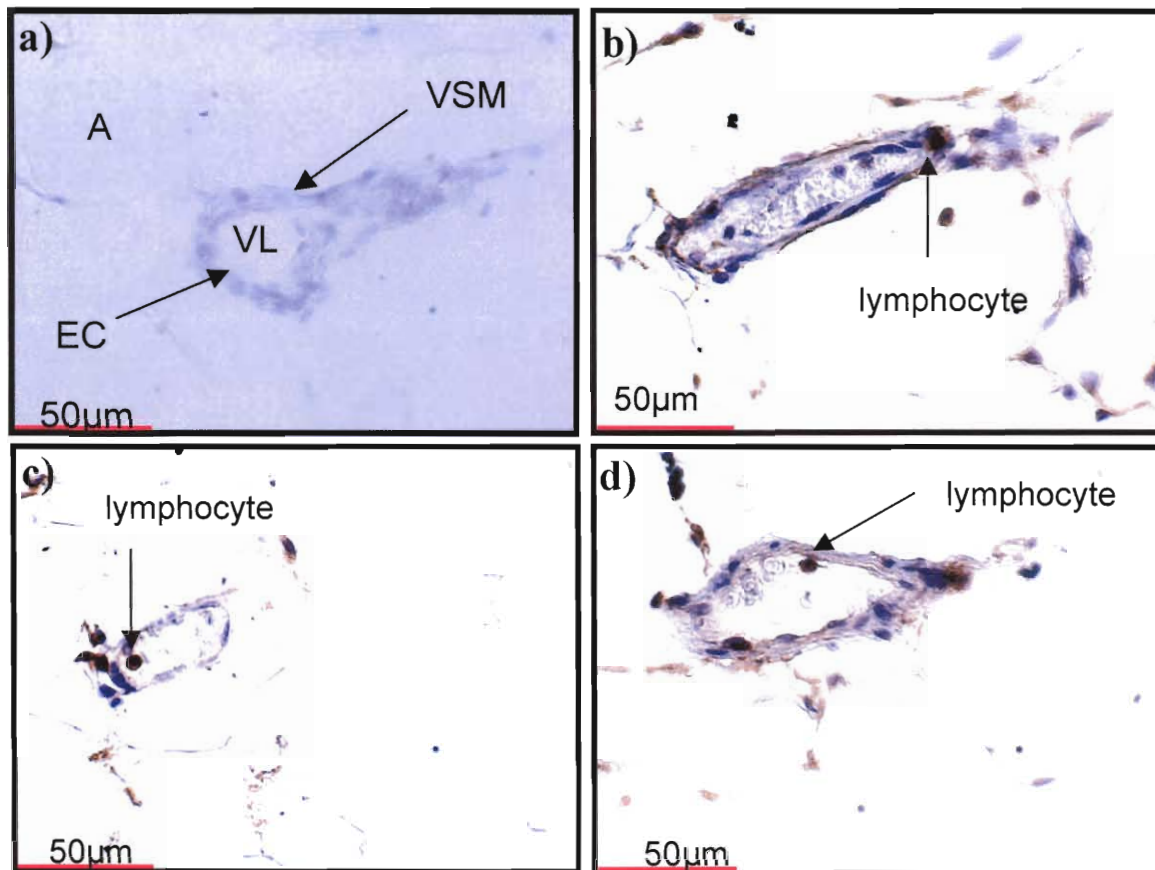


Figure 13. Visual representation of CD99 staining from NNP, NP, and PE patients.

a) IgG negative control, b) NNP with minimal staining visible, c) NP with minimal staining, d) PE shows minimal staining throughout the vessel as well, with most concentrated on the endothelium and vascular smooth muscle. Magnification x400.

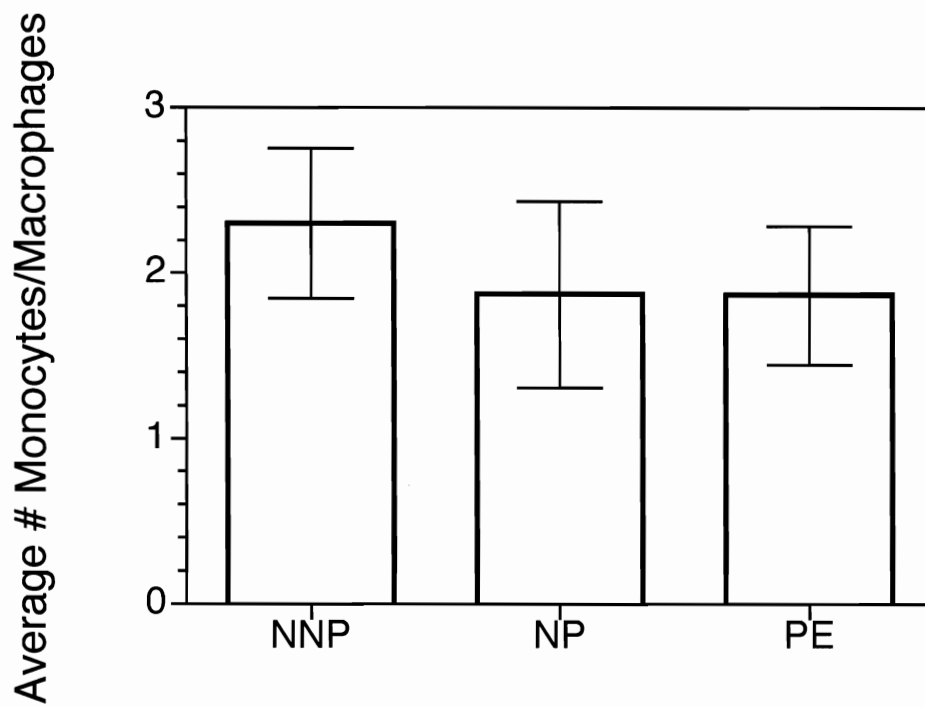


Figure 14. Comparison of CD14 staining per field of view among groups.

There was no CD14 vessel staining. CD14 positive cells were present in fat tissue outside of vessels, but there were no statistically significant differences among the groups when counted per field of view ( $P > 0.05$ ). Data represent mean  $\pm$  SE.

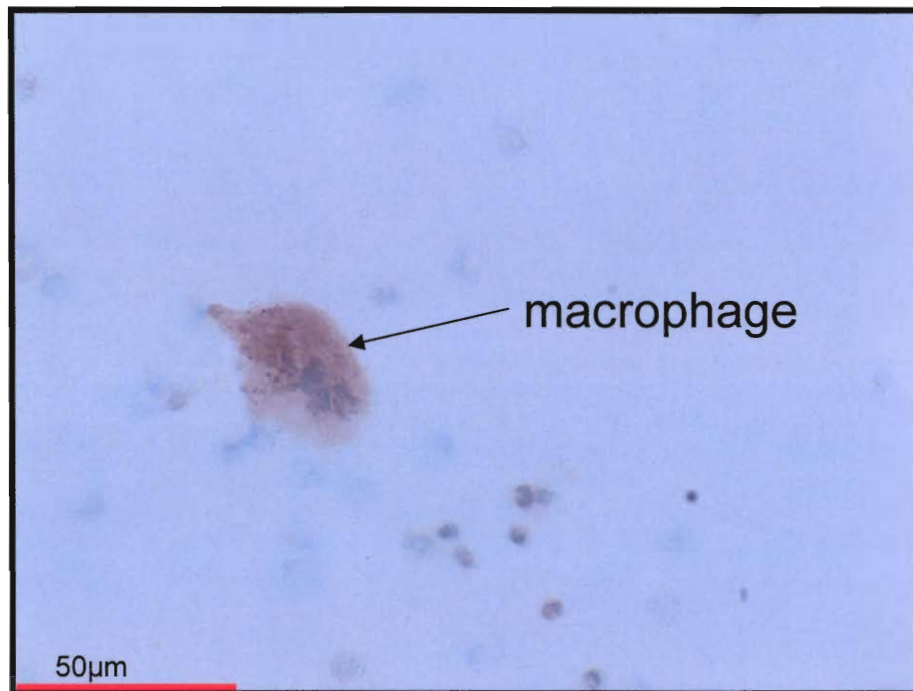


Figure 15. CD14 positive control staining example.

Monocyte blood smear showed positive brown staining for CD14.

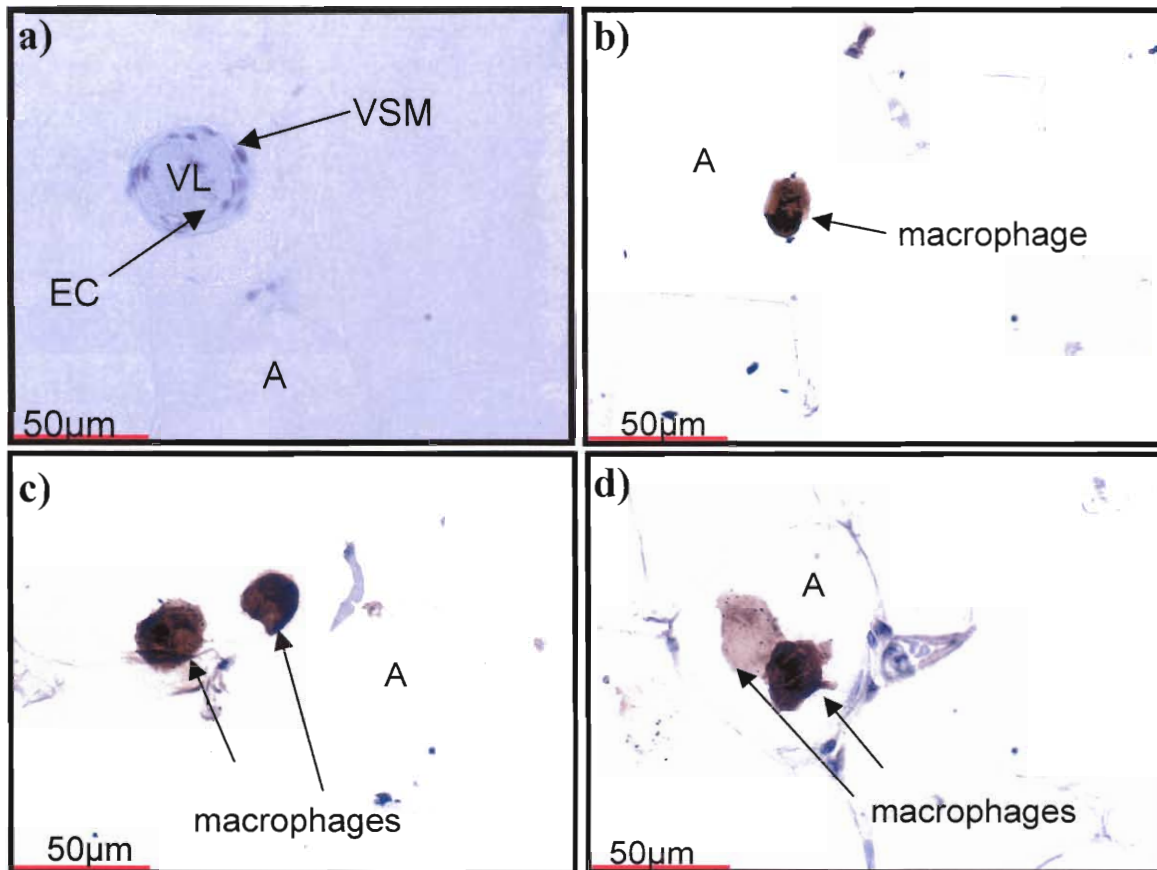


Figure 16. Visual representation of CD14 staining from NNP, NP, and PE patients.

Staining was found outside vessels within the adipocyte cells. a) IgG negative control, b) NNP with minimal staining all found outside in the adipocytes, c) NP with minimal staining, d) PE shows minimal staining as well. Magnification x400.

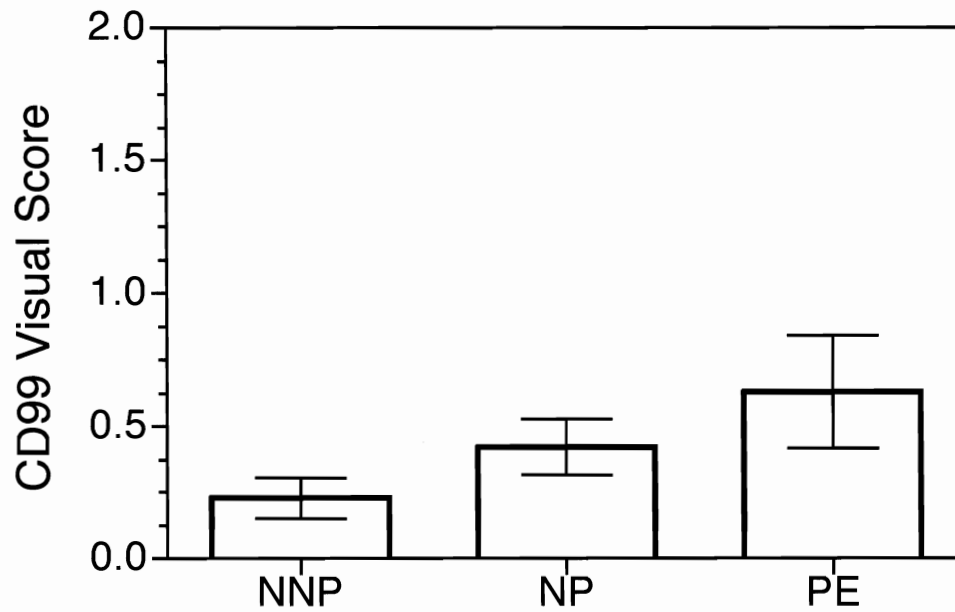


Figure 17. Comparison of CD99 visual score for NNP, NP and PE.

There were no statistically significant differences among groups for CD99 visual scoring ( $P > 0.05$ ). Data represent mean  $\pm$  SE.



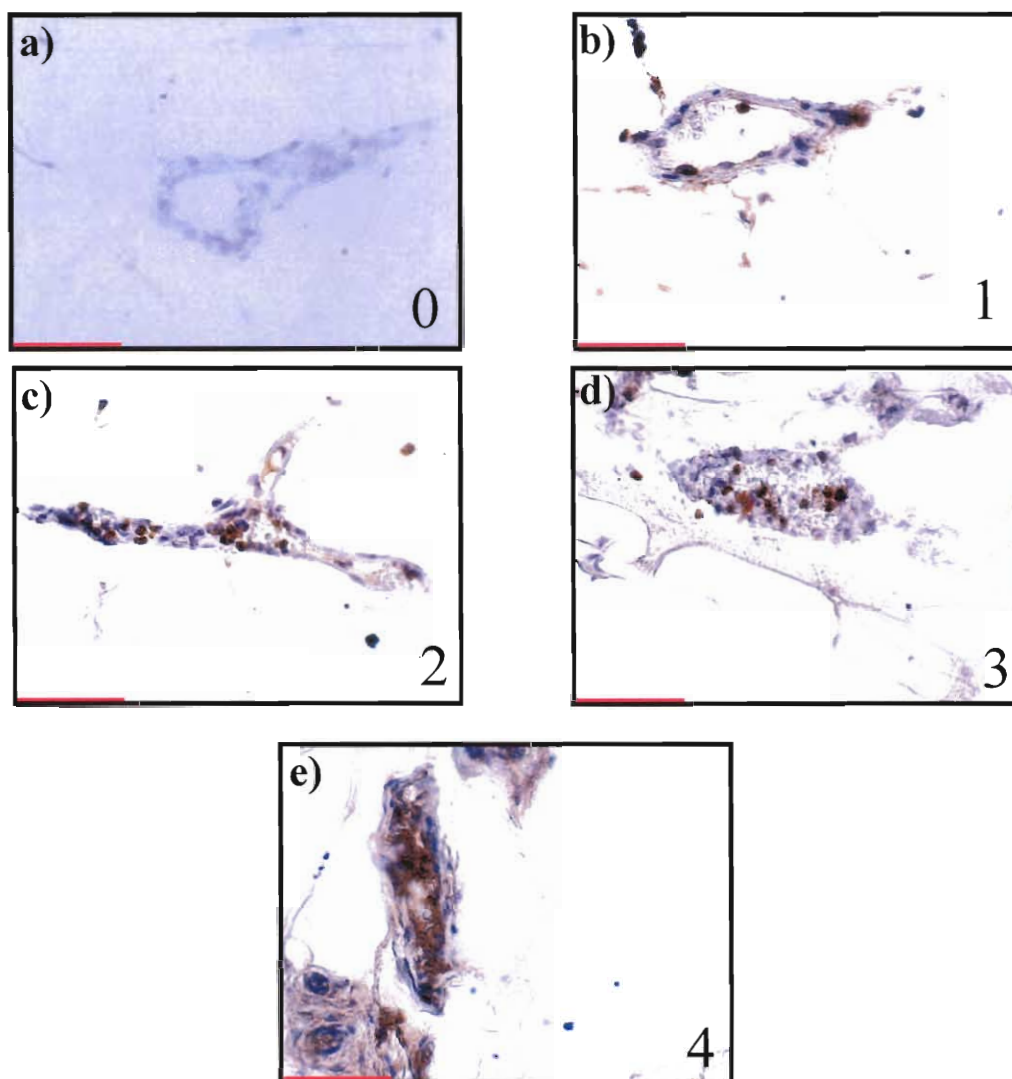


Figure 18. Visual scoring examples.

a) Score of 0, b) score of 1, c) score of 2, d) score of 3, e) score of 4. Magnification x400.

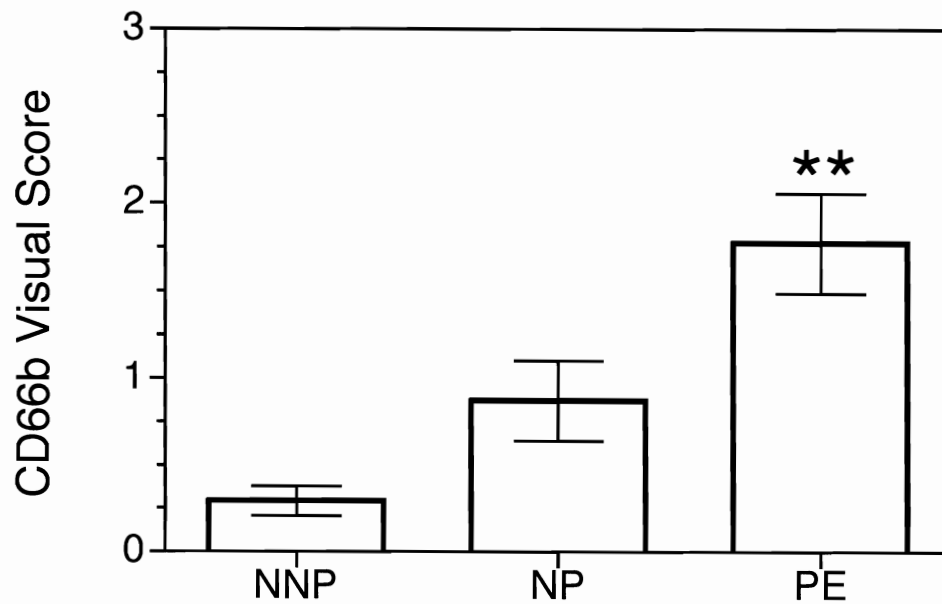


Figure 19. Comparison of CD66b visual score for NNP, NP and PE.

CD66b had significantly higher visual scoring for PE than for NP ( $P < 0.01$ ) and NNP ( $P < 0.001$ ). There was no statistical difference between NP and NNP ( $P > 0.05$ ). Data represent mean  $\pm$  SE.

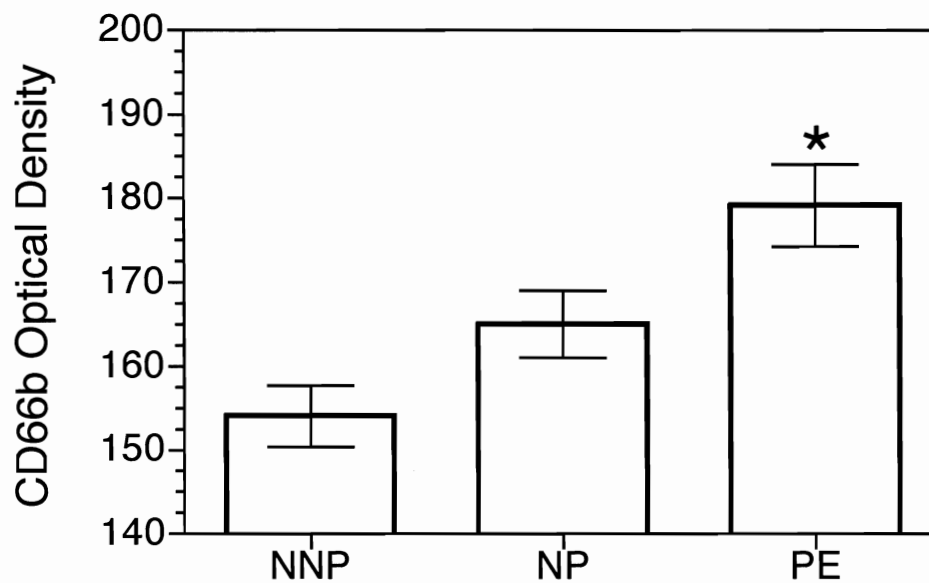


Figure 20. Comparison of CD66b optical densities for NNP, NP and PE.

CD66b had significantly higher optical density for PE than for either NP ( $P < 0.05$ ) or NNP ( $P < 0.01$ ). There was no statistical difference between NP and NNP ( $P > 0.05$ ). Data represent mean  $\pm$  SE.

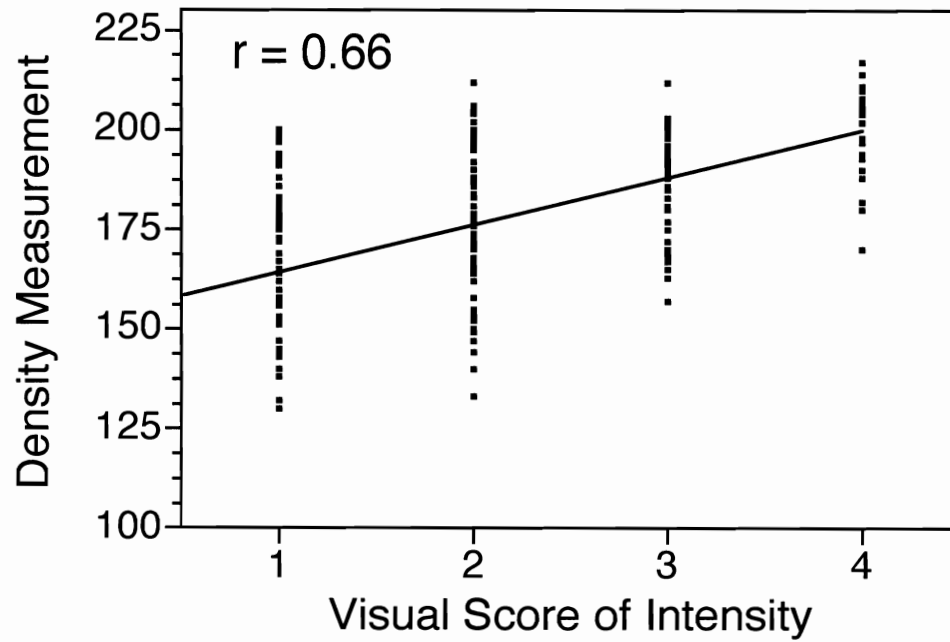


Figure 21. Visual score and density measurement correlation for CD66b staining.

There was a positive correlation with an  $r = 0.66$  for CD66b visual scores and density measurements.

## General Discussion

There was significantly more neutrophil infiltration into the systemic vasculature in preeclamptic women than in normal pregnant and normal non-pregnant women. There was, however, no significant difference in the infiltration of lymphocytes into the maternal systemic vascular system in preeclamptic women as compared to normal pregnant and normal non-pregnant women. Monocytes/macrophages were not found infiltrated into the vasculature at all. These data indicate that neutrophils are the class of leukocyte most likely to cause vascular cell dysfunction in preeclamptic women.

Preeclampsia, still a major cause of maternal and fetal morbidity, has been linked strongly with vascular damage and increased vascular reactivity<sup>24</sup>. Leukocytes, such as neutrophils<sup>25</sup>, lymphocytes<sup>11</sup>, and monocytes/macrophages<sup>14,26</sup> have also been linked with vascular damage and reactivity. Greer et al suggested that neutrophils play a major role in preeclampsia and hypothesized that human neutrophil elastase leads to vascular damage, especially in preeclamptic women<sup>25</sup>. These investigators also found an increase in plasma neutrophil elastase, and white cell activation and degranulation in preeclamptic women compared to normal pregnant women. There was also more plasma neutrophil elastase in normal pregnant than normal non-pregnant women. This study was the first to suggest that neutrophils were activated in pregnancy. Neutrophils also release proteases, which can harm endothelial cells and produce toxic oxygen free radicals leading to membrane lipid

peroxidation, endothelial cell damage and increased vascular permeability and reactivity<sup>25</sup>.

Neutrophil activation is possibly a secondary reaction triggered by maternal immunological mechanisms. Another study determined if superoxide generation by neutrophils was elevated in preeclampsia. They determined that preeclamptic patients had more neutrophil superoxide production than either normal pregnant or normal non-pregnant women<sup>27</sup>, and concluded that this superoxide generation is not just a result of normal pregnancy but stimulated by an activator in preeclamptic women<sup>25</sup>.

One study took plasma samples to determine whether leukocytes were activated in preeclamptic patients' systemic circulation by analyzing neutrophil elastase, monocyte neopterin, and activation of complement-complexes. Complement, neutrophil and macrophage activation were all found elevated in preeclamptic women compared to normal pregnant women. They suggested that preeclamptic damage might be explained by complement induced release of substances from activated leukocytes<sup>26</sup>. Circulating immune complexes were also found elevated in preeclamptic and normal pregnant women<sup>28</sup>. These investigators suggested that circulating immune complexes exist in preeclampsia and that this is immunologically mediated with an inappropriate immune response of deposited immunoglobulin and complement in the placenta. This study also suggested that saturation of circulating immune complexes and surface receptors of polymorphonuclear leukocytes and platelets was hindering their capacity to clear circulating immune complexes and that inflammatory mediators released by the receptor mediated stimulation of leukocytes and platelets could potentially lead to placental vascular damage found in preeclampsia<sup>28</sup>.

Tsukimori et al studied the cytotoxic effect of preeclamptic serum on endothelial cells<sup>23</sup>. They found that sera from preeclamptic women caused more endothelial cell dysfunction than sera from normal pregnant women or normal non-pregnant women. This conclusion was based on increased release of chromium 51 from endothelial cells treated with preeclamptic sera versus normal pregnant and normal non-pregnant sera. Tsukimori et al suggested two possibilities: 1) there is a serum factor cytotoxic to endothelial cells in preeclamptic serum, or 2) serum factors are more concentrated in preeclamptic women therefore causing the cytotoxic effects. They also found that sera from chronic hypertensive women did not cause endothelial cell damage. Therefore, they concluded that cell injury in preeclampsia was not a result of the rise in blood pressure, but rather other factors.

The cause of endothelial damage in preeclampsia is still unknown, but we do know various factors that may contribute to it. Superoxide anions decrease prostacyclin release<sup>29</sup>, while reactive oxygen radicals alter the arachadonic acid pathway to produce more thromboxane than prostacyclin. This imbalance leads to an increase in vasoconstriction<sup>30</sup> and membrane lipid peroxidation<sup>10, 31</sup>. Increased lipid peroxidation, found in preeclamptic patients<sup>32</sup>, could be responsible for endothelial cell dysfunction<sup>33</sup>. It has been suggested that superoxide generation from neutrophils may be an important factor in causing preeclamptic endothelial cell dysfunction<sup>34</sup>.

Sacks et al found an increase in the number of circulating monocytes and granulocytes in normal pregnancy<sup>18</sup>. They suggested that an overactive innate immune response to pregnancy might cause immunological compromise and therefore

preeclampsia, especially if there is an excessive release of placental debris. Redman et al suggested that placental products and debris move into maternal circulation and cause increased leukocyte activation therefore leading to endothelial cell damage and the symptoms of preeclampsia<sup>35</sup>. Redman et al also found that debris clearance from the maternal circulation creates a systemic inflammatory response in normal pregnancy, especially in the third trimester, and suggested that preeclampsia may result from excessive debris or if a mother's response is more severe to the debris<sup>36</sup>. Poor utero-placental circulation, placental hypoxia, or oxidative stress in preeclampsia could cause more apoptosis and necrosis in the placenta and therefore result in excessive debris in the mother's circulation. Apoptosis is critical in cytotrophoblast turnover and chorionic villi surface renewal in pregnancy, and if increased, such as in preeclampsia, there will be more circulating debris which activates a greater systemic inflammatory response and endothelial activation.

Normal pregnancy has been associated with immune responses in a number of studies. Naccasha et al tested whether normal pregnancy activated the innate immune system and induced inflammatory changes in peripheral blood leukocytes<sup>37</sup>. They discovered that there was an increased baseline for intracellular reactive oxygen species and oxidative burst for granulocytes and monocytes in normal pregnant women and acutely infected women compared to normal non-pregnant women. Sacks et al also suggested that normal pregnancy activates the maternal innate immune response to compensate for the adaptive immune response which is suppressed, and that this helps to keep up the host defense mechanism for infection.



Several studies suggest endothelial cell dysfunction plays an important role in preeclampsia. A specific cause for endothelial dysfunction in preeclampsia is still unknown, yet studies suggest that placentation is not solely to blame, but rather other factors contribute, such as activated neutrophils, especially the reactive oxygen species produced by them causing lipid peroxidation and endothelial cell dysfunction<sup>10, 38</sup>. Endothelial cells are activated by numerous factors which could all be relevant to the pathophysiology of preeclampsia such as: free fatty acids<sup>39</sup>, lipoproteins, oxidized lipoproteins or lipid peroxides<sup>40</sup>, tumor necrosis factor  $\alpha$ <sup>41</sup>, fibronectin degradation products<sup>42</sup> and syncytiotrophoblastic microvillous fragments<sup>43</sup>. Redman et al suggests that the placenta leads to some of the factors listed above in causing dysfunction, but that it does not work alone. For example, they suggested that the normal pregnant inflammatory stimulus became excessive in preeclampsia because of placental hypoxia caused by decreased utero-placental blood flow. This then caused increased maternal circulatory inflammatory stimuli, lipid peroxidation and leukocyte activation<sup>38</sup>. Overall, endothelial dysfunction has been suggested to result from systemic maternal inflammatory responses which affect circulating leukocytes<sup>36</sup>.

Granulocytes, lymphocytes and monocytes all produce reactive oxygen species as part of a nonspecific immune defense mechanism, so all of these classes of leukocytes have the potential to cause cell membrane damage. The present study demonstrates that neutrophils are the leukocyte class most likely to cause vascular and endothelial cell dysfunction in preeclampsia. While there is no specific cure for preeclampsia, with more research we can hopefully apply the findings of this study clinically to find better

preventive measurements.

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## Vita

Kristen Anne Cadden was born on July 7, 1982, in Marin County, California, and is an American citizen. She graduated from Marin Catholic High School, California in 2000 and received her Bachelor of Arts in Psychology from the University of Virginia, Charlottesville, Virginia in 2004. She also completed a certificate program in physiology at Virginia Commonwealth University Medical Center in 2005, and subsequently stayed to use this program towards a Master of Science degree in 2006. During this time at the Virginia Commonwealth University, Kristen was a teaching assistant for an undergraduate physiology course, and held the position of secretary for the Physiology Graduate Student Association. Kristen has participated in both the Women's Health Research Day and the Graduate Student Association Research Symposium at Virginia Commonwealth University and has also displayed her research at a national meeting for the Society for Gynecologic Investigation.