

**INFLAMMATION IN DIABETIC WOMEN WITH CARDIOVASCULAR
DISEASE**

By

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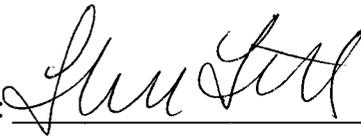


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ABSTRACT

Diabetics have a much greater morbidity and mortality due to coronary heart disease (CHD) than non-diabetics. Furthermore, diabetic women have a 3.8 fold greater risk for CHD compared to diabetic men. Inflammation is now considered a risk factor for cardiovascular disease and also plays a role in diabetes. It is possible that diabetic women with cardiovascular disease (CVD) have a greater inflammatory response and increased interaction between white cells and platelets than diabetic men with CVD or non-diabetic women with CVD. This study tested the hypothesis that platelet-neutrophil conjugates, platelet activation, neutrophil activation, and cytokine production (interleukin-6 (IL-6), tumor necrosis factor (TNF- α), and interleukin-1 (IL-1 β)) are increased in diabetic women with CVD compared to diabetic men with CVD and non-diabetic women with CVD.

Neutrophil activation was assessed by measuring the expression of neutrophil CD11b and the production of Reactive Oxygen Species (ROS). We found that the baseline expression of CD11b and ROS was not statistically different among any of the groups. Platelet activation was quantified by the expression of GPIIb/IIIa and P-selectin. We found that the baseline expression of GPIIb/IIIa was not significantly different among any of the groups. Diabetic women with CVD had a 2 fold greater expression of platelet P-selectin compared to diabetic women without CVD. We also found the platelet-neutrophil conjugate reactivity to platelet activating factor (PAF) was significantly increased by 60% in diabetic and non-diabetic women with CVD in comparison to

diabetic men with CVD. Finally, we found that IL-6 was increased over fourfold in diabetic women with CVD compared to non-diabetic women.

These results indicate that platelets are chronically activated and IL-6 is chronically elevated in diabetic women with CVD compared to diabetic women without CVD and may contribute to thrombosis and the greater severity of coronary heart disease observed in diabetic women. The platelet-neutrophil conjugates may contribute to thrombosis/inflammation and the greater severity of coronary heart disease observed in diabetic women as compared to diabetic men. These aspects of inflammation may indicate one of the processes that exacerbate cardiovascular disease in diabetic women.

CHAPTER 1. INTRODUCTION

DIABETES AND CARDIOVASCULAR DISEASE

Epidemiology of Diabetes Mellitus and Cardiovascular Disease

There are 17 million people in the United States who have diabetes and 95% of those have type 2 diabetes (1). Approximately one third of all cases of type 2 diabetes are undiagnosed and untreated (2). Diabetes has increased morbidity and premature mortality (3). In 1960, 2.6% of adults greater than 45 years old were diagnosed with diabetes. By 1990, 7% of adults greater than 45 years were diagnosed with diabetes (4). This may be due to the increased obesity and physical inactivity. In 1960, 13% of adults were obese and in 1999, the percentage had risen to 27% (5). It is thought that in adding up all the people with diagnosed and undiagnosed diabetes and impaired glucose tolerance (IGT) it totals some 35 million Americans (2). The risk for cardiovascular disease (CVD) is 2 to 8 fold higher in people with diabetes than matched non-diabetic individual. In non-diabetic individuals, the risk of CVD is higher in women than in men (2). Men with diabetes are 2 to 3 times more likely to die from coronary heart disease (CHD) than those without diabetes and the risk for diabetic women is even higher (6-11). According to the American Heart Association, cardiovascular disease was the number one cause of death in women in 2000. In 2000, there were over 945,000 deaths from CVD in the United States. 53% of these deaths due to CVD were in women (12).

Classification of Diabetes Mellitus

Diabetes is a disease in which insulin is not produced or properly used by the body. It is characterized as a chronic metabolic disorder with hyperglycemia and abnormal energy metabolism. Insulin is a hormone secreted by beta cells in the pancreas. This hormone is used by the body to convert sugar and starches to energy. Diabetes is caused by a combination of genetic, autoimmune, and environmental factors. The precise mechanism is unknown but many factors contribute to the development of diabetes including obesity and physical inactivity. There are three major types of diabetes; type 1 diabetes, type 2 diabetes, and gestational diabetes (1).

Type 1 diabetes, previously known as juvenile onset diabetes or insulin dependent diabetes mellitus (IDDM), traditionally affects children and young adults. Approximately 5-10% of people with diabetes have type 1 diabetes. In type 1 diabetes, the body's immune system destroys the beta cells in the pancreas and the patient no longer produces insulin. People initially presenting with type 1 diabetes have three key features; polyuria (excessive urination), polydipsia (excessive thirst), and polyphagia (increased appetite). This deficiency of insulin inhibits the uptake of glucose by the cells in body in turn causing the cells in the body to starve. Since the body is unable to obtain glucose as an energy source it begins to make keto-acids by triggering lipolysis which then releases fatty acid and in turn causes keto-acid synthesis. When the body produces a large amount of ketones the blood's buffering capacity is overwhelmed and the patient can suffer from diabetic ketoacidosis (DKA). DKA is usually the disorder in which

patients with type 1 diabetes present with and is life threatening. Patient with type 1 diabetes are treated with injections of insulin to prevent DKA (1).

Type 2 diabetes, previously known as adult onset diabetes or non-insulin dependent diabetes mellitus (NIDDM), traditionally affected adults but is currently affecting children at an increasing rate. Type 2 diabetes is caused by a combination of genetics, inactivity, and obesity. Type 2 diabetes is characterized by peripheral insulin resistance, impaired regulation of hepatic gluconeogenesis, and a relative impairment of beta-cell function. In the early stages patients have hyperinsulinemia without hyperglycemia which eventually leads to beta-cell failure. The beta-cells are no longer able to compensate for the tissue resistance and the insulin levels are inadequate to maintain the euglycemia. The patient then is characterized by hyperglycemia, which contributes to the long-term microvascular complication of diabetes: retinopathy, nephropathy, and neuropathy. Patients with diabetes are also at risk for macrovascular complications: coronary artery disease, peripheral vascular disease, and strokes. Patients with type 2 diabetes are usually asymptomatic, but can present with polyuria, polydipsia, polyphagia, fatigue and blurred vision (1).

The hyperglycemia that is present in patients with diabetes can be evaluated through testing the glycated hemoglobin. Glycated hemoglobin measures the time-averaged blood glucose of the previous three months. It measures how much glucose adheres (glycates) to the hemoglobin portion of the red blood cell (13). Advanced glycation end

products (AGEs) are a result of advanced protein glycosylation. AGEs accumulate in tissues as a function of time and sugar concentrations. AGEs can cause permanent damage in extracellular matrix components, stimulate cytokine, stimulate reactive oxygen species production, and modify intracellular proteins (14).

Diabetes and Cardiovascular Complications

Cardiovascular disease is the number one killer of patients with diabetes. CVD accounts for up to approximately 89% of the deaths of people with type 2 diabetes (15). The relative risk of death due to cardiovascular events in people with type 2 diabetes is 3 fold higher than in non-diabetics (16). When adjusted for risk factors, the risk rate for increased mortality is 2.4 times greater for diabetic men and 3.5 times greater for diabetic women (16,17).

Kuller et al. found that at baseline, diabetics had a higher prevalence of clinical and subclinical CVD and the risk of clinical events was greater in patients with a history of diabetes compared to patients with newly diagnosed diabetes (18). It was determined that there is a higher prevalence of atherosclerosis in diabetic versus non-diabetic patients (19). Diabetics have an enhanced coagulability putting them at an increased risk for thrombosis formation (20). Glycosylation of proteins could possibly affect the arterial wall physiology and risk of diabetes (21). Elezi et al. determined that patients with diabetes have a poorer clinical outcome at one year after successful stent placement compared to non-diabetic patients. Diabetic patients had a higher incidence of death,

myocardial infarction, reintervention, and increased risk for restenosis (22). Iribarren et al. determined that poor glycemic control (evaluated by the level of hemoglobin A_{1C}) may be associated with an increased risk of heart failure in diabetic patients (23).

Wolfe et al. reported that coronary artery calcification was greater in type 2 diabetics than matched non-diabetics (24). Devereux et al. found that men and women with diabetes mellitus had a larger left ventricular mass and wall thicknesses and smaller left ventricular fractional shortening in comparison to non-diabetic counterparts. They also determined that the pulse pressure/stroke volume and arterial stiffness was higher in patients with diabetes (25).

The link between diabetes and cardiovascular disease is a subject of intense investigation. Possible mechanisms that explain the pathophysiology of ischemic heart disease in diabetes include the atherogenic properties of insulin, abnormalities in lipid metabolism, effects of hyperglycemia, and the disruption in the coagulation system. Diabetic patients also suffer from other risk factors including obesity, advanced age, and/or hypertension (26). Patients with diabetes usually have dyslipidemia including increased very-low-density lipoprotein, decreased high-density lipoprotein (HDL), and increased low-density lipoprotein (LDL) (27,28). The platelets of diabetic patients tend to be activated with increased thromboxane A₂ release and platelet aggregability (29,30). Platelets in diabetic patient have a 50% reduction in the life span (26). Patients with diabetes also have an increase of von Willebrand factor, fibrinogen, d-dimer, thrombin, plasminogen activator

inhibitor-1, and activity of factor VII (26,31). All of these components alter the coagulation status (26).

Cardiovascular Complications in Women Compared to Men

The difference in pathology of diabetes and cardiovascular disease between men and women was neglected for many years, but the importance is finally being recognized. Several reports indicate a worse prognosis for women with CHD than men with CHD (32). The death rate due to cardiovascular disease in the United States was reduced, but the reduction is much less for women than men (12). The absolute number of deaths due to CVD in women is higher than deaths due to CVD in men (12). The estimated costs of CVD in men and women exceeded \$350 billion in 2000 (12). Coronary heart disease (CHD) and stroke is the leading killer of women in the United States. One in two women will eventually die of heart disease or stroke (12). In the Framingham Heart Study, two thirds of sudden deaths due to CHD in women occurred without previous symptoms, thus stressing primary prevention (32). Cardiovascular disease in women presents 10 to 15 years later than in men and one of the hypotheses is that ovarian hormones play a protective role (33).

Diabetes mellitus is an even stronger risk factor for CHD in women than in men. Diabetes is associated with three to sevenfold elevation in CHD risk among women compared with a two to threefold elevation among men (34). Another study demonstrated that the prevalence rate ratio for CHD in individuals with type 2 diabetes

compared to non-diabetics was 4.6 in women compared to 1.8 in men (35). Howard et al. examined different risk factors in diabetic and non-diabetic men and women. They found that waist-to-hip ratios, LDL cholesterol, HDL cholesterol, apoB, apoA1, fibrinogen and LDL size were significantly greater in diabetic women in comparison to diabetic men (36).

CARDIOVASCULAR DISEASE AND MENOPAUSE

Menopause

Menopause is marked by the termination of the reproductive phase in a women's life. At this point almost all of the oocytes have undergone atresia. The term "menopause" denotes the final menstruation and the average age of menopause in the United States is 50-51 years of age. Various physiologic and hormonal changes occur during this period including a decrease in estrogen and increase in FSH and LH. The fall in estradiol leads to symptoms of vasomotor flushing, sweats, mood changes, and depression. There are two pathophysiologic consequences of the decrease in estrogen. The first concerns the cardiovascular system. Once menopause has occurred, the protective benefits of estrogen on the lipid profile and the vascular endothelium are gone. The other pathophysiologic effect is that menopause accelerates bone resorption because estrogen plays an important role in regulating osteoclast activity. The increased bone resorption leads to osteopenia and then osteoporosis (37).

Role of Estrogen in Cardiovascular Disease

After menopause women have an enormous increase in incidence of cardiovascular disease. It is thought that the lack of estrogen plays a significant role in the pathogenesis. Several studies have reported that women who have undergone bilateral oophorectomy are at increased risk of developing cardiovascular disease relative to premenopausal women and women who underwent hysterectomy without oophorectomy (38-40). This indicates that endogenous ovarian hormones possibly have a role in preventing cardiovascular disease (41). The mechanism as to how postmenopausal hormone replacement therapy (HRT) is associated with reduced risk of cardiovascular disease remains obscure.

The beneficial effects of exogenous estrogen on cardiovascular disease include lowering body weight (42-44), increasing HDL cholesterol and lowering LDL cholesterol (42,45-47), lowering fasting insulin and glucose levels (42,46), decreasing blood pressure (48), decreasing insulin resistance (48), and possible regression of atherosclerotic plaques (49). Estrogen also has favorable changes in thrombotic markers such as plasminogen activator inhibitor, fibrinogen, and D-dimer (50). Along with all the good, estrogen also increases the plasma triglyceride by 24 to 67% (51,52). The indirect effects of estrogen include decrease in LDL oxidation. The direct effects of estrogen also include increased cardiovascular production of nitric oxide from the endothelial cells, smooth muscle cells, and myocardial cells (16). Estrogen can also inhibit vascular smooth muscle and myocardial growth and remodeling. Estrogen can influence endothelial cell proliferation

resulting in increased angiogenesis. Another direct effect of estrogen is increased platelet nitric oxide production resulting in reduced platelet aggregation (16).

Estrogen receptor- α and estrogen receptor- β , are expressed on cardiovascular cells, reproductive tissues, bone, liver, and brain (53). Estrogen receptor- α is reported on vascular smooth muscle cells and endothelial cells (54). There are two established mechanisms that estrogen can take to exert their action. One is a rapid, more transient effect that occurs within a few minutes after the exposure to estrogen and is independent of gene expression, including increased dilatation and increased nitric oxide production (54). The other is a longer term effect of estrogen that involves gene expression including increased endothelial cell growth and decreased smooth muscle cell growth (53,54).

The effects of estrogen on lipoproteins, carbohydrate metabolism, hemostasis, and vessel wall tone support a cardioprotective role for estrogen (55). Estrogen's effect on lipoprotein is one of the major benefits of estrogen. When a woman goes through menopause, the low-density lipoprotein cholesterol level increases from 100-120 to 140 mg/dL (56). Postmenopausal women have a higher LDL cholesterol level than age-matched men (57). It was demonstrated in the Postmenopausal Estrogen/Progestin Interventions (PEPI) trial and multiple other studies that postmenopausal estrogen decreases the LDL by about 10% and increased the HDL cholesterol by 10% (45,58,59).

Estrogen's effect on carbohydrate metabolism includes decrease of fasting blood sugar, decrease of fasting insulin, and increase insulin sensitivity (60,61). Salomaa et al. demonstrated that estrogen significantly lowered fasting and two hour insulin levels and fasting blood glucose levels (62). After menopause, there is a progressive increase in insulin resistance (63).

Estrogen also effects hemostasis. Men and women with higher fibrinogen levels have significantly greater frequency of CHD according to one study (64). In the PEPI trial, fibrinogen was increased in patients not taking HRT (45). Marchien van Baal et al. demonstrated in women after 12 months of HRT a 15% decrease in plasma levels of endothelin-1, a 21% decrease in soluble thrombomodulin, a 14% decrease in von Willebrand factor, a 12% decrease in clottable fibrinogen, and a 5% decrease in soluble E-selectin. There was no significant change in endothelium-independent vasodilatation and plasma levels of vWF-propeptide, VCAM-1, fibrinogen antigen, and CRP. This study supports the hypothesis that long-term HRT improves endothelial function and possibly decreases cardiovascular risk (65). It was also demonstrated that estrogen decreases platelet aggregation and increases blood flow by increasing local production of prostacyclin (66).

Estrogen also effects vascular reactivity and tone. Reis et al. determined that intravenous estrogen given to postmenopausal women increases coronary artery blood flow by 23%, decreased coronary artery resistance by 15%, and increased coronary artery cross-

sectional area by 20% (67). Estrogen was also observed to increase the production of prostacyclin in the vascular wall, decrease thromboxane A₂ synthesis by platelets, and increase the production of nitric oxide, causing vasodilatation (55). Studies have suggested that estradiol-17 β may cause vasorelaxation by increasing the release of endothelium-derived relaxing factor (EDRF-nitric oxide) (63). Angerer et al. examined the distensibility of carotid arteries in women given hormone replacement therapy for 48 weeks. They found that estrogen and progestin did not influence the distensibility of central arteries (68). In various animal studies, estrogen replacement was demonstrated to inhibit the development of atherosclerotic lesions (69-71). It was observed in atherosclerotic coronary arteries of premenopausal women that there is a significantly decreased expression of estrogen receptors compared to normal arteries (72). Zanger et al. demonstrated that hormone therapy significantly decreased the serum levels of cell adhesion molecule E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1. It was also demonstrated that CRP and IL-6 were elevated, but not significantly (73).

Estrogen may act as a calcium channel blocker and an antioxidant; both are possibly mechanisms that could protect against CAD (74). In a study by Bodel et al., respiratory bursts following phagocytosis were suppressed when estradiol was present. This study demonstrated suppression in some of the normal response of leukocytes to phagocytosis, i.e. increased respiration, glucose oxidation, and granule lysis, when cells were incubated with estradiol (75). Rosenson et al. determined in a randomized, double blind study,

plasma viscosity was significantly decreased with estrogen replacement therapy. The authors hypothesize that this could be a mechanism in which ERT plays a protective role in postmenopausal women (76). Noriko et al. determined that estradiol replacement at physiological doses in ovariectomized female rats decreased the rate of apoptosis of endothelial cells by approximately 50%. Progesterone did not influence the effects of estradiol on endothelial cell apoptosis (77).

Effects of Hormone Replacement Therapy on Cardiovascular Disease

Many observational studies report a significant reduction of CHD with use of estrogen replacement therapy (ERT) with or without progesterone. A meta-analysis of more than 30 studies determined that the relative risk of CHD for women who used estrogen was 0.65 of the rate for women who never used estrogen (78). Another meta-analysis of the majority of observational epidemiological studies demonstrated a 35% reduction in CHD events among users of ERT alone and similar results for HRT. (79) Another meta-analysis of studies examining the effects of estrogen on cardiovascular disease determined that estrogen therapy had a relative risk of 0.56 (80). Klein et al. determined that women with more than 18 years of endogenous estrogen exposure had a statistically significant 20% decrease in cardiovascular mortality compared with those who had 13 years or less of exposure. Therefore, the age of menopause is related to cardiovascular disease mortality (81).

The Nurses' Health Study (NHS) reported a two-fold increased risk of myocardial infarction (MI) or coronary heart disease death within the first year after initiation of hormone therapy in women with prior MI compared with nonusers of hormones (82). The study determined that for short-term current users the relative risk for major coronary disease was increased 25% compared to non-users. After longer-term hormone use, the rate of second events was lower in current users than in never-users. There was no difference observed between the estrogen alone and the estrogen combined with progestin groups (83). In the 10-year follow-up of the Nurses' Health Study, the relative risk of major coronary disease in women who currently used estrogen was 0.56 compared with women who had never used hormones (84). In conclusion, on the 20 year follow up the relative risk for a second event among current users of hormone therapy was 0.65 compared to women who never used hormones (83). The limitation of all these observational studies include the healthy-user bias, compliance bias, surveillance bias, and healthy-survivor bias (55). It is also thought that women who take HRT are healthier (selection bias). The monitoring and treatment may be more intensive for women taking HRT (prevention bias). Taking a medication every day is significantly associated with survival benefit (compliance bias) (53). In the Healthy Women's Study, women who decided to take hormones had significantly increased HDL, decreased blood pressure, decreased fasting insulin, decreased body weight, decreased alcohol intake, and increased physical activity. These factors may exaggerate the protective effects of estrogen (82).

The Heart and Estrogen/Progestin Replacement Study (HERS) was a large randomized, double blind, placebo-controlled clinical trial of estrogen replacement therapy for secondary prevention of coronary heart disease (incidence include prior history of MI, coronary revascularization, or angiographic evidence of CHD) in postmenopausal women. HERS did not demonstrate a reduced rate of coronary heart disease events after 4.1 years. This study enrolled 2763 postmenopausal women with CHD. The two groups were not significantly different at the onset of the study. The risk of nonfatal myocardial infarction or death from CHD was 52% higher after the first year in the HRT group compared to the placebo. Women taking HRT did have a 14% decrease in LDL cholesterol compared to a 3% decrease in the placebo group in the first year of the study. There was an increase of 8% in HDL cholesterol and 10% in triglyceride in the HRT group compared to 2% reduction in HDL and 2% increase in triglyceride in the placebo group. At the end of the study, there was no significant difference in total mortality between the groups. Women in the HRT group did have a significantly greater number of venous thromboembolic events in comparison to the placebo group at the end of the study. A possible explanation of these unexpected results include inadequate duration of follow-up, adverse effects of the progesterone, estrogen having an early risk and a late benefit, a population too old to benefit from therapy, or that HRT is ineffective in preventing cardiovascular events in women with established disease (59). In results similar to the HERS study, there was a study in which men with documented MI were given estrogen. This design provided an opportunity to evaluate the effects of estrogen as secondary prevention of CVD in men. The relative hazards for 0 to 4 months were 1.58

and 0.96 for 13 to 60 months. These results indicate that estrogen increased cardiovascular events during the first four months but after sixty months, there was no effect of estrogen, positive or negative (85).

The most recent randomized controlled trial, Women's Health Initiative, examined the risks and benefits of estrogen plus progestin in healthy postmenopausal women. They determined that the overall health risks, including CHD and stroke risk, exceeded the benefits from the use of estrogen plus progestin after a 5.2 year follow-up. They concluded that this regimen should not be initiated or continued for primary prevention of CHD (86). An in depth review of all the literature determined that postmenopausal hormone replacement therapy is not indicated for the purpose of cardiac disease prevention (87).

The Estrogen Replacement and Atherosclerosis (ERA) Trial tested the effect of ERT and HRT on the progression of atherosclerosis in postmenopausal women with documented coronary stenosis. This study found no benefit of estrogen and progesterone on the angiographic progression of the disease after 3.2 years of follow-up (88). Angerer et al. determined that 1 year of HRT did not slow the progression of subclinical atherosclerosis in postmenopausal women (89).

Measurement of carotid artery intima-medial thickness (IMT) has proven to be a good marker of subclinical atherosclerotic cardiovascular disease (CVD). An increased carotid

IMT is associated with an increased risk of myocardial infarction and stroke in patients without a prior history of CVD (90,91). Dwyer et al. determined that in women who had undergone bilateral oophorectomy, the intima-media thickness was significantly related to years since hysterectomy and over 90% of the women had a history of hormone replacement therapy use. This finding of increased intima-media thickness in the presence of HRT supports the thought that estrogens do not have a beneficial effect on atherosclerosis (41).

Since it appears that hormone replacement therapy does not have the cardiovascular preventative effect SERMs (selective estrogen receptor modulator) were developed. Gerdien et al. examined raloxifene (a SERM) as an alternative to postmenopausal hormone replacement. Raloxifene exerts the beneficial effects on bone and cardiovascular risk factors and acts as an antagonist on the endometrium and breast tissue. They found that raloxifene lowered the level of LDL cholesterol and fibrinogen and the results were similar to the results of estrogen (92).

Another possibility is that testosterone, not estrogen, is the hormone that is protecting women from cardiovascular disease prior to menopause. Golden et al. took a group of women with no history of HRT and examined the endogenous postmenopausal hormone levels in women with and without significant carotid atherosclerosis. There was no association between atherosclerosis and the levels of estrone, dehydroepiandrosterone

sulfate, or androstenedione. They did find that patients with higher levels of testosterone had lower risk of atherosclerosis (49).

Effects of Cardiovascular Disease on Postmenopausal Diabetic Women

Women with diabetes have a five to sixfold higher risk of CHD compared to non-diabetic women (56,93). The question remains as the effect of hormone replacement therapy on cardiovascular disease in diabetic women. Diabetics are half as likely to be prescribed HRT compared to those in the general population (94) even though there are no adverse effects of HRT on blood pressure and glycaemic control in diabetic women (95,96).

Recent studies on diabetic postmenopausal women have demonstrated an improvement of insulin sensitivity and glycemic control after treatment with estrogen (97). There is also a decrease of LDL and an increase of HDL observed in the same women (97,98). A significant decrease of HbA_{1C} was observed in postmenopausal diabetic women that were treated with estrogen (97,98). The PEPI trial determined that patients taking ERT with or without progesterone had decreased fasting levels of glucose and insulin (99).

In a study by Robinson et al., the question of the effects of hormone replacement therapy on lipid profile of diabetic patients was examined. It was discovered that LDL cholesterol levels were similar between diabetic and non-diabetic women on HRT. It was also demonstrated that the HDL-raising effects of HRT were blunted in diabetic women

compared to non-diabetic women and triglycerides were increased to a greater degree in diabetic women in comparison to non-diabetic women (100).

DIABETES, CARDIOVASCULAR DISEASE, AND INFLAMMATION

Inflammation is now considered a primary cardiovascular risk factor and a recent study of predictors of cardiovascular risk ranks markers of inflammation, such as C-reactive protein, as comparable to markers of cholesterol (101-103). It is known that inflammation mediates the formation of atherosclerotic plaque, the major cause of cardiovascular dysfunction. One of the links between diabetes and cardiovascular disease is inflammation. Hayden et al. hypothesized that type 2 diabetes mellitus is a vascular disease (atherosclerosis) and that NOS, NO, and redox stress, components of inflammation, play a causative role in type 2 diabetes, in turn causing atherosclerosis (104). Vozarova et al. reported that a high white blood cell (WBC) count predicts the development of type 2 diabetes and a worsening of insulin action in Pima Indians. The WBC count was positively correlated with measures of adiposity and fasting plasma insulin concentration (105). Studies by Thorand et al. and Barzilay et al. also found that low-grade inflammation (in particular C-reactive protein) was associated with an increased risk of type 2 diabetes mellitus in middle-aged men and the elderly (106,107). Earl et al. reported that men and women with a higher WBC and sedimentation rate had a greater risk of developing diabetes (108).

Atherosclerosis in Diabetes

Atherosclerosis develops at an earlier age in patients with diabetes and involves the coronary vessels more extensively (13). The endothelial cell layer acts as a barrier to separate circulation factors and the arterial wall. It also functions as an anticoagulant and fibrinolytic surface (109). Factors which are commonly increased in diabetes, like glucose, free fatty acids, lipoproteins, and derivatives of glycation and oxidation, can all damage endothelial cells (110). In the atherogenic process, the adhesion of leukocytes to the vascular endothelium involves many adhesion molecules (vascular adhesion molecule-1, VCAM-1, intercellular adhesion molecule-1, ICAM-1, E-selectin, and P-selectin), which are increased in people with diabetes (111-113). These aid the migration of circulating monocytes into the arterial intima. There they undergo differentiation and activation resulting in a fatty streak. Endothelial cells and macrophages both produce cytokines and growth factors that cause smooth muscle cells to migrate from the media to the intima where they proliferate and form a fibrous cap. Then cell death occurs which results in a large lipid core with necrotic tissue, macrophages, and a thin fibrous cap, which can result in a plaque rupture. Some evidence indicates an increased amount of macrophages in the atherosclerotic lesions of diabetic patients. Possibly this results from an increased recruitment of macrophages into the vessel wall by the higher levels of cytokines (109).

Hyperglycemia adds another complicating factor to atherosclerosis. Hyperglycemia causes an increase of oxidative stress and decrease of nitric oxide, leading to apoptosis

and impaired function of the endothelium. Hyperglycemia causes glycation of most molecules in the arterial wall, which produces AGEs (advanced glycation end products). Then complexes are formed and irreversible substances that are highly injurious to the integrity and function of the vessel walls. This can be done via three pathways; AGE cross-bridges formed between macromolecules, AGE accumulation into the vessel wall via trapping blood components, or by disrupting the cell function that involve receptor and non-receptor pathways. The vasculature is then full of these pathogenic substances that in turn activate local inflammation and hypertrophy. Resting T lymphocytes (CD4, CD8) have AGE receptors constitutively expressed and their activation in the vessel wall may also trigger a low-grade inflammation (109).

Neutrophil Function in Diabetes

An elevated total white blood cell (WBC) count is considered a risk factor for atherosclerosis. WBCs are believed to contribute to vascular injury and atherosclerotic progression (114,115). Chong et al. determined that there was a direct association of WBC count and the incidence of coronary heart disease and stroke and with cardiovascular disease mortality (after adjustment for age, sex, and race) (116).

Neutrophils are a main type of inflammatory cells and once activated they release reactive oxygen species, including hydrogen peroxide, contributing to endothelial damage and cardiovascular disease (117,118). Neutrophils are also known to release cytokines (119). Okouchi et al. determined that hyperinsulinemia increased neutrophil

transendothelial migration in a dose-dependent response and this could then possibly accelerate atherosclerosis (120). Salas et al. determined that in ischemia-reperfusion in diabetic rats, there was a significantly larger amount of leukocyte adhesion and migration (121). Studies have demonstrated that hyperglycemia and AGEs decrease neutrophil function, possibly contributing to the dysfunctional host defense in diabetic patients (122,123).

Both ROS production and the expression of CD11b are linked to diabetes and CVD. Wong et al. determined that AGEs caused a dose-dependent increase of the neutrophil respiratory burst in response to a secondary mechanical stimulus (124). Mohanty et al. demonstrated that glucose stimulated ROS generation by neutrophils (125). Yasunari et al. determined that monocytes and neutrophil oxidative stress was increased in patients with both hypertension and diabetes. They also determined that there was a significant correlation between neutrophil oxidative stress and HbA_{1C} (126). Sampson et al. determined that acute glycemia caused an increase in monocyte Mac-1 (CD11b) expression in type 2 diabetic patients, but there was no significant change in neutrophil expression of Mac-1 after the glucose load (127). Hu et al. demonstrated that at clinical concentrations of insulin, leukocyte CD11b expression is increased, but leukocyte respiratory bursts are decreased (128).

Platelet Function in Diabetes

Platelets are a known component of inflammation. Platelets are known to be the source of various inflammatory mediators including thromboxane A₂ and P-selection (129,130). These inflammatory mediators then can upregulate the complement system or cytokines (131,132). Activated platelets adhere to the endothelium and induce inflammatory responses of the endothelium, which in turn contributes to the early steps of arteriosclerosis (133,134).

Platelet adhesion and platelet aggregation were observed to be increased in diabetes mellitus. Platelet reactivity is suggested as a potential mechanism of the accelerated atherosclerosis and increased incidence of thrombosis observed in diabetes (135,136). Gresele et al. determined that acute, short-term hyperglycemia caused an increased activation of platelets exposed to high shear stress. They hypothesized that hyperglycemia in type 2 diabetes mellitus may cause vascular occlusions via platelet activation (137). Davi et al. demonstrated that in type 2 diabetes there is an increase platelet in vivo thromboxane production. This increase of thromboxane might then mediate the increased fibrinogen binding and aggregation of platelets in patients with diabetes (29). Endler et al. determined that an increased mean platelet volume, which is an indicator of more reactive platelets, places patients with coronary artery disease at an increased risk of myocardial infarction (30).

GPIIb/IIIa is a receptor expressed by platelets when they are activated. Various GPIIb/IIIa inhibitors are an area of active research in preventing complications linked to percutaneous coronary intervention. Various studies have demonstrated that administration of a glycoprotein IIb/IIIa antagonism is effective to prevent ischemic complication of percutaneous coronary intervention in women and diabetic patients. These results are comparable to that of their male counterparts (138-140).

P-selectin was also demonstrated to play an active role in cardiovascular disease and diabetes. The primary interaction between platelets and leukocytes is via the P-selectin receptor on the platelets and the P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes (141-146). Nomura et al. determined that platelet P-selectin expression was increased in diabetic patients (147). Zhou et al. determined that administration of a P-selectin antagonist decreased the accumulation of leukocytes and decreased neointimal formation after balloon injury in diabetic rats (148).

Platelet-Neutrophil Conjugates in Diabetes

The primary interaction between platelets and leukocytes is via the P-selectin receptor (also known as the platelet activation dependent granule external membrane/granule membrane protein 140 (PADGEM/GMP-140) or CD62) on the platelets and the P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes (141-146). Rinder et al. demonstrated that monocytes have a competitive advantage over neutrophils when binding activated platelets (141). Platelets and endothelial cells both express P-selectin.

P-selectin, which is stored in α -granules of platelets and in Weibel-Palade bodies of endothelial cells, is translocated to the cellular surface within 5 to 15 minutes after stimulation with histamine, thrombin, or oxygen radicals (149-153). The adhesion between P-selectin and PSGL-1 is stabilized by binding of Mac-1 (CD11b/CD18) to a counter-receptor on platelets (144,145,154,155). The expression of Mac-1 is a result of tyrosine phosphorylation and mitogen-activated protein kinase activation after PSGL-1 ligation (155-157). The binding of platelets to leukocytes was demonstrated in vitro to cause the expression of pro-inflammatory cytokines, the production of oxidative bursts, upregulate expression of cell adhesion molecules like Mac-1, induce neutrophil activation, and generate signals that promote integrin activation (132,158-161).

Platelet-neutrophil conjugates are observed in various cardiovascular disease states. Neumann et al. determined that after an acute myocardial infarction (AMI) leukocyte-platelet adhesion was increased and remained elevated for at least five days after PTCA. They also determined that binding activated platelets induced IL-1 β release by leukocytes. It was concluded that the platelet-leukocyte conjugates might contribute to inflammatory responses in acute coronary syndromes (132). Morse et al. reported that during cardiopulmonary bypass (CPB), patients have significantly activated platelets (increase of P-selectin) and neutrophils (increase of CD11b) (162). In another study examining CPB, Rinder et al. demonstrated that monocyte-platelet conjugates were increased significantly during CPB and neutrophil-platelet conjugates were increased only slightly (163). Gawaz et al. reported platelet-leukocyte adhesion was increased in

patients with symptomatic coronary heart disease when compared to normal controls. The study also determined that when stimulated, patients with symptomatic coronary heart disease had a hyper-reactive response, possibly indicating an increased risk of an acute thrombotic event (164).

The role of platelet-neutrophil conjugates in ischemia-reperfusion injury was also investigated. Lefer et al. examined the effects of neutrophils and platelets separately and together in causing cardiac dysfunction in perfused rat hearts after ischemia and reperfusion (I/R). It was demonstrated that I/R hearts perfused with neutrophils or platelets alone exhibited a decreased of 10% to 12% of heart functions but when neutrophils and platelets were perfused together, the various heart functions decreased by 50% to 60%. This study also demonstrated that blocking of P-selectin decreased the synergism between the platelets and neutrophils. The possible mechanism of the decrease in cardiac function in this experiment includes neutrophil and platelet aggregates obstructing the flow of the coronary microvessels. Platelets and neutrophils can also release many toxic components, which could then decrease the coronary flow and the cardiac contractility (165). Kogaki et al. found that the presence of activated platelets increased neutrophil adhesion and migration after hypoxia-reoxygenation and that this was inhibited with an anti-P-selectin antibody. They hypothesized that the increased neutrophil adhesion and migration via P-selectin resulted in subsequent neutrophil activation (153).

There is a complex interaction that occurs between platelets and neutrophils. Activated platelets act as stimuli for neutrophils, but platelets may also inhibit neutrophil activation. On the other hand, neutrophils too may inhibit and activate platelets. This cross talk may affect the development of thrombotic and inflammatory diseases (166). Platelets not only play a role in hemostasis but also play a role in inflammation via releasing substances with physiological potential and by interacting with leukocytes and vascular endothelial cells involved in modulation of the inflammatory reaction (167). There are several components released by stimulated platelets that induce neutrophil activation and chemotaxis. For example, platelet factor 4 increases endothelial ICAM-1 (168), and platelet-derived growth factor are chemotactic substances for human neutrophils (169,170). Thromboxane A₂ (also released from platelets) may enhance neutrophil adhesion to the endothelial wall (171). P-selectin, which is responsible for the adhesion of platelets to neutrophils, could possibly optimize the signaling between the two cells (166).

Activated neutrophils produce free radicals, which in turn are potent activators of platelets. Superoxide anion is a strong platelet aggregant and increases platelet's response to thrombin (172,173). Hydrogen peroxide is also a platelet agonist, but is weaker (174). Proteolytic enzymes, which are released from activated neutrophils, cause the exposure of the active fibrinogen-binding site of the GPIIb/IIIa complex resulting in platelet aggregation (175). This evidence of platelet stimulation of neutrophils and neutrophil stimulation of platelets suggests a positive feedback loop leading to further

stimulation and amplification of tissue injury (166). Aziz et al. demonstrated that short term incubations of neutrophils with stimulated platelets causes an increase of oxidative bursts from the neutrophil (131).

There is very little investigation of platelet-neutrophil conjugates in diabetic patients. Kaplar et al. determined there was a significant increase in the number of monocyte-platelet aggregates in type 2 diabetics compared to the control group. They also found a correlation between elevation of postprandial serum glucose levels and platelet-monocyte aggregate formation in type 2 diabetics (176). Another way platelets and leukocytes can form conjugates is via the GPIIb/IIIa receptor. The use of abciximab, GPIIb/IIIa antagonist, was demonstrated to significantly reduce major cardiac events and target-vessel revascularization at 6 months among diabetic patients after stenting (177).

One hypothesis is that platelet aggregation occurring in the atherosclerotic coronary arteries may contribute to acute myocardial ischemia. After coronary artery occlusion and reperfusion, leukocytes then become activated and release oxygen free radicals and proteolytic enzymes. This could then possibly cause endothelial and myocardial injury (178). One oxygen free radical, superoxide, causes endothelial injury, which then results in increased smooth muscle contraction and decreased endothelium contraction (179). The vascular injury from the oxygen free radicals may then cause a decrease of coronary blood flow reserve and coronary reactivity after reperfusion (180,181). Possible physical obstruction of the microvascular bed by activated leukocytes and decreased neutrophil

deformability are possible factors in which leukocytes contribute to vascular injury seen in ischemic coronary artery disease (178,182).

Platelet-neutrophil interaction localizes both cells to the same location and also creates an optimal environment for the exchange of mediators and metabolites (141). Binding of platelets to leukocytes modulates a variety of leukocyte function including cytokine synthesis (183-185), surface expression of adhesion receptors, such as CD11/CD18 (154,186), and oxidative burst (178).

The Role of Estrogen in Humoral and Cellular Immune Response

Females have a stronger humoral and cellular immune response than males. Estrogens stimulate the B cell response and in contrast, androgens and progesterone depress it (187,188). Jose et al. observed that in ovariectomized female mice there was an acceleration of the cartilage breakdown associated with increased production of IL-1 and granulomatous tissue in the cartilage. It was demonstrated that treatment with estradiol and androgen, but not progesterone, reversed the effects (189). Shirai et al. demonstrated in ovariectomized rats that treatment with exogenous ovarian steroids decreased the granulomatous inflammatory process in the lungs (190). Lundgren et al. demonstrated that estrogen treatment decreased the accumulation of exudates in addition to the number of inflammatory cells during the implantation of a foreign object in rats. It was also observed that progesterone increased the accumulation of exudates (191).

CYTOKINES

Cytokines are soluble polypeptides released from cells of the immune system. Pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF- α), interleukin-1 (IL-1), and interleukin-6 (IL-6) are major mediators in the induction of the acute phase response (119).

Function of Interleukin-6

Many vascular cells including endothelial cells, smooth muscle cells, T lymphocytes, macrophages/monocytes, fibroblasts, osteoblasts, adipose tissue, and chondrocytes are also primarily identified with IL-6 production (192-194). The production of IL-6 by all these different cells can be stimulated by IL-1 β and TNF- α (192). Estrogens inhibit IL-6 secretion from different cell types (195).

IL-6 has multiple actions, including promotion of acute phase reactants/proteins, initiating hematopoiesis in particular B and T lymphocyte differentiation, stimulation of fever, and platelet activation (196-201). Nomra et al. observed that IL-6 increased activation via P-selection expression and platelet-derived microparticles (196). Studies by Oleksowicz et al. demonstrated that with human platelets, IL-6 increased agonist-induced platelet aggregation, increased α -granule and thromboxane β 2 secretion, and increased platelet cytoskeletal assembly (202,203).

Function of Tumor Necrosis Factor- α

TNF- α is synthesized by activated macrophages, endothelial cells and the myocardium (204). TNF- α exerts its action by influencing energy balance and is associated with weight loss, hypermetabolism, and increased resting energy expenditure in infectious or malignant diseases (205-207). TNF- α also causes fever, hypotension, acute phase protein response, lipoprotein lipase inhibition, and release of stress hormones (193). It is also thought that TNF- α increases the transendothelial migration of monocytes and promotes the expression of proteolytic enzymes by macrophages and smooth muscle cells within the atherosclerotic plaques (208-210).

TNF- α has a complex relationship with diabetes. TNF- α exerts its action on insulin signaling and lipid metabolism and is proposed as the link between obesity and insulin resistance and possibly type 2 diabetes (211,212). TNF- α is also involved in beta cell damage leading to type 1 diabetes causing insulin resistance (213,214). It was demonstrated that incubating muscle cells and adipose cells with TNF- α results in significant impairment of insulin action (215).

Function of Interleukin-1 β

IL-1 production is increased during acute illness in response to antigen, bacterial toxins, or tissue injury. It responds during illness by causing fever, sleep, neutrophil leukocytosis, production of acute phase proteins, and endocrine changes (193).

Receptors of IL-1 induce the release of secondary substances that cause inflammation and tissue remodeling. IL-1 stimulates the production of prostaglandins and nitric oxide, both of which are highly inflammatory. IL-1 causes production of chemokines that aid the entry of neutrophils, macrophages, and lymphocytes into the tissue (216). In a study that examined mice in which the gene for IL-1 receptor antagonist was knocked out, it was observed that there was a lethal arterial inflammation characterized by transmural infiltration of neutrophils, macrophages, and T lymphocytes. The lesions demonstrated the effects of IL-1 on endothelial cells and smooth-muscle cells. The authors concluded that IL-1 receptor antagonist produced in healthy mice protects them from the effects of interleukin-1 (217).

Cardiovascular Disease and Cytokines

Cytokine activity is implicated in various aspects of the pathogenesis in cardiovascular disease including atherosclerosis (218-220), acute myocardial infarction (221), congestive heart failure (222-226), myocarditis (222,224), and allograft rejection (227). Cardiovascular disease is characterized by a chronic low-level inflammatory process with increased circulating concentrations of pro-inflammatory cytokines (such as IL-6, TNF- α , IL-1 β) (133).

Kanda et al. demonstrated that plasma levels of IL-6 and IL-6R were significantly increased in patients after acute myocardial infarctions, but not in patients with angina pectoris (228). In another study, after acute myocardial infarctions, Pannitteri et al.

observed that IL-6 was increased, but there was no significant rise of TNF- α (229). Yazdani et al. determined that IL-6 was significantly increased up to two fold in patients with unstable angina compared with patients with stable angina. They also found that there was no significant difference between the levels of IL-1 in patients with unstable angina when compared to patients with stable angina and healthy controls. It was determined that one month after percutaneous coronary intervention, there was no significant difference of IL-6 between the unstable and stable angina group and healthy control (230).

The pro-inflammatory cytokines TNF- α and IL-1 β are implicated in the pathogenesis of cardiovascular disease. Cain et al. demonstrated that TNF- α and IL-1 β decreased human myocardial function in a dose-dependent fashion, separately and synergistically, affecting systolic more than diastolic function (231). It was also determined that an elevated level of IL-6 was associated with a poorer prognosis in patients with congestive heart failure (232).

Tsutamoto et al. found TNF- α and IL-6 were increased in patients with congestive heart failure compared with normal subjects (233). Roig et al. determined that TNF- α and IL-6 were increased in plasma of patients with heart failure. In patients with idiopathic dilated cardiomyopathy, a higher serum IL-6 was associated with lower ejection fraction and worse prognosis. The authors hypothesized that IL-6 may contribute to chronic myocardial damage and play an important role in disease progression (234).

A number of studies reported IL-6 to be increased after CABG, valvular surgery, and other cardiac surgery in a number of studies (235-240). Nomura et al. found that levels of IL-6 and IL-1 β were elevated preoperatively prior to vascular surgery. TNF- α and IL-1 β levels returned to normal levels 7 days postoperatively, but IL-6 was still increased after 7 days (196). Suzuki et al. determined that patients with stable angina pectoris undergoing elective coronary angioplasty, IL-6 was significantly increased one and six hours after the procedure (3.6 and 4.4-fold, respectively) in patients that later presented with restenosis compared to patients that did not have restenosis (241).

In opposition to prior studies, Woodward et al. determined that plasma IL-6 levels did not correlate with major cardiovascular risk factors (except age and smoking) or with prevalent cardiovascular disease (242).

Atherosclerosis and Cytokines

The pathogenesis of atherosclerosis involves many different cytokines, particularly the pro-inflammatory cytokines, IL-6, IL-1 β , and TNF- α . Evidence that IL-6 plays an active role in cardiovascular disease is demonstrated in a study by Huber et al. This investigation injected Apo-E-deficient mice with IL-6 or saline. Fatty streak lesions were 1.9-5.1 fold larger in the IL-6 treated mice compared to the saline treated mice. This demonstrated that exogenously administered IL-6 significantly enhanced fatty lesion development in the atherosclerosis-prone, but not in the atherosclerosis-resistant animals.

This suggests that IL-6 likely has an active role in the atherosclerosis disease process (192). It was determined that there were large quantities of IL-6 found in human atherosclerotic plaques, typically in macrophage rich area (243). IL-1 β was also observed in atherosclerotic lesions (218,219).

Nicklin et al. examined mice in which the gene for IL-1 receptor antagonist was knocked out. It was observed that there was a lethal arterial inflammation characterized by transmural infiltration of neutrophils, macrophages, and T lymphocytes. The inflammatory nature of the lesions was consistent with the biologic effects of IL-1 on endothelial cells and smooth-muscle cells. IL-1 receptor antagonist produced in healthy mice protects them from the effects of interleukin-1 (217).

Diabetes and Cytokines

It is understood that cytokines play a significant role in diabetes. There is evidence that inflammation is an initiating factor in insulin insensitivity and pro-inflammatory cytokines are the key linkage between inflammation and insulin insensitivity (211,244-246). Several studies reported an increase in circulating cytokines in patients with diabetes (247,248). This increase in cytokine production could then lead to inappropriate metabolic effects causing arteriosclerosis. IL-6 and TNF- α were significantly increased in type 2 diabetic patients compared to normal subjects in a study by Pickup et al. The diabetic patients were observed to have higher body mass indices, glycated hemoglobin percentage (HbA_{1C}), and plasma total cholesterol and triglyceride concentration. Five of

the twenty diabetic patients in this study had microvascular (including retinopathy and nephropathy) and/or macrovascular disease (including myocardial infarction, angina, peripheral vascular disease, or stroke) and there was no significant difference in comparison to diabetic patients without vascular disease (247). In contrast, Mooradian et al. reported that diabetes mellitus was not associated with significant changes in serum TNF- α or IL-1 β (249).

Bastard et al. observed obese non-diabetic and diabetic patients with high insulin resistance had increased IL-6 and TNF- α in comparison to lean controls. IL-6, not TNF- α , was found to be proportional to insulin resistance and blood glucose. Thus, severe diabetes is associated with increased IL-6. When the obese patients were placed on a very low-calorie diet (VLCD) for a three-week period, the IL-6 decreased significantly in both adipose tissue and serum. TNF- α , however, was unchanged. They found serum concentration of IL-6 and TNF- α were significantly correlated with BMI. The authors concluded that the circulating IL-6 possibly reflected adipose tissue production and insulin resistance (248). Vozarova et al. also reported that plasma IL-6 relates positively to adiposity and negatively to insulin sensitivity (250). A study by Kato et al. determined that IL-6 production was positively regulated by hyperglycemia (251).

TNF- α has a complex relationship with diabetes. TNF- α exerts its action on insulin signaling and lipid metabolism by suppressing expression of the insulin sensitive glucose transporter GLUT-4, insulin receptor substrate-1 (IRS-1), and insulin receptor

(245,252,253). This causes an excessive free fatty acids (FFA) flux from hypertrophied insulin-resistant adipocytes (245,252,253). This mechanism demonstrates a possible link between TNF- α , obesity, insulin resistance, and type 2 diabetes (211,244-246). It was also demonstrated that incubating rat skeletal muscle cells with TNF- α results in significant impairment of insulin action (215).

Estrogens and Androgens and Cytokines

The observation of the role of estrogens and androgens in the expression of different cytokines is evident. Caulin-Glaser et al. investigated whether 17 β -estradiol (E₂) could inhibit cytokine-mediated endothelial cell adhesion molecule transcriptional activation. They determined that E₂ inhibited IL-1 mediated membrane E-selectin, vascular cell adhesion molecule-1 induction, and intercellular adhesion molecule-1 hyperinduction (254). Stein et al. attempted to elucidate how estrogen inhibited IL-6. They demonstrated that the mechanism of IL-6 gene repression by estrogen is different from that of activation of promoters with estrogen receptor binding sites (255).

Dehydroepiandrosterone (DHEA) is a biologically active adrenal androgen and is a predominant source of estrogen in postmenopausal women. DHEA serves as a precursor to estrogen. Haden et al. demonstrated that as DHEA decreased, IL-6 increased with age (256). Cantatore et al. determined that both IL-1 β and IL-6 were significantly increased in untreated women with total hysterectomy and oophorectomy, but no increase in IL-1 β

and IL-6 was observed in patients treated with estrogen with or without progesterone or in patients that did not have a oophorectomy (257).

Many studies were performed on the function of estrogen bone formation, but these experiments still give us information about the interaction of estrogen and cytokines. IL-6 is an essential mediator of bone loss and is caused by loss of estrogen. In a study of ovariectomized mice, estrogen's inhibition of osteoclast formation is mediated by estrogen's direct inhibition of IL-6 gene expression (258). In a study on rodents, oophorectomy and orchidectomy resulted in an increased IL-6 production by bone marrow stromal cells. They also demonstrated an increase in osteoclastogenesis and a decrease in bone density. Administration of estrogens and androgens prevented these events (259,260). Animal studies have demonstrated that ovariectomized mice did not develop osteoporosis if an antibody to IL-6 was administered (258,261).

Bellido et al. determined that both testosterone and dihydrotestosterone inhibited IL-6 production of bone marrow cells. They also determined that orchidectomy in mice caused an increase production of osteoclasts in the bone marrow which could be prevented by androgen replacement or administration of an IL-6 neutralizing antibody. They determined that in IL-6 deficient mice, orchidectomy did not increase the osteoclast number. This evidence indicates that IL-6 increases osteoclastogenesis and that male sex hormones can inhibit the expression of IL-6 gene (262).

Bismar et al. determined that IL-6 secretion is significantly increased in human bone marrow after menopause or after discontinuation of estrogen replacement therapy. It also determined that IL-1 β and TNF- α were higher in patients after menopause or after discontinuation of estrogen replacement therapy, but there was not significance (241). In opposition to the other data, a study by Kassem et al compared the bone marrow plasma and conditioned medium *in vitro* levels of IL-1 β and IL-6 in post-menopausal women on estrogen replacement therapy and untreated women and found no significant difference between the two groups (263). In conclusion, estrogen decreases cytokine levels, but this response has not been investigated in diabetic women.

SUMMARY

The objective of this research project is to test the hypothesis that chronic inflammation contributes to the severity of cardiovascular disease in diabetic women. Very little is known as to why there is a 3.8 fold increased risk of cardiovascular disease in diabetic women compared to diabetic men. Of particular interest is the inflammatory response and its effect on cardiovascular disease. The inflammatory response complicates cardiovascular disease and plays a role in diabetes. It is now known that diabetic men exhibit increased inflammation as demonstrated by our lab (264). Since inflammation amplifies ischemia-reperfusion injury, it is important to investigate if this complication exists in diabetic women. Inflammatory response will be defined as the activation of neutrophils, the activation of platelets, the increased interaction between platelets and neutrophils and the increased production of cytokines.

Aim #1. To investigate the severity of neutrophil and platelet activation in diabetic and non-diabetic men and women with cardiovascular disease.

Aim #2. To investigate neutrophil-platelet conjugates in diabetic and non-diabetic men and women with cardiovascular disease.

Aim #3. To investigate pro-inflammatory cytokines, IL-6, TNF- α , and IL-1 β in diabetic and non-diabetic women with cardiovascular disease.

In men with type II diabetes, our lab found that there is an increased neutrophil ROS (reactive oxygen species) production, which indicates an increased activity. There are also increased platelet-leukocyte interactions, which may further amplify ROS production (264). These factors may cause microvascular and organ injury in the setting of ischemia-reperfusion in the heart (265). Platelets are known to mediate the activation of leukocytes. Our lab found that when the platelet and neutrophil interaction is inhibited, ROS production is decreased (266). These experiments were performed in men, but very little is known about the cell-mediated inflammatory response in diabetic women. Activation of inflammation may be a result of many factors, but one such factor is the release of cytokines. It is known that the majority of type II diabetic patients are obese. With obesity and the hypertrophy of the adipose cells, there is the release of IL-6. The release of IL-6 is increased by IL-1 β and TNF- α (244). All of these factors mediate an inflammatory response and are expected to be increased in diabetic patients. Examining a panel of cytokines, IL-6, IL-1, and TNF, and determining the difference in diabetic

versus non-diabetic and female versus male patients may lead to a better understanding as to why inflammation is increased in diabetics. This information would initiate the development of a model of inflammatory activation in diabetic patients and may lead to more effective treatments of the inflammatory response.

CHAPTER 2. NEUTROPHIL AND PLATELET ACTIVATION IN DIABETIC WOMEN AND MEN WITH CARDIOVASCULAR DISEASE

INTRODUCTION

Diabetes poses a significant risk factor for cardiovascular disease in both men and women, but the incidence of Type 2 diabetes is markedly greater in women (36). It is known that diabetic women have a significantly greater risk and severity of cardiovascular disease than diabetic men. Diabetic women have an elevated base line risk, and have more cardiovascular risk factors than men (48). From the Framingham Heart Study, diabetic women have an age-adjusted risk ratio of 3.8 for coronary heart disease (CHD) compared to diabetic men. Despite the importance of complicating factors in the severity of heart disease in diabetes, little is known about the interrelationships of risk factors and cardiovascular disorders in diabetes, particularly in women. Of particular interest is the inflammatory response and its effect on cardiovascular disease. The inflammatory response complicates cardiovascular disease and plays a causative role in diabetes (264). Since inflammation plays a role in diabetes and CVD, it is important to investigate this complication in diabetic women and if there is a difference between diabetic women and diabetic men or non-diabetic women.

An enhanced inflammatory response may contribute to the greater recurrence of myocardial infarction and ischemic heart failure in diabetes. From recent ischemia-reperfusion studies, it appears that diabetic rats suffer excessive reperfusion injury

following ischemia (267). Neutrophils accumulate in the coronary microcirculation following ischemia, amplifying reperfusion injury in diabetic rat hearts (265,268). In male, diabetic, heart patients it was determined that there is a chronic increase in neutrophil ROS (reactive oxygen species) production. Activated platelets and their interaction with leukocytes may influence this injury. Diabetic platelets demonstrate enhanced aggregation and an increase in coagulation (264). These experiments were performed in men, but very little is known about the cell-mediated inflammatory response in diabetic women, or how it compares to the inflammatory response of men.

The aim of this study was to investigate the severity of neutrophil activation and platelet activation in diabetic men and diabetic women with CVD. For this purpose, we used whole blood samples obtained from diabetic women and diabetic men and analyzed the blood using a flow cytometer. We evaluated the expression of CD11b on neutrophils and the ROS production in neutrophils. We also examined the expression of GPIIb/IIIa and P-selectin by platelets. We examined all of these components in non-diabetic women without cardiovascular disease, non-diabetic women with cardiovascular disease, diabetic women without cardiovascular disease, diabetic women with cardiovascular disease, non-diabetic men with cardiovascular disease, and diabetic men with cardiovascular disease. We found that the baseline expression of CD11b and the baseline production of ROS were not statistically different among any of the groups. We found that the baseline expression of GPIIb/IIIa was not significantly different among any of the groups, but diabetic women with CVD had a greater expression of platelet P-selectin compared to

diabetic women without CVD. Platelet activation may contribute to thrombosis/inflammation and the greater severity of coronary heart disease observed in diabetic women.

RESEARCH DESIGN AND METHODS

Subjects

Four groups of women were studied; non-diabetic women without cardiovascular disease (NDW-CVD) (n=24), non-diabetic women with cardiovascular disease (NDW+CVD) (n=27), diabetic women without cardiovascular disease (DW-CVD) (n=20), and diabetic women with cardiovascular disease (DW+CVD) (n=27). Two groups of men were studied; non-diabetic men with cardiovascular disease (NDM+CVD) (n=8) and diabetic men with cardiovascular disease (DM+CVD) (n=18). These patients were enrolled from the patients attending the Cardiology Clinic at the Veterans Administration Hospital (Tucson, AZ) and the Diabetes Clinic at the University of Arizona Medical Center (Tucson, AZ). The protocol was approved by the University of Arizona Institutional Review Board. Patient's characteristics are summarized in Table 2.1 and their medications are summarized in Table 2.2. Fifteen milliliters of blood were drawn in a citrate vacutainer from all the patients. The blood was processed immediately to minimize any artificial activation. The severity of diabetes was determined by the patient's hemoglobin A_{1C}. A subject was considered to have cardiovascular disease if they had a previous cardiovascular event (myocardial infarction, CABG (myocardial

infarction, coronary artery bypass graft (CABG), stroke, percutaneous coronary intervention (PCI), or some combination).

Neutrophil Activation

Neutrophil activation was determined according to the method of McCarthy and Macey (269). In this procedure, leukocytes were identified by gently mixing citrated whole blood with an equal volume of a solution containing LDS-751 (Molecular Probes). The whole blood/LDS mixture was divided into 250 μ l aliquots in round bottom polypropylene tubes (Falcon 2052, Becton Dickinson). The expression of CD11b and the intracellular production of ROS were measured. CD11b adhesion molecule expression was measured by incubating saturating concentrations of a FITC (fluorescein isothiocyanate)-conjugated antibody against CD11b (Serotec) with the whole blood/LDS mixture for 15 minutes in a 37°C water bath (Precision Instruments). For measurement of ROS, aliquots of whole blood/LDS were incubated with the intracellular ROS fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA, 80 μ M, Molecular Probes) for 15 minutes in a 37°C water bath. After incubating all samples for 15 minutes, some of the blood samples were stimulated with inflammatory mediators PAF [10⁻⁷M] (Platelet Activation Factor, a cytokine) and fMLP [10⁻⁷M] (formyl-Methyl-Leucylphenylalanine, which mimics a bacterial infection) and were incubated for an additional 10 minutes at 37°C. After 25 minutes of total incubation, samples were diluted

with 4mls of ice-cold PBS (Phosphate-buffered saline), kept on ice, and covered until analysis.

All samples were analyzed by flow cytometry (Becton Dickinson FACScan Clinical Flow Cytometer). A 488-nm argon laser light was used for excitation and fluorescence emission was detected as forward scatter (FSC), which measures the cell size, and side scatter (SSC), which measures cell granularity. The fluorescence emission was detected in FL1 (FITC Anti-CD11b or DCF) or FL3 (LDS-751). A threshold fluorescence was set on the LDS-751 signal and this allowed event collection from leukocytes without the interference from erythrocytes. FSC and SSC were then used to gate the neutrophil population of the leukocytes and this allowed data acquisition of only the neutrophils, a specific subpopulation of leukocytes. Data was acquired and stored in list-mode and 5,000 events were collected. The data was analyzed with WinMDI 2.8 Flow Cytometry Analysis Software. Data was expressed as total fluorescence intensity (TFI = % of positive events x mean channel of fluorescence).

Platelet Activation

Platelet function, in the stimulated and unstimulated states, was also determined and the protocol for platelet labeling in whole blood was modified from Abrams et al. (270). All platelets in whole blood were labeled by a PerCP-conjugated antibody against CD61 (Becton-Dickinson). CD61 is also known as GPIIIa, a subunit of GPIIb/IIIa, and is

expressed in the resting and activated conditions of the platelet. To determine the activation of the platelets, two markers were used; P-selectin and the activated form of GPIIb/IIIa. The P-selectin receptor was measured with a PE (phycoerythrin)-conjugated antibody against CD62P (Becton-Dickinson). The GPIIb/IIIa receptor was measured with a FITC-conjugated antibody against PAC-1 (GPIIb/IIIa) (Becton-Dickinson). All antibodies were used at a concentration of 20µl undiluted antibody/ 10^6 platelets. For all measurements, whole blood aliquots of 5µl were incubated with 20µl of PerCP-conjugated anti-CD61 monoclonal antibody (mAB) in polystyrene round bottom tubes (Falcon 2052, Becton Dickinson). To test for expression of P-selectin and GPIIb/IIIa, 20µl of the respective antibodies were added to tubes in addition to 5µl of the samples. To examine the expression of these antibodies under stimulation, 450µl of whole blood was combined with 50µl of PAF (10^{-7} M, Sigma) and incubated for two minutes. Five micro-liters of the stimulated blood were added to respective tubes and all samples were covered with aluminum foil and incubated for 20 minutes at room temperature. Ice-cold 1% paraformaldehyde (pH 7.4, Sigma) was then added to each tube and they were kept covered on ice until analysis.

Measurements of platelet P-selectin and GPIIb/IIIa were made using flow cytometry (Becton Dickinson FACScan Clinical Flow Cytometer). A 488-nm argon laser light was used for excitation and the fluorescence emission detected in FL1 (FITC PAC-1), FL2 (PE Anti-CD62P), and FL3 (PerCP Anti-GPIIIa). In order to identify only the platelets in the whole blood samples, a threshold was set on FL3 to include only those events that

stained positive for PerCP anti-CD61. This allows one to examine platelets in a whole blood solution without having to separate out the other types of cells. The total number of platelets acquired from each blood sample was 5,000 events and the data was stored in list mode. The data was analyzed with WinMDI 2.8 Flow Cytometry Analysis Software. Data was expressed as total fluorescence intensity (TFI = % of positive events x mean channel of fluorescence).

Data Analysis

Data was collected in notebooks and transferred to a Computer Spreadsheet Format (Excel for Windows). The summary results are represented as mean \pm standard error of the mean (SEM). Comparisons among the four groups were made with an ANOVA (Sigma-Stat 3.0). A Dunn's and Holm-Sidak Post hoc test was performed (Sigma-Stat 3.0). A Chi squared test was performed to determine if the percentages in table 2.1 and 2.2 were significantly related (Sigma-Stat 3.0). $P < 0.05$ was considered as statistically significant.

RESULTS

Patient Characteristics

Table 2.1 summarizes the characteristics of the six groups of women and men studied. There was a significant difference of age in the NDW+CVD and DW+CVD groups compared to the NDW-CVD and DW-CVD groups ($p < 0.05$). There was also a significant difference in body mass index (BMI) of the NDW+CVD group compared to

DW-CVD group ($p < 0.05$) and DW-CVD group compared to NDW-CVD group ($p < 0.05$). There was no significant difference in the duration of diabetes among the three diabetic groups. The glycated hemoglobin (HbA_{1C}) was significantly greater in DW-CVD and DW+CVD compared to NDW-CVD ($p < 0.05$). The glycated hemoglobin (HbA_{1C}) was also significantly greater in DW-CVD compared to NDW+CVD and DW+CVD compared to NDW+CVD ($p < 0.05$). There was a significant difference in hypertension and hypercholesteremia among the groups. There were no significant differences in smoking among any of the groups. The various drug therapies that the patients were receiving are summarized in Table 2.2. There was no significant difference in the percentages of women taking HRT among any of the groups. There was no significant difference in the percentages of women taking HRT or statins among any of the groups. There was a significant difference in the percentages of women taking beta blockers, diuretics, nitrates, ACE inhibitors, antioxidants, aspirin, and hypoglycemic agents.

Neutrophil Activation-CD11b Expression

Neutrophil activation was defined as expression of CD11b adhesion molecule. Figure 2.2 reports the neutrophil activation measured in diabetic and non-diabetic women with and without cardiovascular disease. There was no significant difference of the baseline expression of CD11b among the groups of women, indicating no chronic effects of diabetes. To test the response of the blood to an acute stimulus, we tested the reaction to fMLP and PAF. After stimulation with fMLP and PAF there was a marked increase in CD11b expression. There was also no significant difference of expression of CD11b

after stimulation with fMLP among the groups of women. After the blood was stimulated with PAF, non-diabetic women with CVD and diabetic women with CVD had significantly lower expression of CD11b in comparison to non-diabetic women without CVD ($P < 0.05$). Figure 2.3 reports the neutrophil activation measured in diabetic and non-diabetic men and women with cardiovascular disease. There was no significant difference of the baseline expression of CD11b among the groups of women and men. There was also no significant difference of expression of CD11b after stimulation with fMLP or PAF among the groups of women and men.

Neutrophil Reactivity to Acute Stimulation-CD11b Expression

As an indicator of the reactivity to acute stimulation, we calculated the ratio of PAF stimulated to unstimulated expression of CD11b. Figure 2.4 reports the neutrophil reactivity to acute stimulation (fMLP or PAF) measured in diabetic and non-diabetic women with and without cardiovascular disease. There was no significance difference of the neutrophil reactivity of CD11b expression with fMLP and PAF among the groups of women. Figure 2.5 reports the neutrophil reactivity to acute stimulation (fMLP or PAF) measured in diabetic and non-diabetic men and women with cardiovascular disease. There was no significance difference of the neutrophil reactivity of CD11b expression with fMLP and PAF among the groups of women and men.

Neutrophil Activation-ROS Production

Neutrophil activation was defined as the production of ROS. The chronic effects of diabetes and cardiovascular disease are represented in the unstimulated values. The acute response to stimulus was measured after stimulation with fMLP and PAF. Figure 2.6 reports the ROS production measured in diabetic and non-diabetic women with and without cardiovascular disease. There was no significance difference of the baseline production of ROS among the groups of women. There was also no significance difference of production of ROS after stimulation with fMLP among the groups of women. After the blood was stimulated with PAF, non-diabetic women with CVD had significantly lower production of ROS in comparison to non-diabetic women without CVD ($P < 0.05$). Figure 2.7 reports the ROS production measured in diabetic and non-diabetic men and women with cardiovascular disease. There was no significance difference of the baseline production of ROS among the groups of women and men. There was also no significance difference of production of ROS after stimulation with fMLP or PAF among the groups of women and men.

Neutrophil Reactivity to Acute Stimulation -ROS Production

As an indicator of the reactivity to acute stimulation, we calculated the ratio of PAF stimulated to unstimulated production of ROS. Figure 2.8 reports the neutrophil reactivity to acute stimulation (fMLP or PAF) measured in diabetic and non-diabetic women with and without cardiovascular disease. The neutrophil reactivity of ROS production (with fMLP or PAF) was significantly lower in non-diabetic women with

CVD and diabetic women with CVD in comparison to non-diabetic women without CVD ($P < 0.05$). Figure 2.9 reports the neutrophil reactivity to acute stimulation (fMLP or PAF) measured in diabetic and non-diabetic men and women with cardiovascular disease. There was no significance difference of the neutrophil reactivity of ROS production (with fMLP or PAF) among the groups of women and men.

Platelet Activation-GPIIb/IIIa Expression

Platelet activation was defined as expression of GPIIb/IIIa adhesion molecule. The chronic effects of diabetes and cardiovascular disease are represented in the unstimulated values. The acute response to stimulus was measured after stimulation with PAF. Figure 2.10 reports the GPIIb/IIIa expression measured in diabetic and non-diabetic women with and without cardiovascular disease. There was no significant difference of the baseline expression of GPIIb/IIIa and expression of GPIIb/IIIa after stimulation with PAF among the groups of women. Figure 2.11 reports the GPIIb/IIIa expression measured in diabetic and non-diabetic men and women with cardiovascular disease. There was no significant difference of the baseline expression of GPIIb/IIIa among the groups of women and men. There was significantly lower expression of GPIIb/IIIa after stimulation with PAF in non-diabetic women with CVD in comparison to diabetic men with CVD ($P < 0.05$).

Platelet Activation-P-selectin Expression

Platelet activation was defined as expression of P-selectin. The chronic effects of diabetes and cardiovascular disease are represented in the unstimulated values. The acute

response to stimulus was measured after stimulation with PAF. Figure 2.12 reports the P-selectin expression measured in diabetic and non-diabetic women with and without cardiovascular disease. Diabetic women with CVD had significantly increased baseline expression of P-selectin in comparison diabetic women without CVD ($P<0.05$). There was no significant difference of P-selectin expression after stimulation with PAF among the groups of women. Figure 2.13 reports the P-selectin expression measured in diabetic and non-diabetic men and women with cardiovascular disease. There was no significant difference of the baseline expression of P-selectin and expression of P-selectin after stimulation with PAF among the groups of women and men.

Platelet Reactivity to Acute Stimulation

As an indicator of the reactivity to acute stimulation, we calculated the ratio of PAF stimulated to unstimulated expression of GPIIb/IIIa and P-selectin. Figure 2.14 reports the platelet reactivity to acute stimulation (with PAF) measured in diabetic and non-diabetic women with and without cardiovascular disease. The platelet reactivity of GPIIb/IIIa expression with PAF was significantly lower in diabetic women with CVD in comparison to diabetic women without CVD ($P<0.05$). The platelet reactivity of P-selectin expression with PAF was significantly lower in diabetic women with CVD in comparison to diabetic women without CVD ($P<0.05$). Figure 2.15 reports the platelet reactivity to acute stimulation (with PAF) measured in diabetic and non-diabetic men and women with cardiovascular disease. There was no significance difference of the platelet reactivity of GPIIb/IIIa or P-selectin among the groups of women and men.

DISCUSSION

Coronary heart disease (CHD) and stroke are the leading killers of women in the United States. One in two women will eventually die of heart disease or stroke. Several reports document that women with CHD have a worse prognosis for men with CHD. This might indicate an increased severity of illness at presentation (12,32). Diabetes mellitus is an even stronger risk factor for CHD in women than in men. Diabetes is associated with three to sevenfold elevation in CHD risk among women compared with a two to threefold elevation among men (34). Another report indicated that the prevalence rate ratio for CHD in individuals with type 2 diabetes was 4.6 in women compared to 1.8 in men (35). There must be some difference in pathogenesis of cardiovascular disease in diabetes to explain the marked discrepancy observed in diabetic men versus diabetic women and diabetic women versus non-diabetic women.

Inflammation is now considered a primary cardiovascular risk factor and a recent study of predictors of cardiovascular risk ranks markers of inflammation, such as C-reactive protein, as comparable to plasma cholesterol (101,102). It is known that inflammation mediates the formation of atherosclerotic plaques, the major cause of cardiovascular dysfunction. Inflammation is associated with diabetes. A study by Vozarova et al. reported that a high white blood cell (WBC) count predicts the development of type 2 diabetes and a decrease of insulin action in Pima Indians. The authors hypothesize that a chronic activation of the immune system may play a role in the pathogenesis of type 2

diabetes (105). Studies by Thorand et al. and Barzilay et al. also found that low-grade inflammation (in particular elevated C-reactive protein) was predictive of an increased risk of type 2 diabetes mellitus in middle-aged men and the elderly (106,107). Hayden et al. hypothesized that type 2 diabetes mellitus is a vascular disease (atherosclerosis) and that NOS, NO, and redox stress, components of inflammation, play a causative role in type 2 diabetes, in turn causing atherosclerosis (104). Biondi-Zoccai et al. reviews the role of inflammation in diabetes and atherothrombosis. They state diabetics have increased dyslipidemic abnormalities, increased oxidative stress, and increased prothrombotic abnormalities, all contributing to increased atherosclerosis and atherothrombosis (103).

An enhanced inflammatory response may contribute to the greater recurrence of myocardial infarction and ischemic heart failure in diabetes. We hypothesized that platelet and neutrophil activation, both components of inflammation, are increased in diabetic patients, particularly diabetic women. We examined two markers of neutrophil activation, ROS production and the expression of the adhesion molecule CD11b. We found no significant difference in the baseline expression of CD11b in any of the groups. However, when the blood was stimulated with PAF, women with CVD, regardless of their diabetic status had significantly lower expression of CD11b in comparison to the healthy age-matched control women. This indicates a possible blunting of the inflammatory response to a stimulus. When examining the ROS production, again there was no significant difference in the baseline expression of ROS among any of the groups.

When the blood was stimulated with PAF or fMLP, women with CVD regardless of their diabetic status, had significantly lower stimulation ratio of the production of ROS in comparison to the healthy age-matched controls. These results also support the possibility of blunting of the inflammatory response to a stimulus in women with CVD.

In addition to examining neutrophil activation, we also examined platelet activation. We examined two markers of platelet activation; the expression of the P-selectin and GPIIb/IIIa receptors. We found no significant difference in the baseline expression of GPIIb/IIIa among any of the groups, but when examining P-selectin, we found the expression of P-selectin to be significantly higher in diabetic women with CVD in comparison to diabetic women without cardiovascular disease. We did not find any differences of the expression of GPIIb/IIIa or P-selectin when comparing women to men.

Atherosclerosis in Diabetes

The luminal endothelial cell layer separates circulating factors and blood cells from the arterial wall. It also functions as an anticoagulant and fibrinolytic surface (109). Factors which are commonly increased in diabetes, like glucose, free fatty acids, lipoproteins, and derivatives of glycation and oxidation, can all damage endothelial cells (110). In the atherogenic process, the adhesion of leukocytes to the vascular endothelium involves many adhesion molecules (vascular adhesion molecule-1, VCAM-1, intercellular adhesion molecule-1, ICAM-1, E-selectin, and P-selectin), which are increased in people with diabetes (111-113). These adhesion molecules aid the migration of circulating

monocytes into the arterial intima. There they undergo differentiation and activation resulting in a fatty streak. Endothelial cells and macrophages both produce cytokines and growth factors that cause smooth muscle cells to migrate from the media to the intima where they proliferate and form a fibrous cap. Then cell death occurs which results in a large lipid core with necrotic tissue, macrophages, and a thin fibrous cap, which can result in a plaque rupture. There is some evidence that there is an increased amount of macrophages in the atherosclerotic lesions of diabetic patients, possibly due to an increased recruitment of macrophages into the vessel wall by the higher levels of cytokines (109).

Hyperglycemia adds another complicating factor to atherosclerosis. Hyperglycemia causes an increase of oxidative stress, decrease of nitric oxide, which then leads to apoptosis and impaired function of the endothelium. Hyperglycemia causes glycation of most molecules in the arterial wall, which produces AGEs (advanced glycation end products). Complexes are then formed and irreversible substances that are highly injurious to the integrity and function of the vessel walls. AGEs can cause injury via three pathways; AGE cross-bridges formed between macromolecules, AGE accumulation into the vessel wall via trapping blood components, or by disrupting the cell function that involve receptor and non-receptor pathways. The vasculature is then full of these pathogenic substances that in turn activate local inflammation and hypertrophy. Resting T lymphocytes (CD4, CD8) have AGE receptors constitutively expressed and there activation in the vessel wall may also trigger a low-grade inflammation (109).

Neutrophil Activation in Diabetes

Neutrophils are one of the main types of inflammatory cells and once activated they release reactive oxygen species, including hydrogen peroxide, contributing to endothelial damage and cardiovascular disease (117,118). Neutrophils are also known to release cytokines (119). A study by Okouchi et al. determined that hyperinsulinemia increased neutrophil transendothelial migration in a dose-dependent response and this could then possibly accelerate atherosclerosis (120). In a study by Salas et al., it was determined that in ischemia-reperfusion in diabetic rats, there was a significantly larger amount of leukocyte adhesion and migration (121). Studies have demonstrated that hyperglycemia and AGEs decrease neutrophil function, possibly contributing to the dysfunctional host defense in diabetic patients (122,123).

Both ROS production and the expression of CD11b are linked to diabetes and CVD. ROS is produced when neutrophils are activated. A study by Wong et al. determined that AGEs caused a dose-dependent increase of the neutrophil respiratory burst in response to a secondary mechanical stimulus (124). A study by Mohanty et al. demonstrated that glucose stimulated ROS generation by neutrophils (125). Yasunari et al. determined that monocytes and neutrophil oxidative stress was increased in patients with both hypertension and diabetes. They also determined that there was a significant correlation between neutrophil oxidative stress and HbA_{1C} (126). We found that the baseline production of ROS was not different among any of the groups. When the blood samples

were stimulated with fMLP and PAF, the ROS production was increased. When the blood was stimulated with PAF, non-diabetic women with CVD had significantly lower production of ROS in comparison to the healthy age-matched control women. Diabetic women with CVD had a trend of increased production of ROS in comparison to the healthy age-matched controls. The neutrophil reactivity of ROS to acute stimulation (fMLP and PAF) was significantly decreased in non-diabetic and diabetic women with CVD. This indicates a blunting of the production of ROS in women with cardiovascular disease in comparison to women without cardiovascular disease.

The expression of CD11b is increased during neutrophil and monocyte activation. Sampson et al. determined that acute glycemic caused an increase in monocyte Mac-1 (CD11b) expression in type 2 diabetic patients, but there was no significant change in neutrophil expression of Mac-1 after the glucose load (127). Hu et al. demonstrated that at clinical concentrations of insulin, leukocyte CD11b expression is increased, but leukocyte respiratory bursts are decreased (128). We found that the baseline expression of CD11b was not different among any of the groups. When the blood samples were stimulated with fMLP and PAF, the CD11b expression was increased. In women with CVD, when the blood was stimulated with PAF, regardless of their diabetic status had significantly lower expression of CD11b in comparison to the healthy age-matched control women. The response of CD11b expression and ROS production to PAF possibly indicates a blunting of the inflammatory response to a stimulus in women with cardiovascular disease.

Platelet Activation in Diabetes

Platelets are a known component of the inflammatory response. It is known that platelets are the source of various inflammatory mediators including thromboxane A₂ and P-selection (129,130). These inflammatory mediators then can upregulate the complement system and cytokines (131,132). Activated platelets adhere to the endothelium and induce inflammatory responses of the endothelium, which in turn contributes to the early steps of arteriosclerosis (133,134).

Platelet adhesion and platelet aggregation are increased in diabetes mellitus. Platelet reactivity was suggested as a potential mechanism of the accelerated atherosclerosis and increased incidence of thrombosis observed in diabetes (135,136). Gresele et al. determined that acute, short-term hyperglycemia caused an increased activation of platelets exposed to high shear stress. They hypothesized that hyperglycemia in type 2 diabetes mellitus may cause vascular occlusions via platelet activation (137). Davi et al. demonstrated that in type 2 diabetes there is an increase platelet in vivo thromboxane production. Thromboxane may then mediate the increased fibrinogen binding and aggregation of platelets in patients with diabetes (29). Endler et al. determined that an increased mean platelet volume, which is an indicator of more reactive platelets, places patients with coronary artery disease at an increased risk of myocardial infarction (30). Lefer et al. examined the effects of neutrophils and platelets separately and together in causing cardiac dysfunction in perfused rat hearts after ischemia and reperfusion (I/R). It

was demonstrated that I/R hearts perfused with neutrophils or platelets alone exhibited a decreased of 10% to 12% of heart functions and when neutrophils and platelets were perfused together, the various heart functions decreased by 50% to 60%, suggesting platelet-neutrophil interactions may amplify the neutrophil-mediated I/R injury (165).

In this study, we examined two components of platelet activation; the expression of GPIIb/IIIa and the expression of P-selectin. Platelet P-selectin and GPIIb/IIIa adhesion proteins are expressed on the surface of activated platelets and mediate platelet-platelet and platelet-leukocyte interactions. Various GPIIb/IIIa inhibitors are an area of active research in preventing complications linked to percutaneous coronary intervention. Various studies have demonstrated that administration of a glycoprotein IIb/IIIa antagonism is effective to prevent ischemic complication of percutaneous coronary intervention in women and diabetic patients, results comparable to that of their male counterparts (138-140). We found the baseline expression of GPIIb/IIIa was not significantly different among any of the groups, but we did find a trend for the GPIIb/IIIa expression to be increased in diabetic women with CVD. We also measured the expression of GPIIb/IIIa after stimulating the blood with PAF. Among the groups of women, there was no significant difference of the expression of GPIIb/IIIa after stimulation with PAF.

P-selectin is also demonstrated to play an active role in cardiovascular disease and diabetes. The primary interaction between platelets and leukocytes is via the P-selectin

receptor on the platelets and the P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes (141-146). Nomura et al. determined that platelet P-selectin expression was increased in diabetic patients (147). Zhou et al. determined that administration of a P-selectin antagonist decreased the accumulation of leukocytes and decreased neointimal formation after balloon injury in diabetic rats (148). We measured the baseline expression of P-selectin and found diabetic women with cardiovascular disease to have a significantly increased expression of P-selectin in comparison to diabetic women without cardiovascular disease. The blood was also stimulated with PAF and the expression of P-selectin did increase, but there was no statistical significance among the groups.

The population of women and men in this study have different demographics and are on different medications. There was a significant increase in the age of the women with cardiovascular disease in comparison to women without cardiovascular. This increase of age could be responsible for the blunting of CD11b expression and ROS production after simulation with PAF that was observed in the groups of women with cardiovascular disease. Another influence on the blunting effect that was observed could be the different medications that the patients were on. Overall, patients with cardiovascular disease are on more medications than patients that do not have cardiovascular disease. In particular, there are a higher percentage of women with CVD on aspirin. Statins are known to reduce inflammation via decreasing concentrations of C-reactive protein and upregulating endothelial nitric oxide synthase (271,272). We found that the proportions of patient on statins are not significantly different among groups, making statins less likely to

influence the results. We did find that when separating the patients on statins from the patients not on statins, diabetic women with CVD not on statins did have a significantly higher ROS production and P-selectin expression compared to those on statins. We also found diabetic men with CVD not on statins did have a significantly higher CD11b expression compared to those on statins.

Conclusion

Diabetic patients have an increased risk of cardiovascular disease; which is much more severe in diabetic women than men. Little is known why there are these extreme differences observed between diabetic men versus diabetic women and diabetic women versus non-diabetic women. One of the links between diabetes and cardiovascular disease is inflammation. We hypothesized that neutrophil and platelet activation is increased in diabetic women with cardiovascular disease compared to non-diabetic women with cardiovascular disease and diabetic men with cardiovascular disease.

In this study, we examined some components of inflammation, platelet and neutrophil activation, in six groups of diabetic and non-diabetic patients with and without cardiovascular disease. We did not find neutrophil activation to be increased in diabetic women compared to diabetic men, nor did we find neutrophil activation to be increased in diabetic women compared to non-diabetic women. An increased CD11b expression would contribute to greater neutrophil adhesion in cardiac vessels in the diabetic heart following ischemia and reperfusion. Once the neutrophils have adhered to the vessel,

they may contribute to microvascular damage and cardiac dysfunction through the release of reactive oxygen species.

We did find platelet expression of P-selectin to be increased in diabetic women with CVD compared to diabetic women without CVD. In addition, there was a trend for the GPIIb/IIIa to be increased in diabetic women with CVD. Excessive platelet activation in diabetes may contribute to the pathogenesis of thrombosis-induced ischemic events. Platelet-leukocyte adhesion (via P-selectin) may serve to recruit neutrophils and monocytes to sites of cardiovascular damage and thus promote the inflammatory response. Binding could cause neutrophil activation resulting in increased free radical production. Increased activation of platelet GPIIb/IIIa may contribute to excessive platelet aggregation and thrombus formation, which can lead to increased occurrence of thromboembolic events in diabetic patient. Significantly increased platelet activation may explain hypercoagulability and increased incidence of thromboembolism in diabetes.

An increase in platelet and neutrophil activation in diabetic patients could contribute to the more severe cardiovascular disease in diabetic patients, particularly women. These results did not elucidate any differences between men and women, but we did find platelet activation increased in diabetic women with cardiovascular disease. The reasons men and women have different severity of cardiovascular disease in the presence of diabetes lies in another aspect of the disease, possibly another component of inflammation. An enhanced inflammatory response (via platelet activation) may

contribute to the greater recurrence of myocardial infarction, thromboembolic events, and ischemic-reperfusion injury in diabetes women compared to non-diabetic women.

Table 2.1. Demographics of the women and men.

Patient Characteristics	NDW-CVD (n=24)	NDW+CVD (n=27)	DW-CVD (n=20)	DW+CVD (n=27)	NDM+CVD (n=8)	DM+CVD (n=18)
Age (years)	58.5 ± 1.7	66.7 ± 2.4* **	55.7 ± 2.6	66.0 ± 2.0* **	63.8 ± 3.4	64.9 ± 2.5
Body Mass Index (kg/m ²)	26.8 ± 1.0	28.7 ± 1.4**	36.9 ± 1.8*	29.8 ± 1.3	28.2 ± 1.3	29.2 ± 1.0
Duration of Diabetes (years)	N/A	N/A	9.2 ± 1.5	13.6 ± 2.8	N/A	14.5 ± 3.4
HbA1c (%)	5.4 ± 0.1	5.2 ± 0.2**	8.2 ± 0.4*	6.9 ± 0.3* ***	5.9 ± 0.4	7.4 ± 0.4
Hypertension (%) ψ	8%	74%	55%	70%	50%	83%
Hypercholesteremia (%) ψ	25%	67%	53%	70%	100%	56%
Current Smoker (%)	0%	19%	10%	15%	38%	22%
Prior Cardiovascular Events (%)						
Myocardial Infarction ψ	0%	56%	0%	63%	63%	73%
Stroke ψ	0%	15%	0%	19%	38%	17%
PTCA ψ	0%	19%	0%	33%	25%	75%
CABG ψ	0%	19%	0%	26%	75%	33%

*P<0.05 compared to NDW-CVD group

**P<0.05 compared to DW-CVD group

***P<0.05 compared to NDW+CVD group

 ψ The proportions are significantly related (P<0.05)

Table 2.2. Distribution of medications in women and men.

Medication (%)	NDW-CVD (n=24)	NDW+CVD (n=27)	DW-CVD (n=20)	DW+CVD (n=27)	NDM+CVD (n=8)	DM+CVD (n=18)
Beta Blockers ψ	0%	50%	10%	44%	50%	53%
Diuretics ψ	4%	25%	10%	67%	50%	71%
Nitrates ψ	4%	15%	0%	26%	0%	53%
ACE Inhibitors ψ	4%	25%	65%	41%	63%	41%
Positive Inotropes	0%	5%	5%	19%	0%	12%
Ca ²⁺ Channel Blockers ψ	0%	15%	10%	30%	38%	29%
Aspirin ψ	21%	55%	25%	74%	63%	59%
Anti-coagulants	0%	15%	10%	4%	25%	0%
Antioxidants ψ	50%	25%	10%	15%	0%	0%
Hypoglycemic Agents ψ	0%	0%	65%	22%	0%	29%
Statins	12%	29%	25%	37%	75%	38%
HRT	50%	70%	55%	48%	N/A	N/A

ψ The proportions are significantly related (P<0.05)

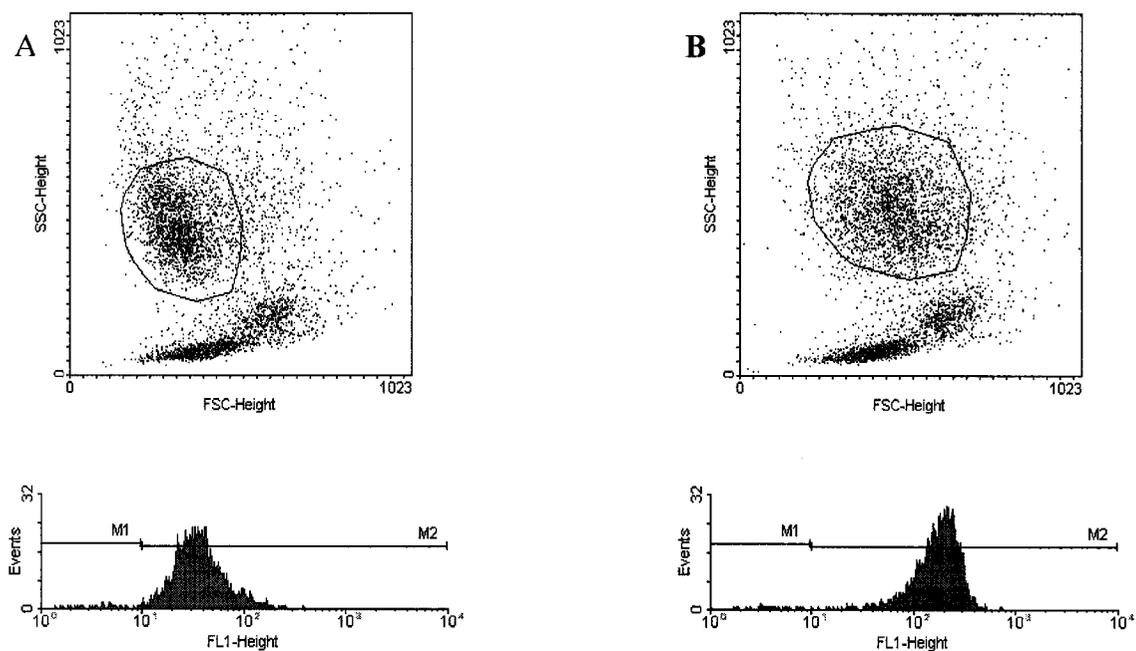


Figure 2.1. Representative FACS plots to assess neutrophil activation in a diabetic woman with cardiovascular disease. (A) Unstimulated blood (TFI: 46.4). (B) Stimulated blood with fMLP (TFI: 205.6).

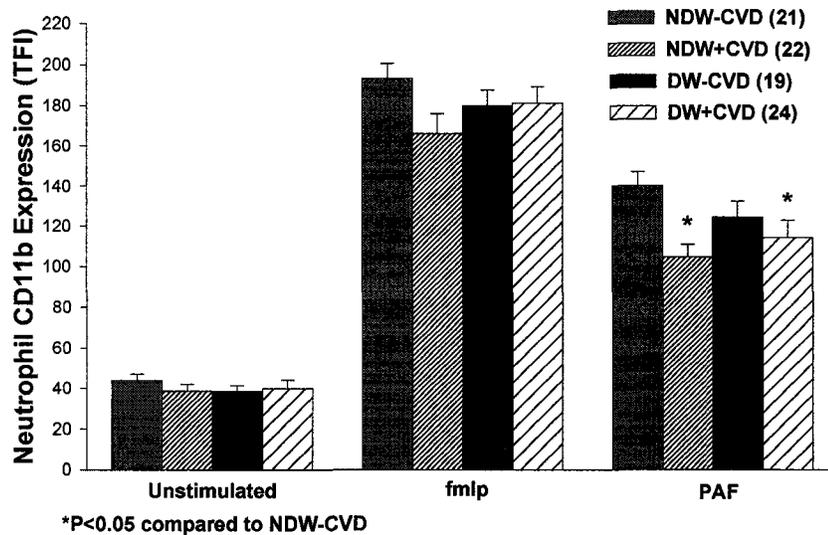


Figure 2.2. Summary of neutrophil CD11b expression for women in the study. Measurement of CD11b expression in unstimulated blood, CD11b expression after stimulation with fMLP, and CD11b expression after stimulation with PAF. Comparison of non-diabetic women without cardiovascular disease (NDW-CVD) versus non-diabetic women with cardiovascular disease (NDW+CVD) versus diabetic women without cardiovascular disease (DW-CVD) versus diabetic women with cardiovascular disease (DW+CVD). CD11b expression after stimulation with PAF was significantly lower in NDW+CVD and DW+CVD in comparison to NDW-CVD ($P < 0.05$).

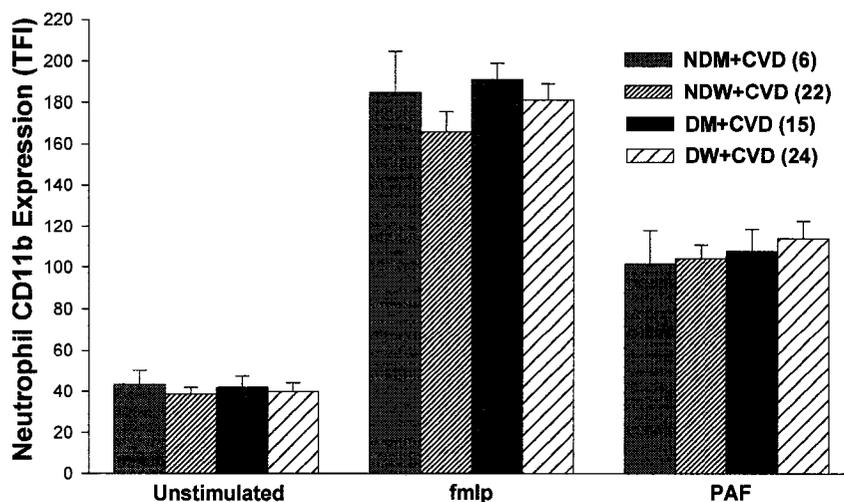


Figure 2.3. Summary of neutrophil CD11b expression for women and men in the study. Comparison of non-diabetic men with cardiovascular disease (NDM+CVD) versus non-diabetic women with cardiovascular disease (NDW+CVD) versus diabetic men with cardiovascular disease (DM+CVD) versus diabetic women with cardiovascular disease (DW+CVD). There is no significant difference within the treatment groups.

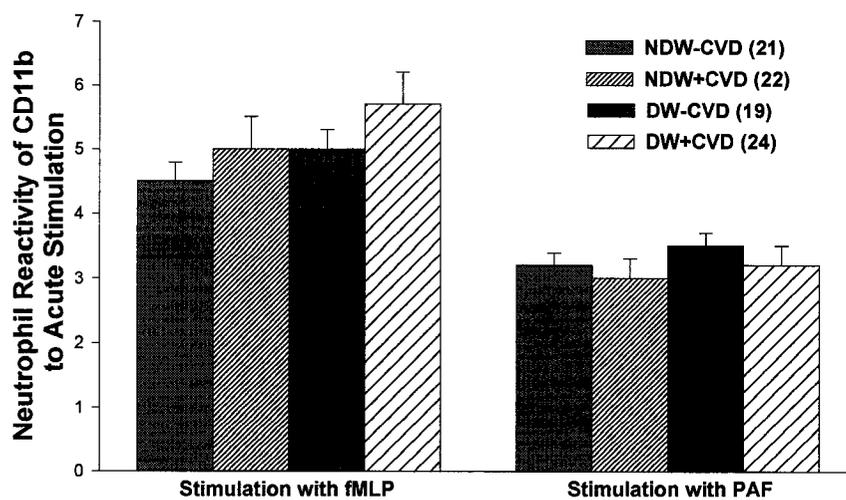


Figure 2.4. Summary of neutrophil reactivity of CD11b to acute stimulation (with fMLP and PAF) for women in the study. Comparison of non-diabetic women without cardiovascular disease (NDW-CVD) versus non-diabetic women with cardiovascular disease (NDW+CVD) versus diabetic women without cardiovascular disease (DW-CVD) versus diabetic women with cardiovascular disease (DW+CVD). There is no significant difference among any of the groups.

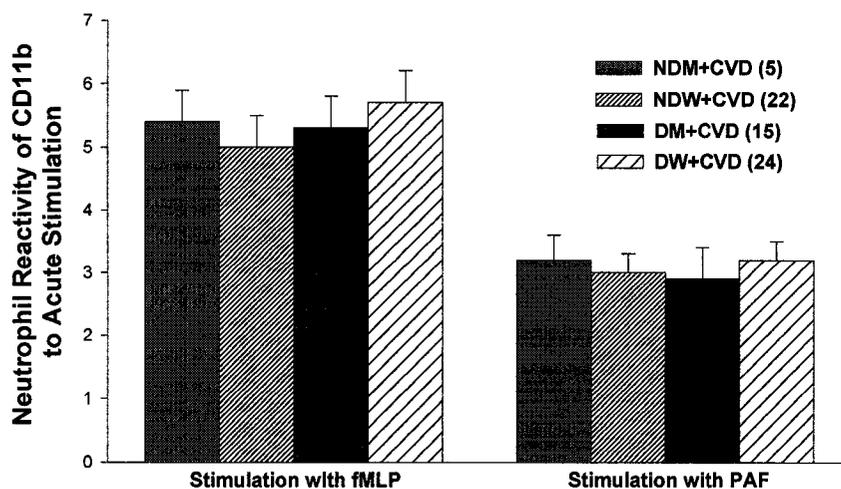


Figure 2.5. Summary of neutrophil reactivity of CD11b to acute stimulation (with fMLP and PAF) for women and men in the study. Comparison of non-diabetic men with cardiovascular disease (NDM+CVD) versus non-diabetic women with cardiovascular disease (NDW+CVD) versus diabetic men with cardiovascular disease (DM+CVD) versus diabetic women with cardiovascular disease (DW+CVD). There is no significant difference among any of the groups.

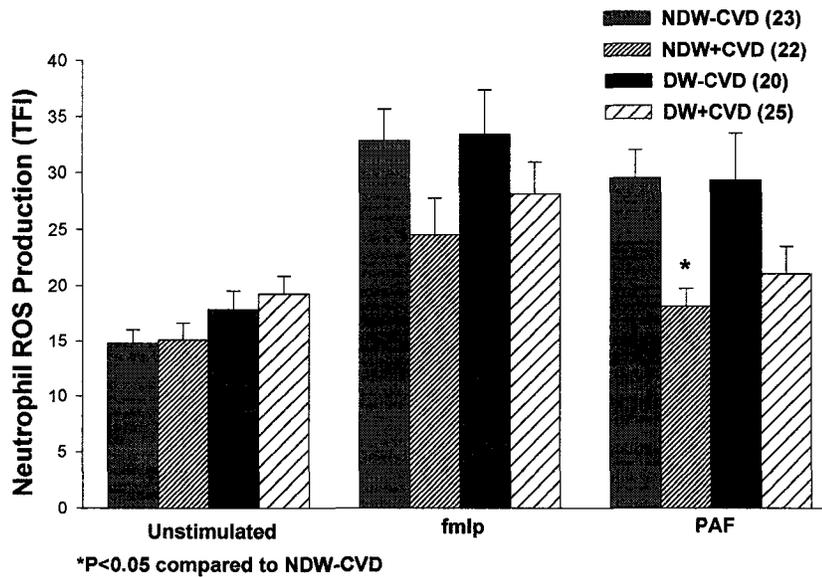


Figure 2.6. Summary of neutrophil ROS production for women in the study. Measurement of ROS production in unstimulated blood, ROS production after stimulation with fMLP, and ROS production after stimulation with PAF. Comparison of non-diabetic women without cardiovascular disease (NDW-CVD) versus non-diabetic women with cardiovascular disease (NDW+CVD) versus diabetic women without cardiovascular disease (DW-CVD) versus diabetic women with cardiovascular disease (DW+CVD). DCF expression after stimulation with PAF was significantly lower in NDW+CVD in comparison to NDW-CVD ($P<0.05$).

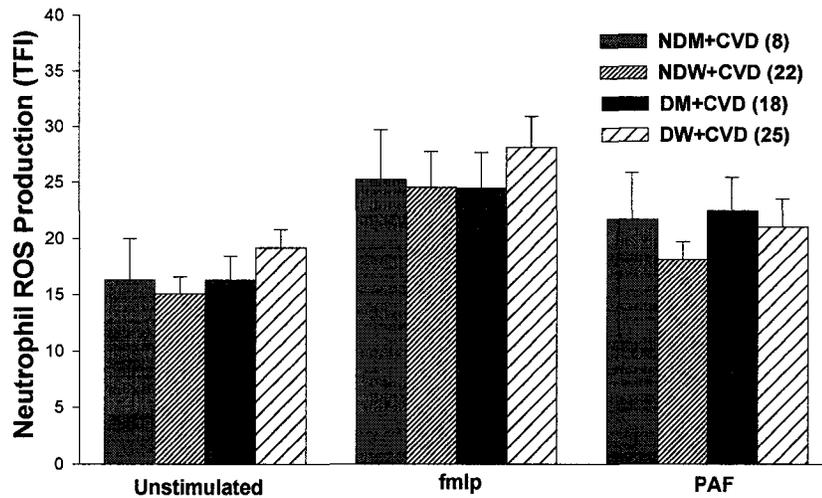


Figure 2.7. Summary of neutrophil ROS production for women and men in the study. Comparison of non-diabetic men with cardiovascular disease (NDM+CVD) versus non-diabetic women with cardiovascular disease (NDW+CVD) versus diabetic men with cardiovascular disease (DM+CVD) versus diabetic women with cardiovascular disease (DW+CVD). There is no significant difference within the treatment groups.

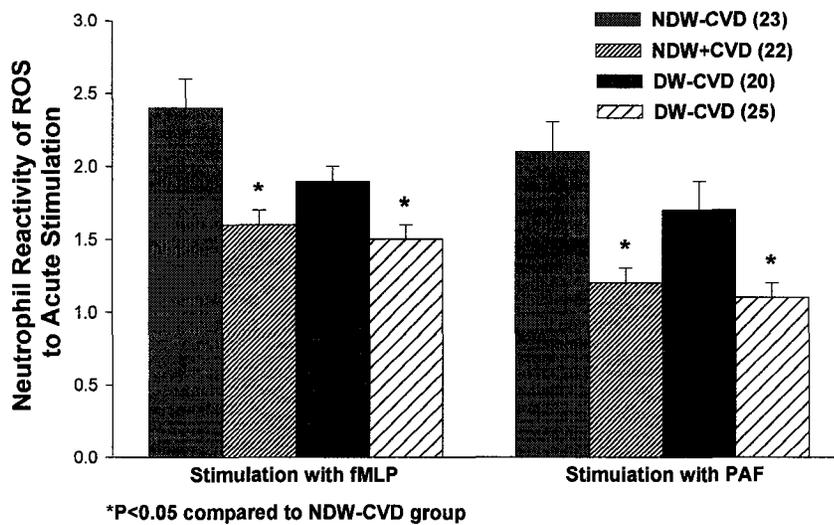


Figure 2.8. Summary of neutrophil reactivity of ROS to acute stimulation (with fMLP and PAF) for women in the study. Comparison of non-diabetic women without cardiovascular disease (NDW-CVD) versus non-diabetic women with cardiovascular disease (NDW+CVD) versus diabetic women without cardiovascular disease (DW-CVD) versus diabetic women with cardiovascular disease (DW+CVD). The stimulation ratio with fMLP was significantly lower in NDW+CVD and DW+CVD in comparison to NDW-CVD ($P<0.05$). The stimulation ratio with PAF was significantly lower in NDW+CVD and DW+CVD in comparison to NDW-CVD ($P<0.05$).

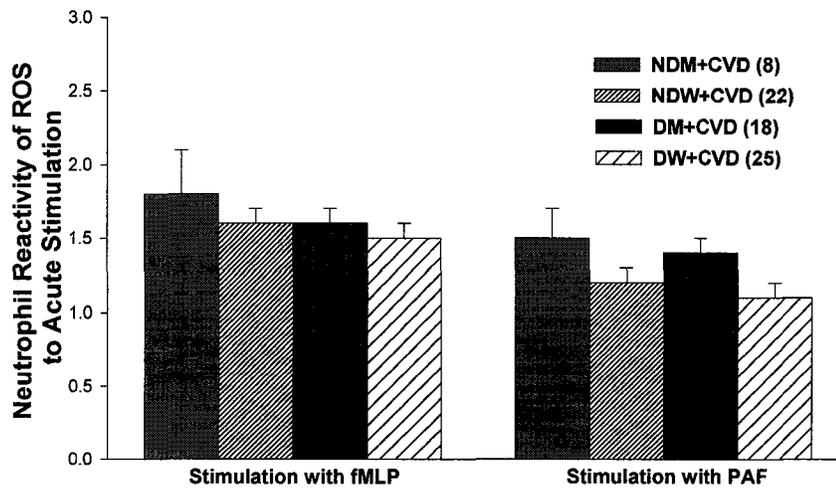


Figure 2.9. Summary of neutrophil reactivity of ROS to acute stimulation (with fMLP and PAF) for women and men in the study. Comparison of non-diabetic men with cardiovascular disease (NDM+CVD) versus non-diabetic women with cardiovascular disease (NDW+CVD) versus diabetic men with cardiovascular disease (DM+CVD) versus diabetic women with cardiovascular disease (DW+CVD). There is no significant difference among any of the groups.

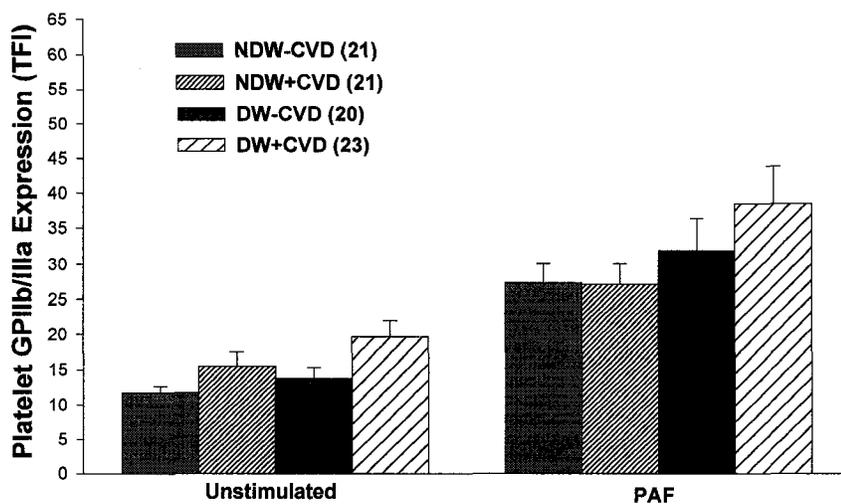


Figure 2.10. Summary of platelet GPIIb/IIIa for women in the study. Measurement of GPIIb/IIIa expression in unstimulated blood and GPIIb/IIIa expression after stimulation with PAF. Comparison of non-diabetic women without cardiovascular disease (NDW-CVD) versus non-diabetic women with cardiovascular disease (NDW+CVD) versus diabetic women without cardiovascular disease (DW-CVD) versus diabetic women with cardiovascular disease (DW+CVD). There is no significant difference among any of the groups.

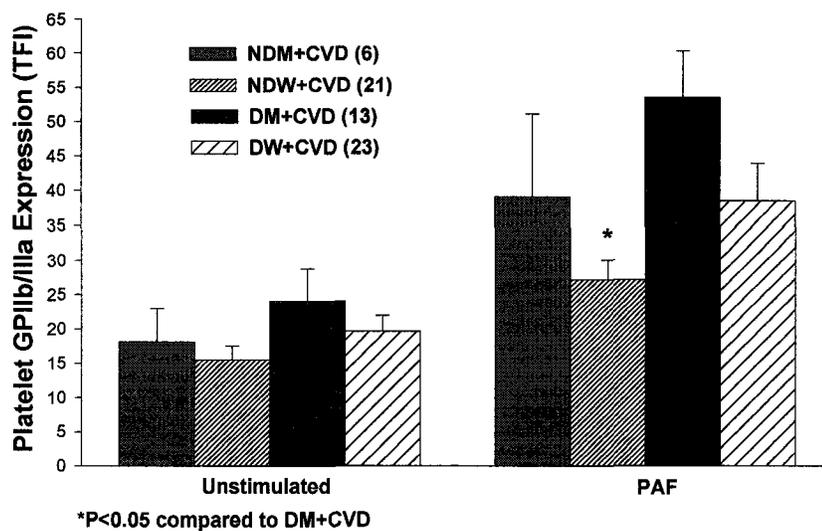


Figure 2.11. Summary of platelet GPIIb/IIIa for women and men in the study. Comparison of non-diabetic men with cardiovascular disease (NDM+CVD) versus non-diabetic women with cardiovascular disease (NDW+CVD) versus diabetic men with cardiovascular disease (DM+CVD) versus diabetic women with cardiovascular disease (DW+CVD). The GPIIb/IIIa expression after stimulation with PAF was significantly lower in NDW+CVD in comparison to DM+CVD ($P<0.05$).

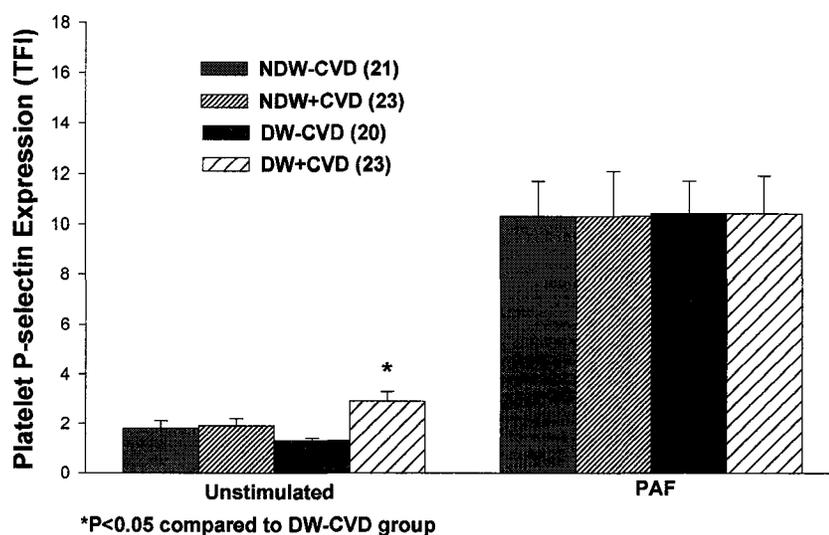


Figure 2.12. Summary of platelet P-selectin for women in the study. Measurement of P-selectin expression in unstimulated blood and P-selectin expression after stimulation with PAF. (A) Comparison of non-diabetic women without cardiovascular disease (NDW-CVD) versus non-diabetic women with cardiovascular disease (NDW+CVD) versus diabetic women without cardiovascular disease (DW-CVD) versus diabetic women with cardiovascular disease (DW+CVD). The P-selectin expression in unstimulated blood was significantly higher in DW+CVD in comparison to DW-CVD ($P < 0.05$).

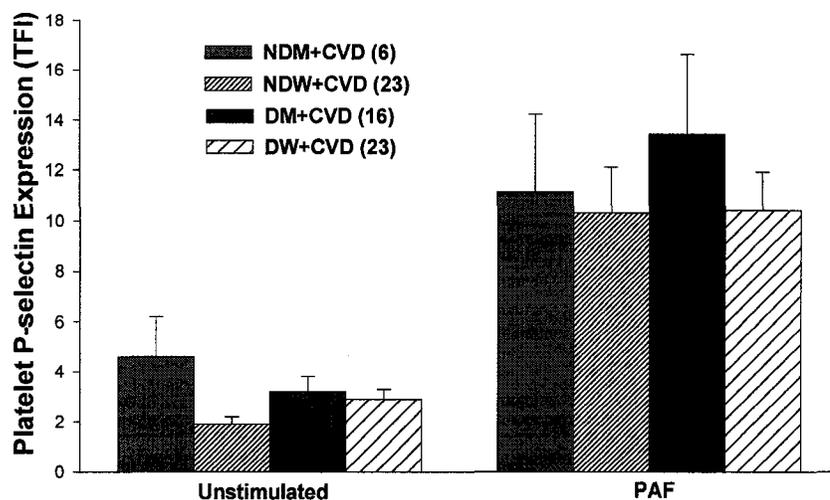


Figure 2.13. Summary of platelet P-selectin for women and men in the study. Comparison of non-diabetic men with cardiovascular disease (NDM+CVD) versus non-diabetic women with cardiovascular disease (NDW+CVD) versus diabetic men with cardiovascular disease (DM+CVD) versus diabetic women with cardiovascular disease (DW+CVD). There is no significant difference among any of the groups.

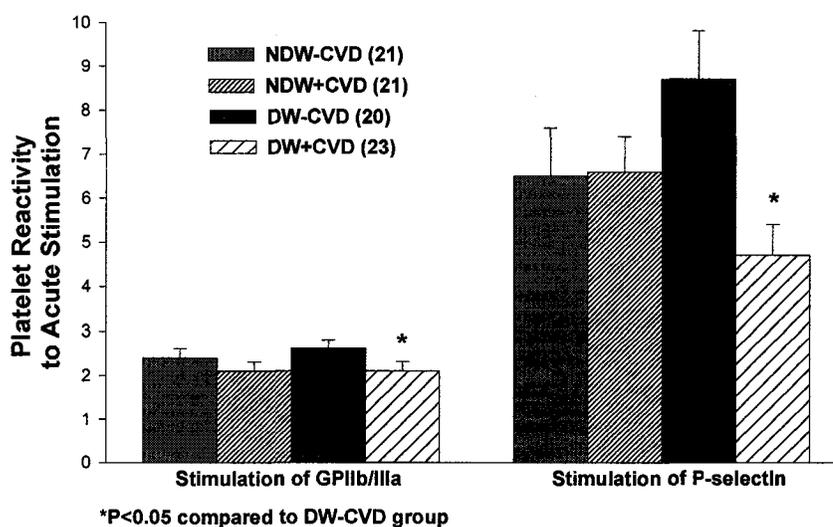


Figure 2.14. Summary of platelet reactivity to acute stimulation (with PAF) for women in the study. The platelet reactivity of GPIIb/IIIa and the platelet reactivity of P-selectin. Comparison of non-diabetic women without cardiovascular disease (NDW-CVD) versus non-diabetic women with cardiovascular disease (NDW+CVD) versus diabetic women without cardiovascular disease (DW-CVD) versus diabetic women with cardiovascular disease (DW+CVD). The stimulation ratio of GPIIb/IIIa after stimulation with PAF was significantly lower in DW+CVD in comparison to DW-CVD ($P<0.05$). The stimulation ratio of P-selectin after stimulation with PAF was significantly lower in DW+CVD in comparison to DW-CVD ($P<0.05$).

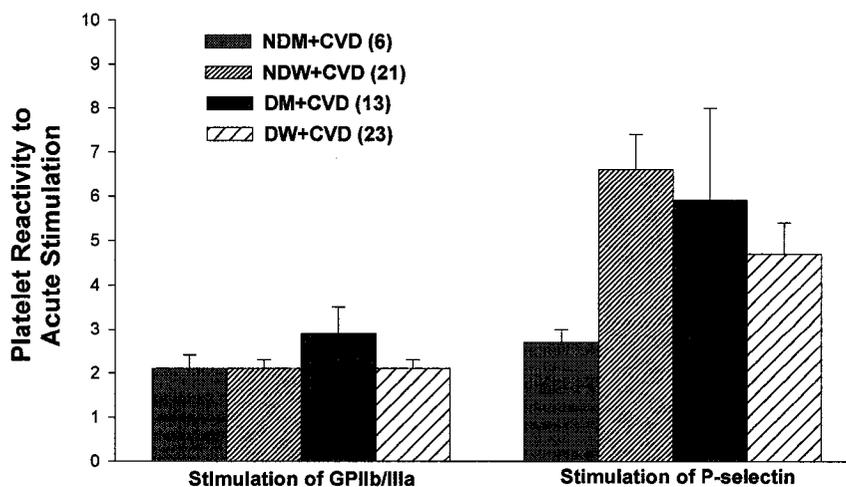


Figure 2.15. Summary of platelet reactivity to acute stimulation (with PAF) for women and men in the study. Comparison of non-diabetic men with cardiovascular disease (NDM+CVD) versus non-diabetic women with cardiovascular disease (NDW+CVD) versus diabetic men with cardiovascular disease (DM+CVD) versus diabetic women with cardiovascular disease (DW+CVD). There is no significant difference among any of the groups.

**CHAPTER 3. PLATELET-NEUTROPHIL CONJUGATE FORMATION IS
INCREASED IN DIABETIC WOMEN WITH CARDIOVASCULAR DISEASE
COMPARED TO DIABETIC MEN WITH CARDIOVASCULAR DISEASE**

INTRODUCTION

Diabetes poses a significant risk factor for cardiovascular disease in both men and women, but the incidence of type 2 diabetes is markedly greater in women (36). It is known that diabetic women have a significantly greater risk and severity of cardiovascular disease than diabetic men. Diabetic women have an elevated base line risk, and have more cardiovascular risk factors than men (48). From the Framingham Heart Study, diabetic women have an age-adjusted risk ratio of 3.8 for coronary heart disease (CHD) compared to diabetic men. Despite the importance of complicating factors in the severity of heart disease in diabetes, little is known about the interrelationships of risk factors and cardiovascular disorders, particularly in diabetic women. Of particular interest is the inflammatory response and its effect on cardiovascular disease. The inflammatory response complicates cardiovascular disease in several ways (101), but its role in diabetes is unclear. It was observed that diabetic men with cardiovascular disease exhibit chronic inflammation (264). Since inflammation amplifies atherosclerosis and ischemia-reperfusion injury, it is important to investigate if this complication is exacerbated in diabetic women.

An enhanced inflammatory response may contribute to the greater rate, recurrence and severity of myocardial infarction and ischemic heart failure in diabetes. From recent laboratory ischemia-reperfusion studies, it appears that reperfusion injury following ischemia is exacerbated in type 1 diabetes (267). Neutrophils accumulate in the coronary microcirculation following ischemia, amplifying reperfusion injury in diabetic hearts (265,268). In men with type 2 diabetes, it was determined that there is a chronic increase in neutrophil ROS (reactive oxygen species) production, which indicates an increased neutrophil activity. Activated platelets and their interaction with leukocytes may influence this injury. Diabetic platelets demonstrate enhanced platelet aggregation and an increase in coagulation (264). There are also increased platelet-leukocyte interactions, which may further amplify ROS production (264). Platelets are known to mediate the activation of leukocytes. It was demonstrated that when the platelet and neutrophil interaction is inhibited, ROS production is decreased (266). These experiments were performed in men, but very little is known about the cell-mediated inflammatory response in diabetic women, or how it compares to the inflammatory response of men.

The aim of this study was to investigate the severity of platelet-neutrophil conjugates in diabetic men and diabetic women with ischemic heart disease. For this purpose, we used whole blood samples obtained from diabetic women and diabetic men and analyzed the blood using a flow cytometer. We evaluated the formation of platelet and neutrophil conjugates in non-diabetic women without cardiovascular disease, non-diabetic women with cardiovascular disease, diabetic women without cardiovascular disease, diabetic

women with cardiovascular disease, non-diabetic men with cardiovascular disease, and diabetic men with cardiovascular disease. We found no significant difference in platelet-neutrophil conjugates in unstimulated blood. However, when the blood was stimulated with PAF (platelet activating factor), diabetic women without CVD demonstrated an increase in platelet-neutrophil conjugates compared to diabetic women with CVD and non-diabetic women with CVD. We found no significant difference in platelet-neutrophil conjugates after stimulation in comparing women to men. The stimulation ratio was significantly greater in diabetic and non-diabetic women with CVD in comparison to diabetic men with CVD, suggesting diabetic women's blood is hyper-reactive to stimulation compared to diabetic men's blood. These results indicate that platelets and neutrophils in diabetic women have a greater potential for activation compared to diabetic men. Platelet-neutrophil conjugates may contribute to thrombosis/inflammation and the greater severity of coronary heart disease observed in diabetic women as compared to diabetic men.

RESEARCH DESIGN AND METHODS

Subjects

Four groups of women were studied; non-diabetic women without cardiovascular disease (NDW-CVD) (n=23), non-diabetic women with cardiovascular disease (NDW+CVD) (n=25), diabetic women without cardiovascular disease (DW-CVD) (n=20), and diabetic women with cardiovascular disease (DW+CVD) (n=24). Two groups of men were studied; non-diabetic men with cardiovascular disease (NDM+CVD) (n=8) and diabetic

men with cardiovascular disease (DM+CVD) (n=18). These patients were enrolled from the patients attending the Cardiology Clinic at the Veterans Administration Hospital (Tucson, AZ) and the Diabetes Clinic at the University of Arizona Medical Center (Tucson, AZ). The protocol was approved by the University of Arizona Institutional Review Board. Patient's characteristics are summarized in Table 3.1 and their medications are summarized in Table 3.2. Fifteen milliliters of blood were drawn in a citrate tube from all the patients. The severity of diabetes was determined by the patient's hemoglobin A_{1C}. A subject was considered to have cardiovascular disease if they had a previous cardiovascular event (myocardial infarction, coronary artery bypass graft (CABG), stroke, percutaneous coronary intervention (PCI), or some combination).

Platelet-Neutrophil Conjugates

The platelet-neutrophil conjugates were determined by measuring fluorescent markers specific to platelets and leukocytes. The leukocytes were labeled by mixing whole blood with an equal volume of solution containing LDS-751 (Molecular Probes). Platelets were measured as the fluorescence of a PE-conjugated antibody (Pharmingen) against CD42b (GPIb). Through the use of these two different colored probes, it was possible to gate the events using the flow cytometer. These events were then designated the platelet-neutrophil conjugates.

Since P-selectin is the main receptor by which platelets and neutrophils form conjugates, we used an antibody against P-selectin to block the formation of the conjugates in order

to confirm that we were actually observing platelet-neutrophil conjugates. Platelet-neutrophil conjugates were blocked with a purified P-selectin (CD62) antibody (Pharmingen). In this experiment the WB/LDS, GPIb antibody, and anti-P-selection were combined in respective tubes, covered and incubated at room temperature for 15 minutes. To determine the effects of acute stimulation on platelet-neutrophil conjugate formation, PAF was added to respective tubes and incubated for another 10 minutes. After 25 minutes, ice-cold PBS was added and the samples were put on ice until analyzed on the flow cytometer (Becton Dickinson FACScan Clinical Flow Cytometer). Side scatter and Forward scatter were used to back-gate and identify the neutrophil population in the whole blood sample. The neutrophil population was then analyzed for the co-expression of the PE-GPIb probe to obtain a population of platelet and neutrophil conjugates (see figure 3.1). The neutrophils were then analyzed to determine the response to P-selectin blockade and PAF stimulation. Data was acquired and stored in list-mode for analysis of percent positive events and mean channel of fluorescence (264,266).

Estradiol Measurement

We examined the estrogen levels in the women to determine if there was a difference among the groups, possibly influencing the platelet-neutrophil conjugate formation. The blood samples were centrifuged (Megafuge 1.0R, Baxter Scientific Products) at 2,500 RPM for thirty minutes at 4°C. The plasma was pipetted from each sample and then immediately stored at -70°C. Once a sufficient number of samples were collected, they

were removed from the freezer and thawed to room temperature. The estradiol was measured using a double antibody ^{125}I radioimmunoassay from DPC. All samples were run in duplicate. The samples were prepared according to the directions provided by the company. Standard tubes were made and 100uL of Estradiol Antiserum was added to all tubes except the nonspecific binding and the total count tubes. The samples were incubated for 2 hours at room temperature and then 100uL of ^{125}I Estradiol was added to all tubes. The samples were then incubated for one hour at room temperature. Cold precipitating solution was added to all tubes and the samples were incubated for 10 minutes at room temperature. Finally, the samples were centrifuged for 15 minutes at 3000xg and the supernatant was decanted. The precipitate was retained for counting. Each tube was counted for one minute. The values of estradiol were obtained in pg/mL from a logit-log representation of the calibration curve.

Data Analysis

Data were collected in notebooks and transferred to a Computer Spreadsheet Format (Excel for Windows). The summary results are represented as mean \pm standard error of the mean (SEM). Comparisons among the four groups were made with an ANOVA (Sigma-Stat 3.0). A Dunn's and Holm-Sidak Post hoc test was performed (Sigma-Stat 3.0). A Chi squared test was performed to determine if the percentages in table 3.1 and 3.2 were significantly related. $P < 0.05$ was considered as statistically significant.

RESULTS

Patient Characteristic

Table 3.1 summarizes the characteristics of the six groups studied. There was a significant difference of age in the NDW+CVD and DW+CVD groups compared to the NDW-CVD and DW-CVD groups ($p<0.05$). There was also a significant difference in body mass index (BMI) of the NDW+CVD group compared to DW-CVD group ($p<0.05$) and DW-CVD group compared to NDW-CVD group ($p<0.05$). There was no significant difference in the duration of diabetes among the three diabetic groups. The glycated hemoglobin (HbA_{1C}) was significantly greater in DW-CVD and DW+CVD compared to NDW-CVD ($p<0.05$). The glycated hemoglobin (HbA_{1C}) was also significantly greater in DW-CVD compared to NDW+CVD and DW+CVD compared to NDW+CVD ($p<0.05$). There was a significant difference in hypertension and hypercholesteremia among the groups. There were no significant differences in smoking among any of the groups. The various drug therapies that the patients were receiving are summarized in Table 3.2. There was no significant difference in the percentages of women taking HRT or statins among any of the groups. There was a significant difference in the percentages of women taking beta blockers, diuretics, nitrates, ACE inhibitors, antioxidants, aspirin, and hypoglycemic agents.

Platelet-Neutrophil Conjugates

Figure 3.3 reports the percentage of platelet-neutrophil conjugates measured in diabetic and non-diabetic women with and without cardiovascular disease. There is a trend

toward increased platelet-neutrophil conjugates in diabetic women with cardiovascular disease in comparison to the other groups of women, but no statistical significance. Figure 3.4 reports the percentage of platelet-neutrophil conjugates measured in diabetic and non-diabetic men and women with cardiovascular disease. There was no significance among the groups of women and men.

Platelet-Neutrophil Conjugates after Stimulation with PAF

The percentage of platelet-neutrophil conjugates was increased in all groups after stimulation with PAF. Figure 3.5 reports the percentage of platelet-neutrophil conjugates after stimulation measured in diabetic and non-diabetic women with and without cardiovascular disease. The greatest response to PAF stimulation was observed in the diabetic women without CVD. NDW+CVD and DW+CVD had significantly less platelet-neutrophil conjugates after stimulation in comparison to DW-CVD ($p < 0.05$). Figure 3.6 reports the percentage of platelet-neutrophil conjugates after stimulation measured in diabetic and non-diabetic men and women with cardiovascular disease.

Platelet-Neutrophil Conjugates reactivity to PAF

As an indicator of the reactivity to acute stimulation, we calculated the ratio of PAF stimulated to unstimulated conjugates. Figure 3.7 reports the platelet-neutrophil conjugate reactivity to PAF measured in diabetic and non-diabetic women with and without cardiovascular disease. NDW+CVD and DW+CVD had significantly less platelet-neutrophil conjugate reactivity to PAF in comparison to DW-CVD ($P < 0.05$).

Figure 3.8 reports the platelet-neutrophil conjugate reactivity to PAF measured in diabetic and non-diabetic men and women with cardiovascular disease. NDW+CVD and DW+CVD had significantly greater platelet-neutrophil conjugate reactivity to PAF in comparison to DM+CVD ($P < 0.05$).

Platelet-Neutrophil Conjugates after Inhibition with anti-P-selectin

The percentage of platelet-neutrophil conjugates after P-selectin blockade was measured. We performed this experiment to confirm that we were examining platelet-neutrophil conjugates via the p-selectin receptor. P-selectin blockade markedly reduced the conjugate formation. Figure 3.9 reports the platelet-neutrophil conjugates after P-selectin blockade measured in diabetic and non-diabetic women with and without cardiovascular disease. The blockade caused an overall 76% decrease in the conjugate. Figure 3.10 reports the platelet-neutrophil conjugates after P-selectin blockade measured in diabetic and non-diabetic men and women with cardiovascular disease. The blockade caused an overall 75% decrease in the conjugate.

Estradiol Levels

The estradiol levels were measured in diabetic and non-diabetic women with and without cardiovascular disease. There was no significant difference among any of the groups (Figure 3.11). Estradiol in non-diabetic women without cardiovascular disease was 22.1 ± 7.3 . Estradiol in non-diabetic women with cardiovascular disease was 30.6 ± 8.0 .

Estradiol in diabetic women without cardiovascular disease was 30.2 ± 9.3 . Estradiol in diabetic women with cardiovascular disease was 37.0 ± 14.0 .

DISCUSSION

Gender differences in the pathology of cardiovascular disease have not been studied extensively. There was a reduction in the overall death rate due to cardiovascular disease (CVD) in the United States during the last several decades, but the CVD reduction was less for women than men. The absolute number of deaths due to CVD is actually increasing in women. In 2000, the estimated direct and indirect costs of CVD and stroke exceeded \$350 billion. Coronary heart disease (CHD) and stroke are the leading killers of women in the United States. Fifty percent of women will eventually die of heart disease or stroke. Several reports document that a women with CHD has a worse prognosis than men with CHD. This might indicate an increased severity of illness at presentation (12,32). Diabetes mellitus is an even stronger risk factor for CHD in women than in men. Diabetes is associated with three to sevenfold elevation in CHD risk among women compared with a two to threefold elevation among men (32). Another report indicated that the prevalence rate ratio for CHD in individuals with type 2 diabetes was 4.6 in women compared to 1.8 in men (36). Despite the marked difference, little is known about the pathogenesis of cardiovascular disease in diabetes to explain the discrepancy in diabetic men and diabetic women.

One possible explanation is that the inflammatory response is more marked in women. Inflammation is now considered a primary cardiovascular risk factor and a recent study of predictors of cardiovascular risk ranks markers of inflammation, such as C-reactive protein, as comparable to markers of cholesterol (101,102). It is known that inflammation mediates the formation of atherosclerotic plaque, the major cause of cardiovascular dysfunction. An increase in WBC, pro-inflammatory cytokines, and C-reactive protein are all linked to the development of diabetes. A study by Vozarova et al. reported that a high white blood cell (WBC) count predicts the development of type 2 diabetes and a decrease of insulin action in Pima Indians (105). Studies by Thorand et al. and Barzilay et al. also found that low-grade inflammation (in particular increased C-reactive protein) was predictive of an increased risk of type 2 diabetes mellitus in middle-aged and elderly men (106,107). Hayden et al. hypothesized that type 2 diabetes mellitus is a vascular disease (atherosclerosis) and that NOS, NO, and redox stress, components of inflammation, play a causative role in type 2 diabetes, in turn causing atherosclerosis (104).

We hypothesized that one possible element of the pathogenesis of cardiovascular disease in diabetes, platelet-neutrophil conjugates, a result of inflammation, is different in diabetic women and diabetic men. Rinder et al. reports $15\% \pm 1.5\%$ of platelet-neutrophil conjugates in patients prior to cardiopulmonary bypass (273) and we found $15\% \pm 1.5\%$ of platelet-neutrophil conjugates in healthy age matched controls. We did not find the chronic baseline percentage of platelet-neutrophil conjugates to be

significantly different among any of the groups studied. We did however observe that after stimulation with PAF (platelet activating factor), a signaling molecule released with ischemia and reperfusion (274), the percentage of platelet-neutrophil conjugates was significantly less in non-diabetic women with cardiovascular disease and diabetic women with cardiovascular disease compared to diabetic women without cardiovascular disease. These results indicate that there might be some blunting effect after stimulation in women with cardiovascular disease regardless of their diabetic status.

When comparing men to women there were no chronic differences observed. The most intriguing results were found when comparing the response to acute stimulation of platelet-neutrophil conjugates. It was observed that non-diabetic women with cardiovascular disease and diabetic women with cardiovascular disease had a significantly increased reactivity to PAF in comparison to diabetic men with cardiovascular disease. These results suggest that women's blood may be more reactive to acute stimulus that occurs under ischemic conditions than diabetic men's blood. Platelet-neutrophil conjugates may contribute to thrombosis/inflammation and the greater severity of coronary heart disease observed in diabetic women as compared to diabetic men (266,275).

Platelet-Neutrophil Conjugate Formation

The primary interaction between platelets and leukocytes is via the P-selectin receptor (also known as the platelet activation dependent granule external membrane/granule

membrane protein 140 (PADGEM/GMP-140) or P-selectin or CD62) on the platelets and the P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes (141-146). Rinder et al. demonstrated that monocytes have a competitive advantage over neutrophils when binding activated platelets (141). Platelets and endothelial cells both express P-selectin. P-selectin, which is stored in α -granules of platelets and in Weibel-Palade bodies of endothelial cells, is translocated to the cellular surface within 5 to 15 minutes after stimulation with histamine, thrombin, or oxygen radicals (149-153). The adhesion between P-selectin and PSGL-1 is stabilized by binding of Mac-1 (CD11b/CD18) to a counter-receptor on platelets (144,145,154,155). The expression of Mac-1 is a result of tyrosine phosphorylation and mitogen-activated protein kinase activation as a result of PSGL-1 ligation (155-157). The binding of platelets to leukocytes was demonstrated in vitro to cause the expression of pro-inflammatory cytokines, an oxidative burst, increased expression of cell adhesion molecules like Mac-1, induce neutrophil activation, and generate signals that promote integrin activation (132,158-161).

Interaction between Platelets and Neutrophils

There is a complex interaction that occurs platelets and neutrophils. Activated platelets act as stimuli for neutrophils, but platelets may also inhibit neutrophil activation. Conversely neutrophils affect platelets. Platelet and neutrophil cross talk may affect the development of thrombotic and inflammatory diseases (166). Platelets not only play a role in hemostasis but also play a role in inflammation via releasing substances with physiologic potential and by interacting with leukocytes and vascular endothelial cells

involved in modulation of the inflammatory reaction (167). There are several components released by stimulated platelets induce neutrophil activation and chemotaxis. For example, two substances released from platelets, platelet factor 4, which increases endothelial ICAM-1 (168), and platelet-derived growth factor are chemotactic substances for human neutrophils (169,170). Thromboxane A2 (also released from platelets) may enhance neutrophil adhesion to the endothelial wall (171). P-selectin, which is responsible for the adhesion of platelets to neutrophils, could possibly optimize the signaling between the two cells (166).

Activated neutrophils produce free radicals, which in turn are potent activators of platelets. Superoxide anion is a strong platelet aggregant and increases platelet's response to thrombin (172,173). Hydrogen peroxide is also a platelet agonist, but is weaker (174). Proteolytic enzymes, which are released from activated neutrophils, causes the exposure of the active fibrinogen-binding site of the GPIIb/IIIa complex resulting in platelet aggregation (175). This evidence of platelet stimulation of neutrophils and neutrophil stimulation of platelets suggests a positive feedback loop leading to further stimulation and amplification of tissue injury (166). A study by Aziz et al. demonstrated that short term incubations of neutrophils with stimulated platelets causes an increase of oxidative bursts from the neutrophil (131).

Platelet-Neutrophil Conjugates in Cardiovascular Disease

Platelet-neutrophil conjugates were observed in various cardiovascular disease states. Neumann et al. determined that after an acute myocardial infarction (AMI) leukocyte-platelet adhesion was increased and remained elevated for at least five days after PTCA. They also determined that binding activated platelets induced IL-1 β release by leukocytes. It was concluded that the platelet-leukocyte conjugates might contribute to inflammatory responses in acute coronary syndromes (132). A study by Morse et al. reported that during cardiopulmonary bypass (CPB) patients have significantly activated platelets (increase of P-selectin) and neutrophils (increase of CD11b) (162). In another study examining CPB, Rinder et al. demonstrated that monocyte-platelet conjugates were increased significantly during CPB and neutrophil-platelet conjugates were increased only slightly (163). A study by Gawaz et al. reported platelet-leukocyte adhesion was increased in patients with symptomatic coronary heart disease compared to normal controls. It was also determined that when stimulated, patients with symptomatic coronary heart disease had a hyper-reactive response, which could indicate an increased risk of acute thrombotic event (164). We did not observe the baseline concentrations of platelet-neutrophil conjugates to be significantly different among any of the groups studied. Other studies indicate that platelet-neutrophil conjugates are activated in an acute setting, while our results indicates that platelet-neutrophil conjugates are not chronically activated. We did find that non-diabetic women with cardiovascular disease and diabetic women with cardiovascular disease had significantly increased conjugate reactivity compared to diabetic men with cardiovascular disease. This indicates that if a

diabetic women with cardiovascular disease had an acute event, their blood would react more.

Platelet-Neutrophil Conjugates in Diabetes

There are few reports of platelet-neutrophil conjugates in diabetic patients. Kaplar et al. found a significant increase in the number of monocyte-platelet aggregates in type 2 diabetics compared to non-diabetics. They also found in type 2 diabetics a correlation between elevation of postprandial serum glucose levels and platelet-monocyte aggregate formation (176). We found the baseline concentrations of platelet-neutrophil conjugates were not significantly different among any of the groups studied. We also found that after stimulation with PAF, the percentage of platelet-neutrophil conjugates were significantly decreased in non-diabetic women with cardiovascular disease and diabetic women with cardiovascular disease compared to diabetic women without cardiovascular disease. This contrary to previous studies. We would have though patients with diabetes and cardiovascular disease would have a higher amount of platelet-neutrophil conjugates in comparison to any other groups.

Implications of Platelet-Neutrophil Conjugates

Platelet aggregation in atherosclerotic coronary arteries contributes to acute myocardial ischemia. After coronary artery occlusion and reperfusion, leukocytes become activated, sequester in the microcirculation and release oxygen free radicals and proteolytic enzymes. The radicals and enzymes may be responsible for progression of vascular and

cardiomyocyte injury (178). Superoxide radical-mediated endothelial injury causes smooth muscle contraction and decreased endothelium contraction (179). The vascular injury from the oxygen free radicals may then cause a decrease of coronary blood flow reserve and coronary reactivity after reperfusion (180,181). The physical obstruction of the microvascular bed by activated leukocytes and decreased neutrophil deformability are possible factors by which leukocytes contribute to vascular injury seen in ischemic coronary artery disease (178,182,267,268,276).

Platelet-neutrophil interaction localizes both cells but also creates an optimal environment for the exchange of mediators and metabolites (141). Binding of platelets to leukocytes modulates a variety of leukocyte function including cytokine synthesis (183-185), surface expression of adhesion receptors, like CD11/CD18 (154,186), and oxidative burst (178).

The role of platelet-neutrophil conjugates in ischemia-reperfusion injury was also investigated. A study by Lefer et al. examined the effects of neutrophils and platelets separately and together, but not platelet-neutrophil conjugates, in causing cardiac dysfunction in perfused rat hearts after ischemia and reperfusion (I/R). It was demonstrated that I/R hearts perfused with neutrophils or platelets alone exhibited a decreased of 10% to 12% of heart functions and when neutrophils and platelets were perfused together, the various heart functions decreased by 50% to 60%. This study also demonstrated that blocking of P-selectin decreased the synergism between the platelets

and neutrophils. The possible mechanism of the decrease in cardiac function in this experiment includes neutrophil and platelet aggregates obstructing the flow of the coronary microvessels. Platelets and neutrophils can also release many toxic components, which could then decrease the coronary flow and the cardiac contractility (165). Another study by Kogaki et al. found that the presence of activated platelets increased neutrophil adhesion and migration after hypoxia-reoxygenation and this was inhibited with an anti-P-selectin antibody. They hypothesized that the increased neutrophil adhesion and migration via P-selectin and subsequent neutrophil activation (153).

The population of women and men in this study have different demographics and are on different medications. There was a significant increase in the age of the women with cardiovascular disease in comparison to women without cardiovascular. Overall, patients with cardiovascular disease are on more medications than patients that do not have cardiovascular disease. A higher percentage of patients with cardiovascular disease are on aspirin and calcium channel blockers. The influence of medications and age could be a reason why there was no differences observed in the baseline percentages of platelet-neutrophil conjugates. Statins are known to reduce inflammation via decreasing concentrations of C-reactive protein and upregulating endothelial nitric oxide synthase (271,272). We found that the proportions of patient on statins are not significantly different among groups, making statins less likely to influence the results. We did find that when separating the patients on statins from the patients not on statins, non-diabetic

women without CVD not on statins did have significantly higher platelet-neutrophil conjugates compared to those on statins. Although the proportions of patient on hormone replacement therapy are not significantly different among groups, we also examined the differences of estradiol levels in the women to determine if estrogen levels had an influence on inflammation. We found no significant difference in estradiol levels between any of the groups. There was also no correlation between estrogen and the percentage of platelet-neutrophil conjugates. This implies that estrogen does not influence platelet-neutrophil conjugates that we found.

Conclusion

Diabetic patients have an increased risk of cardiovascular disease; which is much more severe in diabetic women, precisely why this occurs is not known. One of the links between diabetes and cardiovascular disease is inflammation. We hypothesized that platelet-neutrophil conjugates are increased in diabetic women with cardiovascular disease compared to non-diabetic women with cardiovascular disease and diabetic men with cardiovascular disease. In this study we decided to examine one component of inflammation, platelet-neutrophil conjugates, in six groups of diabetic and non-diabetic patients with and without cardiovascular disease. We found the baseline concentrations of platelet-neutrophil conjugates were not significantly different among any of the groups studied. After stimulation with PAF, the percentage of platelet-neutrophil conjugates were significantly decreased in non-diabetic women with cardiovascular disease and diabetic women with cardiovascular disease compared to diabetic women without

cardiovascular disease. We also found non-diabetic women with cardiovascular disease and diabetic women with cardiovascular disease had significantly increased conjugate reactivity compared to diabetic men with cardiovascular disease. This indicates an increased propensity for female blood to form conjugates in response to acute stimulation. An increase in platelet-neutrophil conjugates reactivity in diabetic women could potentially be one of the factors causing more severe cardiovascular disease in diabetic patients, particularly women.

These results suggest that one of the potential causes of the difference of severity of cardiovascular disease between diabetic men and diabetic women is the diabetic women's propensity to hyper-react to an acute ischemic stimulus and activate platelet-neutrophil conjugates more than that of diabetic men. The increased number of platelet-neutrophil conjugates may localize both cell types in the microcirculation causing obstruction and ROS release. In addition, the complex activation of each of the cells by the other could occur creating a positive feed back loop of increased inflammatory activation. This increase in inflammation may then possibly increase the severity of thrombosis and cardiovascular disease.

Table 3.1. Demographics of the women and men.

Patient Characteristics	NDW-CVD (n=23)	NDW+CVD (n=25)	DW-CVD (n=20)	DW+CVD (n=24)	NDM+CVD (n=8)	DM+CVD (n=18)
Age (years)	58.5 ± 1.7	66.0 ± 2.5* **	55.7 ± 2.6	65.4 ± 2.2* **	63.8 ± 3.4	64.9 ± 2.5
Body Mass Index (kg/m ²)	26.8 ± 1.0	28.8 ± 1.4**	36.9 ± 1.8*	29.9 ± 1.5	28.2 ± 1.3	29.2 ± 1.0
Duration of Diabetes (years)	N/A	N/A	9.2 ± 1.5	14.3 ± 3.2	N/A	14.5 ± 3.4
HbA1c (%)	5.4 ± 0.1	5.2 ± 0.2**	8.2 ± 0.4*	6.9 ± 0.3* ***	5.9 ± 0.4	7.4 ± 0.4
Hypertension (%) ψ	8%	76%	55%	67%	50%	83%
Hypercholesteremia (%) ψ	25%	64%	53%	71%	100%	56%
Current Smoker (%)	0%	20%	10%	8%	38%	22%
Prior Cardiovascular Events (%)						
Myocardial Infarction ψ	0%	52%	0%	63%	63%	73%
Stroke ψ	0%	12%	0%	17%	38%	17%
PTCA ψ	0%	20%	0%	33%	25%	75%
CABG ψ	0%	20%	0%	25%	75%	33%

*P<0.05 compared to NDW-CVD group

**P<0.05 compared to DW-CVD group

***P<0.05 compared to NDW+CVD group

 ψ The proportions are significantly related (P<0.05)

Table 3.2. Distribution of medications in women and men.

Medication (%)	<u>NDW-CVD (n=23)</u>	<u>NDW+CVD (n=25)</u>	<u>DW-CVD (n=20)</u>	<u>DW+CVD (n=24)</u>	<u>NDM+CVD (n=8)</u>	<u>DM+CVD (n=18)</u>
Beta Blockers ψ	0%	48%	10%	46%	50%	53%
Diuretics ψ	4%	20%	10%	63%	50%	71%
Nitrates ψ	4%	16%	0%	21%	0%	53%
ACE Inhibitors ψ	4%	20%	65%	38%	63%	41%
Positive Intropes	0%	8%	5%	21%	0%	12%
Ca ²⁺ Channel Blockers ψ	0%	16%	10%	29%	38%	29%
Aspirin ψ	21%	56%	25%	79%	63%	59%
Anti-coagulants	0%	12%	10%	4%	25%	0%
Antioxidants ψ	50%	28%	10%	13%	0%	0%
Hypoglycemic Agents ψ	0%	0%	65%	21%	0%	29%
Statins	12%	29%	25%	34%	75%	38%
HRT	50%	64%	55%	42%	N/A	N/A

ψ The proportions are significantly related (P<0.05)

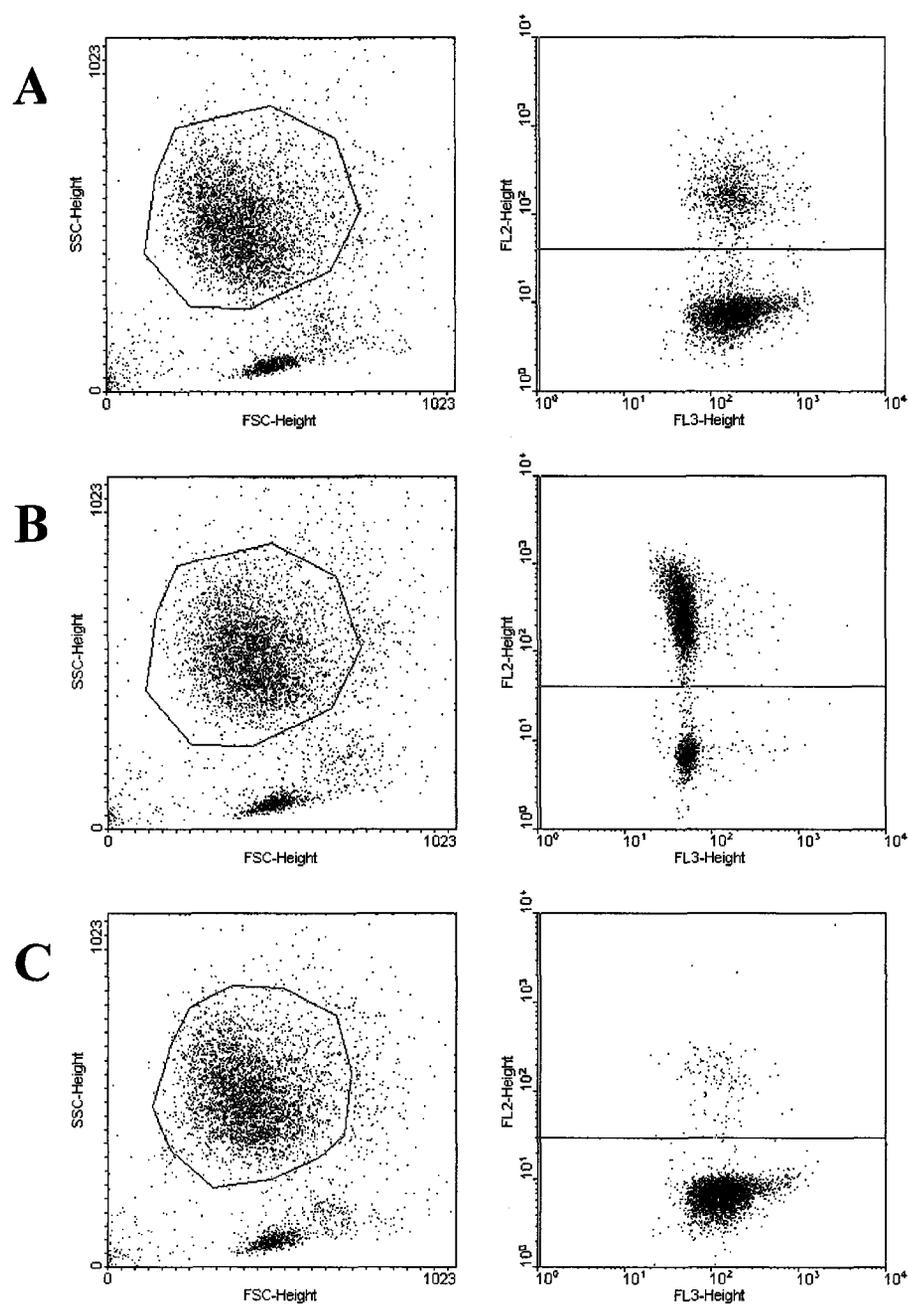


Figure 3.1. Representative FACS plots to assess platelet-neutrophil conjugates in a diabetic woman with cardiovascular disease. (A) Unstimulated blood (22.4% of platelet-neutrophil conjugates). (B) Stimulated blood (75.9% of platelet-neutrophil conjugates). (C) Inhibited blood (3.5% of platelet-neutrophil conjugates).

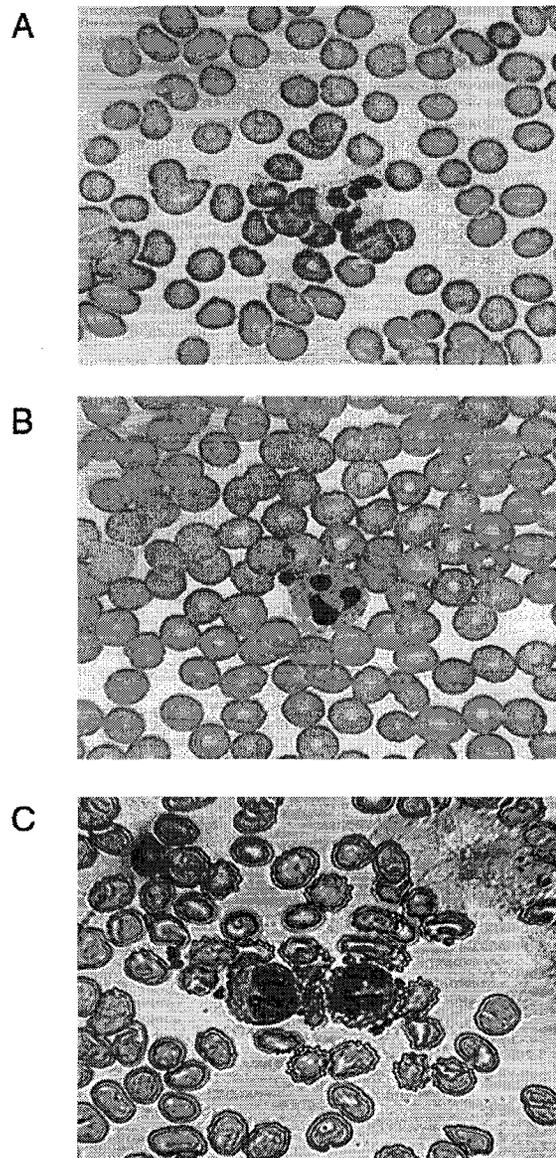


Figure 3.2. Pictures demonstrating platelet-neutrophil interaction. (A) Unstimulated blood from a non-diabetic woman without cardiovascular disease. (B) Stimulated blood (with PAF) from a diabetic man with cardiovascular disease. (C) Stimulated blood (with PAF) from a diabetic woman with cardiovascular disease.

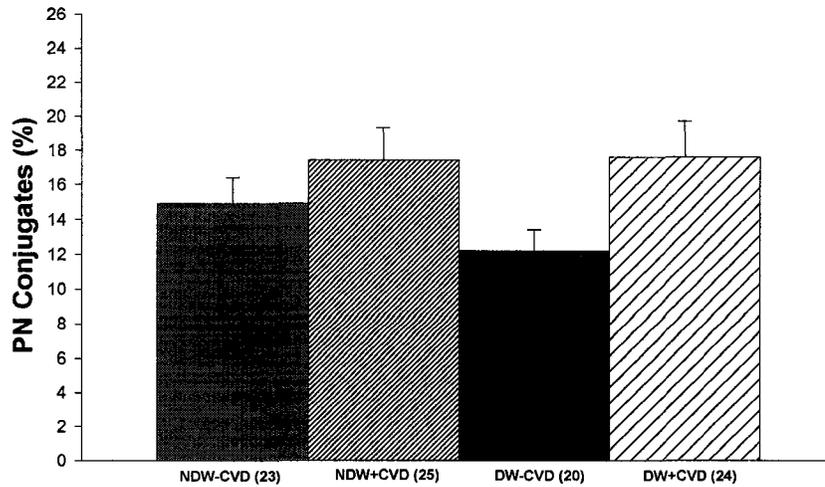


Figure 3.3. Summary of percent of platelet-neutrophil conjugates for women in the study. Comparison of non-diabetic women without cardiovascular disease (NDW-CVD) versus non-diabetic women with cardiovascular disease (NDW+CVD) versus diabetic women without cardiovascular disease (DW-CVD) versus diabetic women with cardiovascular disease (DW+CVD). There is no significant difference among any of these groups.

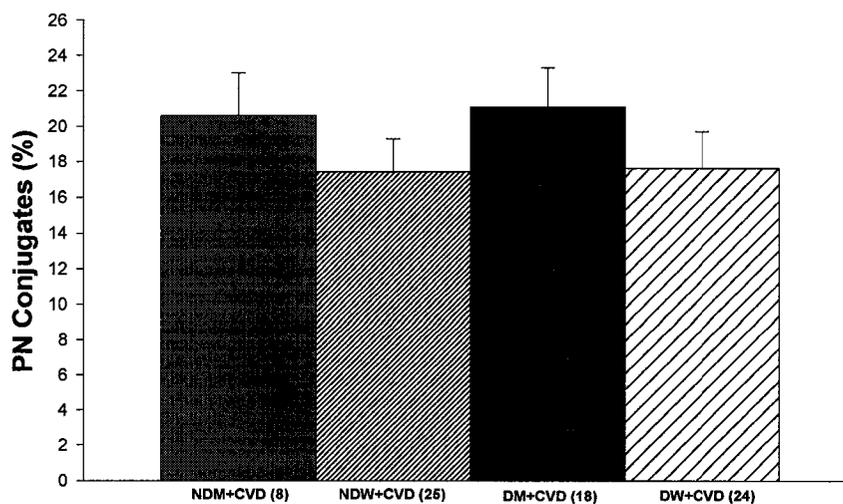


Figure 3.4. Summary of percent of platelet-neutrophil conjugates for women and men in the study. Comparison of non-diabetic men with cardiovascular disease (NDM+CVD) versus non-diabetic women with cardiovascular disease (NDW+CVD) versus diabetic men with cardiovascular disease (DM+CVD) versus diabetic women with cardiovascular disease (DW+CVD). There is no significant difference among any of the groups.

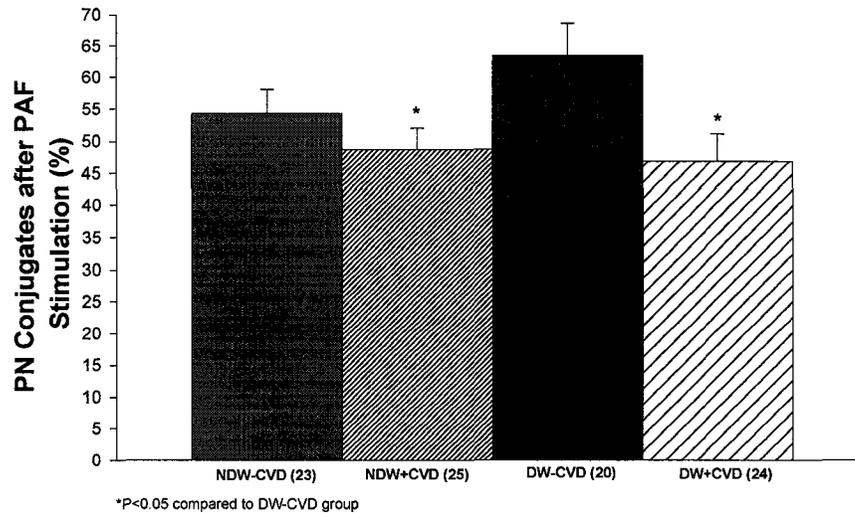


Figure 3.5. Summary of percent of platelet-neutrophil conjugates after stimulation with PAF for women in the study. Comparison of non-diabetic women without cardiovascular disease (NDW-CVD) versus non-diabetic women with cardiovascular disease (NDW+CVD) versus diabetic women without cardiovascular disease (DW-CVD) versus diabetic women with cardiovascular disease (DW+CVD). NDW+CVD and DW+CVD had significantly less platelet-neutrophil conjugates after stimulation in comparison to DW-CVD ($P<0.05$).

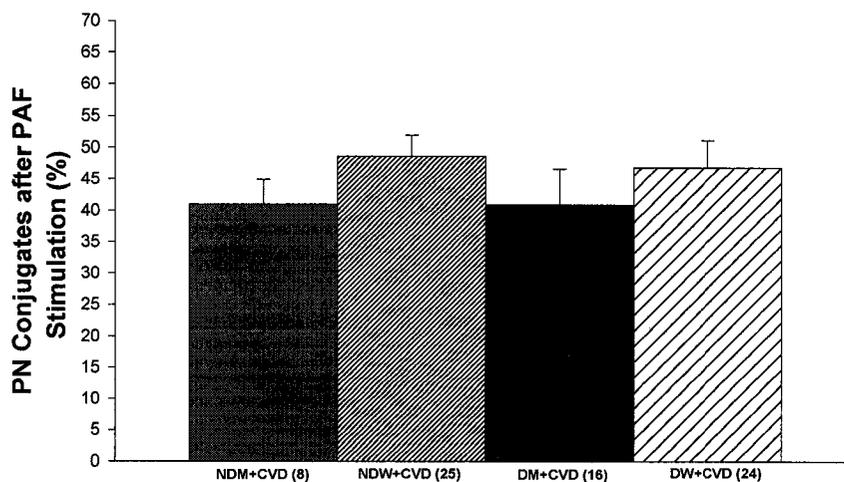


Figure 3.6. Summary of percent of platelet-neutrophil conjugates after stimulation with PAF for women and men in the study. Comparison of non-diabetic men with cardiovascular disease (NDM+CVD) versus non-diabetic women with cardiovascular disease (NDW+CVD) versus diabetic men with cardiovascular disease (DM+CVD) versus diabetic women with cardiovascular disease (DW+CVD). There is no significant difference among any of the groups.

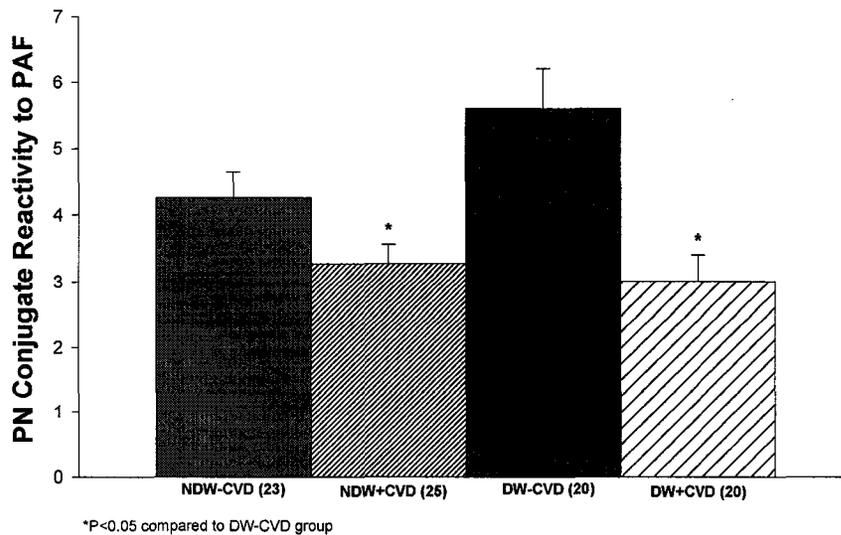


Figure 3.7. Summary of platelet-neutrophil conjugate reactivity to PAF for women in the study. Comparison of non-diabetic women without cardiovascular disease (NDW-CVD) versus non-diabetic women with cardiovascular disease (NDW+CVD) versus diabetic women without cardiovascular disease (DW-CVD) versus diabetic women with cardiovascular disease (DW+CVD). NDW+CVD and DW+CVD had significantly less stimulation ratio of platelet-neutrophil conjugates in comparison to DW-CVD ($P<0.05$).

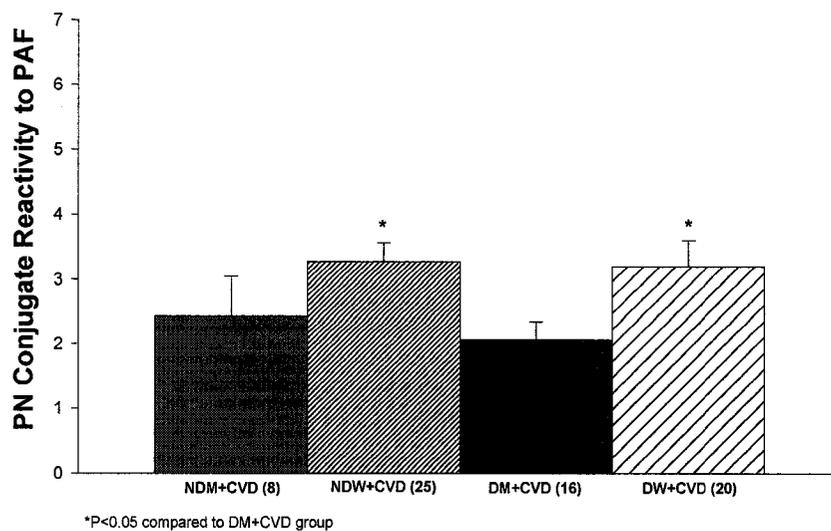


Figure 3.8. Summary of platelet-neutrophil conjugate reactivity to PAF for women and men in the study. Comparison of non-diabetic men with cardiovascular disease (NDM+CVD) versus non-diabetic women with cardiovascular disease (NDW+CVD) versus diabetic men with cardiovascular disease (DM+CVD) versus diabetic women with cardiovascular disease (DW+CVD). NDW+CVD and DW+CVD had significantly greater stimulation ratio of platelet-neutrophil conjugates in comparison to DM+CVD ($P<0.05$).

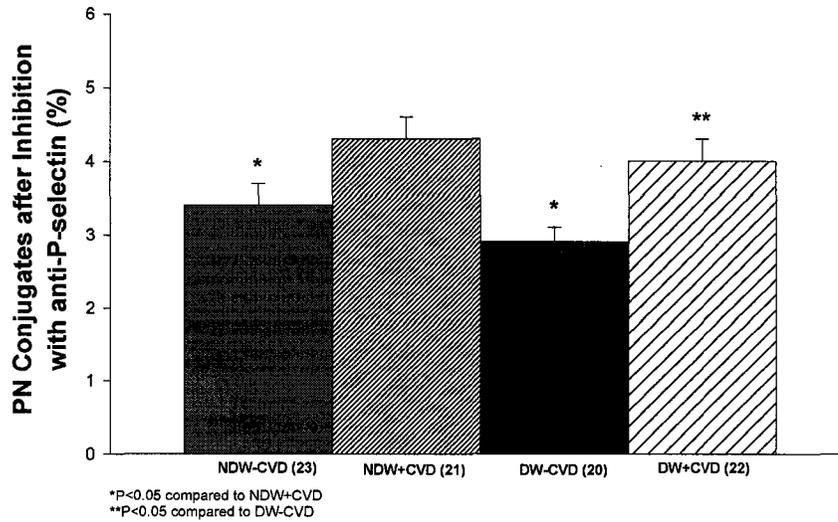


Figure 3.9. Summary of inhibition of platelet-neutrophil conjugates with anti-P-selectin for women in the study. Comparison of non-diabetic women without cardiovascular disease (NDW-CVD) versus non-diabetic women with cardiovascular disease (NDW+CVD) versus diabetic women without cardiovascular disease (DW-CVD) versus diabetic women with cardiovascular disease (DW+CVD). NDW-CVD and DW-CVD had significantly less platelet-neutrophil conjugates in comparison to NDW+CVD ($P<0.05$). DW+CVD had significantly greater platelet-neutrophil conjugates in comparison to DW-CVD ($P<0.05$).

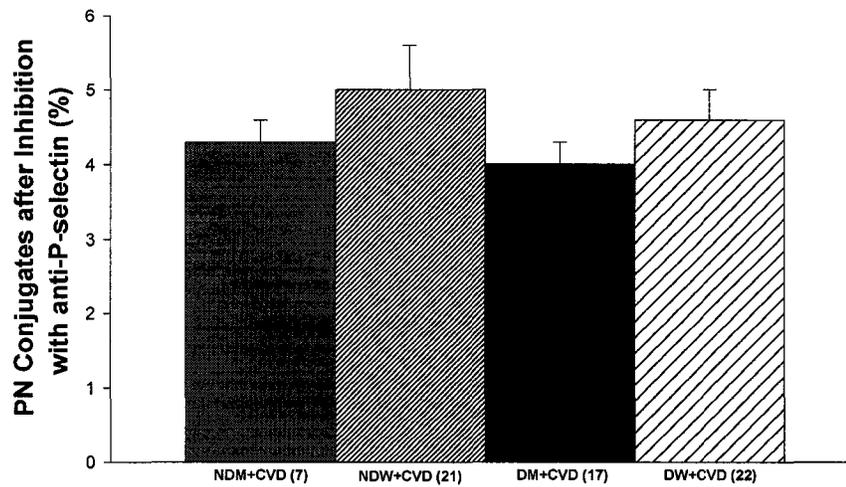


Figure 3.10. Summary of inhibition of platelet-neutrophil conjugates with anti-P-selectin for women and men in the study. Comparison of non-diabetic men with cardiovascular disease (NDM+CVD) versus non-diabetic women with cardiovascular disease (NDW+CVD) versus diabetic men with cardiovascular disease (DM+CVD) versus diabetic women with cardiovascular disease (DW+CVD). There is no significant difference among any of the groups.

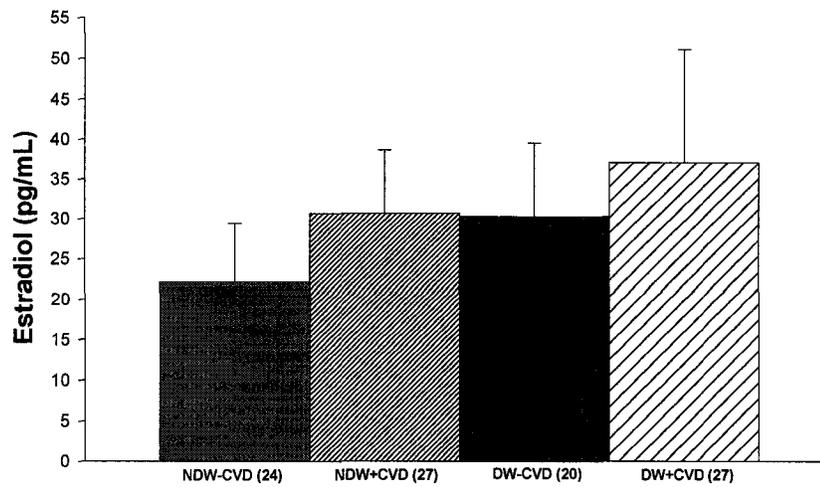


Figure 3.11. Summary of estradiol for all women in the study. Comparison of non-diabetic women without cardiovascular disease (NDW-CVD) versus non-diabetic women with cardiovascular disease (NDW+CVD) versus diabetic women without cardiovascular disease (DW-CVD) versus diabetic women with cardiovascular disease (DW+CVD). There is no significant difference among any of the groups.

CHAPTER 4. PRO-INFLAMMATORY CYTOKINES ARE INCREASED IN TYPE 2 DIABETIC WOMEN WITH CARDIOVASCULAR DISEASE

INTRODUCTION

There is a strong link between diabetes and cardiovascular disease. Cardiovascular disease is the number one killer of patients with diabetes. Men with diabetes are 2 to 3 times more likely to die from CHD than those without diabetes, and the risk for women is even higher (277). Patients with diabetes have increased morbidity and mortality associated with cardiovascular disease. The precise mechanism as to why patients with diabetes have such an increased risk of cardiovascular disease is unclear. It is currently understood that inflammation plays a role in cardiovascular disease (101,102). One hypothesis to explain why diabetic patients have more severe cardiovascular disease is that diabetic patients have an increase in inflammation compared to the non-diabetic patient. One component of inflammation that was reported in both diabetes and cardiovascular disease is the production of pro-inflammatory cytokines, particularly interleukin-6 (IL-6), tumor necrosis factor (TNF- α), and interleukin-1 (IL-1 β).

Cytokines are soluble polypeptides released from cells of the immune system, particularly macrophages. Pro-inflammatory cytokines including, IL-6, TNF- α , and IL-1 β , are major mediators in the induction of the acute phase response (119). Previous studies indicate that IL-6, TNF- α , and IL-1 β are increased in many different types of cardiovascular disease including unstable angina, myocardial dysfunction, chronic heart

failure, myocardial infarction, and different cardiac surgeries (228-231,233,234). All three cytokines play a key role in the development of atherosclerosis (133). It was also demonstrated that IL-6 enhanced the development of atherosclerosis and IL-1 β was associated with the development of a lethal arterial inflammation in mice (192,216).

The role of cytokines in diabetes is also under intense investigation. Studies by Lechleitner et al., Yudkin et al., and McCarty et al. indicate TNF- α may play a role in the development of diabetes by impairing insulin action (211,212,244). IL-6 and TNF- α was observed to be increased in diabetic patients (247,248). In another study, IL-6 was increased in response to acute hyperglycemia (251). There is little investigation of IL-1 β in diabetic patients.

It is apparent that cytokines play a pathologic role in both diabetes and cardiovascular disease. It is possible that cytokine concentrations are additive in patients with diabetes and cardiovascular disease, causing more severe disease. Cytokine concentrations in patients, particularly women, with cardiovascular disease and diabetes combined have not been reported previously. In this study three pro-inflammatory cytokines, IL-6, TNF- α , and IL-1 β were examined via Enzyme Linked-Immuno-Sorbent Assay (ELISA) in four different groups of women: healthy age-matched controls, non-diabetic women with cardiovascular disease, diabetic women without cardiovascular disease, and diabetic women with cardiovascular disease. We found that IL-6 in diabetic women with cardiovascular disease was significantly greater in comparison to healthy age-matched

controls. There was also a trend for TNF- α to be greater in diabetic women with cardiovascular disease and diabetic women without cardiovascular disease in comparison to healthy age-matched controls, but no statistical significance. IL-1 β was not significantly different among the four groups. This study demonstrates an increase of TNF- α and IL-6 in diabetic women with and without cardiovascular disease in comparison to healthy age-matched controls. The additive increase in cytokines suggests a common inflammatory etiology in cardiovascular disease and diabetes in women.

RESEARCH DESIGN AND METHODS

Subjects

Four groups were studied; non-diabetic women without cardiovascular disease (ND-CVD) (n=24), non-diabetic women with cardiovascular disease (ND+CVD) (n=7), diabetic women without cardiovascular disease (D-CVD) (n=19), and diabetic women with cardiovascular disease (D+CVD) (n=12). These subjects were enrolled from the patients attending the Cardiology Clinic at the Veterans Administration Hospital (Tucson, AZ) and the Diabetes Clinic at the University of Arizona Medical Center (Tucson, AZ). The protocol was approved by the University of Arizona Institutional Review Board. Patient's characteristics are summarized in Table 4.1 and their medications are summarized in Table 4.2. The severity of diabetes was determined by the patient's hemoglobin A_{1C}. A subject was considered to have cardiovascular disease if they had a previous cardiovascular event (myocardial infarction, coronary artery bypass graft

(CABG), stroke, percutaneous coronary intervention (PCI), or some combination). Fifteen milliliters of blood were drawn in a citrate tube from all the patients.

IL-6 Measurement

The blood samples were centrifuged in a (Megafuge 1.0R, Baxter Scientific Products) at 2,500 RPM for thirty minutes at 4°C. The plasma was pipetted from each sample and then immediately stored at -70°C. Once a sufficient number of samples were collected, they were removed from the freezer and thawed to room temperature. An ELISA human IL-6 Ultrasensitive kit from Biosource International was used. The samples were prepared according to the directions provided by Biosource International. At first, the hIL-6 standard was reconstituted and diluted. The standard was reconstituted to 2500 /ml with standard diluent buffer. The sample was mixed gently and allowed to sit for ten minutes to ensure complete reconstitution. The standard needed to be used within one hour of reconstitution. Then, 0.010 mL of the reconstituted standard was added to a tube containing 2.490 mL of the standard diluent buffer. This tube was labeled 10.0 pg/mL hIL-6. Then, serial dilutions of the standard were done to ultimately have six tubes labeled 5.0, 2.5, 1.25, 0.62, 0.31, and 0.16 pg/mL hIL-6. Then the streptavidin-HRP was made. This comes in the kit as 50x concentration. 20 µL of the concentrated solution was diluted in 1 mL of streptavidin-HRP diluent for each 8-well strip that was used in the assay. Next, the number of 8-well strips needed for the assay was determined and set aside. 100 µL of the standard diluent buffer was added to the zero wells. 100µL of the standards, samples, and controls were added to the appropriate microtiter wells. The

plate was then covered and incubated for three hours at 37°C. Then the liquid from each well was thoroughly aspirated or decanted. After this, the wells were then washed six times. 100 µL of biotinylated anti-IL-6 solution was added to all wells except the chromogen blank wells. The plate was tapped gently to ensure thorough mixing. The plate was then covered and incubated for 45 minutes at room temperature. The liquid from each well was thoroughly aspirated and each well was washed six times. 100 µL of streptavidin-HRP was added to each well except the chromogen blank and the plate was covered and incubated again for 45 minutes at room temperature. Each well was again thoroughly aspirated and washed six times. Then 100 µL of stabilized chromogen was added to each well and the liquid in the wells turned blue. This was incubated for 30 minutes at room temperature, in the dark. After the final incubation, 100 µL of stop solution was added to each well and the wells turned from blue to yellow. The plates were then read at 450 nm (Vmax, Molecular Devices) having blanked the plate reader against a chromogen blank composed of 100 µL each of stabilized chromogen and stop solution. The plate was read within two hours of adding the stop solution. A standard curve was plotted and all the unknown samples were determined from the standard curve (Figure 4.1).

TNF- α Measurement

The blood samples were centrifuged in a (Megafuge 1.0R, Baxter Scientific Products) at 2,500 RPM for thirty minutes at 4°C. The plasma was pipetted from each sample and then immediately stored at -70°C. Once a sufficient number of samples were collected,

they were removed from the freezer and thawed to room temperature. An ELISA human TNF- α Ultrasensitive kit from Biosource International was used. The samples were prepared according to the directions provided by Biosource International. At first, the hTNF- α standard was reconstituted and diluted. The standard was reconstituted to 380 pg/ml with standard diluent buffer. The sample was mixed gently and allowed to sit for ten minutes to ensure complete reconstitution. The standard needed to be used within one hour of reconstitution. Then, 0.050 mL of the reconstituted standard was added to a tube containing 0.550 mL of the standard diluent buffer. This tube was labeled 32 pg/mL hTNF- α . Then, serial dilutions of the standard were done to ultimately have six tubes labeled 16, 8, 4, 2, 1, and 0.5 pg/mL hTNF- α . Then the streptavidin-HRP was made. This comes in the kit as 100x concentration. 10 μ L of the concentrated solution was diluted in 1 mL of streptavidin-HRP diluent for each 8-well strip that was used in the assay. Then, the number of 8-well strips needed for the assay was determined and set aside. 50 μ L of incubation buffer was added to all wells except for the chromagen blank. 100 μ L of the standard diluent buffer was added to the zero wells. 100 μ L of the standards were added to the appropriate microtiter wells and 50 μ L of standard diluent buffer and sample were added to the remaining wells. The side of the plate was tapped gently to ensure proper mixing. Then 50 μ L of biotinylated anti- TNF- α solution was added to all wells except the chromogen blank wells. The plate was tapped gently to ensure thorough mixing. The plate was then covered and incubated for two hours at 37°C. The liquid from each well was then thoroughly aspirated and each well was washed four times. 100 μ L of streptavidin-HRP was added to each well except the

chromogen blank and the plate was covered and incubated again for 30 minutes at room temperature. Each well was again thoroughly aspirated and washed four times. Then 100 μ L of stabilized chromogen was added to each well and the liquid in the wells turned blue. This was incubated for 30 minutes at room temperature, in the dark. After the final incubation, 100 μ L of stop solution was added to each well and the wells then turned from blue to yellow. The plates were read at 450 nm (Vmax, Molecular Devices) having blanked the plate reader against a chromogen blank composed of 100 μ L each of stabilized chromogen and stop solution. The plate was read within two hours of adding the stop solution. A standard curve was plotted and all the unknown samples were determined from the standard curve.

IL-1 β Measurement

The blood samples were centrifuged in a (Megafuge 1.0R, Baxter Scientific Products) at 2,500 RPM for thirty minutes at 4°C. The plasma was pipetted from each sample and then immediately stored at -70°C. Once a sufficient number of samples were collected, they were removed from the freezer and thawed to room temperature. An ELISA human IL-1 β Ultrasensitive kit from Biosource International was used. The samples were prepared according to the directions provided by Biosource International. At first, the hIL-1 β standard was reconstituted and diluted. The standard was reconstituted to 2500 pg/ml with standard diluent buffer. The sample was mixed gently and allowed to sit for ten minutes to ensure complete reconstitution. The standard needed to be used within one hour of reconstitution. Then, 0.020 mL of the reconstituted standard was added to a

tube containing 2.480 mL of the standard diluent buffer. This tube was labeled 20.0 pg/mL hIL-1 β . Next, serial dilutions of the standard were done to ultimately have six tubes labeled 10, 5, 2.5, 1.25, 0.63, and 0.31 pg/mL hIL-1 β . Then the streptavidin-HRP was made. This comes in the kit as 100x concentration. 10 μ L of the concentrated solution was diluted in 1 mL of streptavidin-HRP diluent for each 8-well strip that was used in the assay. Then, the number of 8-well strips needed for the assay was determined and set aside. 100 μ L of the standard diluent buffer was added to the zero wells. 100 μ L of the standards, samples, and controls and 100 μ L of incubation buffer was added to the appropriate microtiter wells. The plate was covered and incubated for three hours at room temperature. Then the liquid from each well was thoroughly aspirated or decanted. Afterwards the wells were washed four times. Then 100 μ L of biotinylated anti-IL-1B solution was added to all wells except the chromogen blank wells. The plate was tapped gently to ensure thorough mixing. The plate was then covered and incubated for one hour at room temperature. The liquid from each well was thoroughly aspirated and each well was washed four times. 100 μ L of streptavidin-HRP was added to each well except the chromogen blank and the plate was covered and incubated again for 30 minutes at room temperature. Each well was again thoroughly aspirated and washed four times. Then 100 μ L of stabilized chromogen was added to each well and the liquid in the wells turned blue. This was incubated for 30 minutes at room temperature, in the dark. After the final incubation, 100 μ L of stop solution was added to each well and the wells turned from blue to yellow. The plates were read at 450 nm (Vmax, Molecular Devices) having blanked the plate reader against a chromogen blank composed of 100 μ L each of

stabilized chromogen and stop solution. The plate was read within two hours of adding the stop solution. A standard curve was plotted and all the unknown samples were determined from the standard curve.

Data Analysis

Data were collected in notebooks and transferred to a Computer Spreadsheet Format (Excel for Windows). The summary results are represented as mean \pm standard error of the mean (SEM). Cytokine concentrations are expressed as pg/mL. Comparisons among the four groups were made with an ANOVA (Sigma-Stat 3.0). A Dunn's and Holm-Sidak Post hoc test was performed (Sigma-Stat). A Chi squared test was performed to determine if the percentages in table 4.1 and 4.2 were significantly related (Sigma-Stat 3.0). $P < 0.05$ was considered as statistically significant.

RESULTS

Patient Characteristics

Table 4.1 summarizes the characteristics of the four groups of women studied. There was a significant difference of age in the D+CVD group compared to the D-CVD group ($p < 0.05$). There was also a significant difference in body mass index (BMI) of the D-CVD group compared to three other groups ($p < 0.01$). There was no significant difference of the duration of diabetes between the two diabetic groups. The glycated hemoglobin (HbA_{1C}) was significantly greater in diabetic women without CVD compared to non-diabetic women with and without CVD ($p < 0.01$). There are significantly more

patients with hypertension in the ND+CVD and D+CVD group compared to the ND-CVD group. There was no significant difference in hypercholesteremia or percent of smokers among any of the groups. The various drug therapies that the patients were receiving are given in Table 4.2. There was no significant difference in the percentages of women taking HRT or statins among any of the groups. There was a significant difference in the percentages of women taking beta blockers, diuretics, ACE inhibitors, antioxidants, aspirin, and hypoglycemic agents.

IL-6 Production.

Figure 4.2 indicates that IL-6 concentration was graded. In comparison to non-diabetic women without cardiovascular disease, we found that IL-6 was significantly increased in diabetic women with cardiovascular disease (Fig. 2) ($p < 0.05$). IL-6 in non-diabetic women without cardiovascular disease was 0.33 ± 0.06 . IL-6 in non-diabetic women with cardiovascular disease was 0.47 ± 0.11 . IL-6 in diabetic women without cardiovascular disease was 0.96 ± 0.27 . IL-6 was greatest in diabetic women with cardiovascular disease (1.41 ± 0.48).

TNF- α Production

Figure 4.3 indicates that TNF- α also appeared to be graded. There was a trend toward increasing TNF- α production in diabetic women with cardiovascular disease to be greater than the other groups, but the increase did not reach statistical significance. In comparison to non-diabetic women without cardiovascular disease, we found that there

was a trend toward increasing of TNF- α in diabetic women with and diabetic women without cardiovascular disease. TNF- α in non-diabetic women without cardiovascular disease was 2.29 ± 0.37 . TNF- α in non-diabetic women with cardiovascular disease was 2.33 ± 0.89 . TNF- α in diabetic women without cardiovascular disease was 3.93 ± 0.53 . TNF- α was greatest in diabetic women with cardiovascular disease (4.53 ± 1.38). There was no statistical significance between any of the groups.

IL-1 β Activation

Figure 4.4 indicates that there was not a graded response in IL-1 β for the four groups. There was no significance among any of the groups of women. IL-1 β in non-diabetic women without cardiovascular disease was 0.15 ± 0.05 . IL-1 β in non-diabetic women with cardiovascular disease was 0.08 ± 0.08 . IL-1 β in diabetic women without cardiovascular disease was 0.25 ± 0.09 . IL-1 β in diabetic women with cardiovascular disease was 0.22 ± 0.14 .

DISCUSSION

There are 17 million people in the United States who have diabetes and 95% of those have type 2 diabetes (1). The risk for cardiovascular disease (CVD) is 2 to 8 fold higher in people with diabetes than matched non-diabetic individuals (2). Diabetes mellitus is a much stronger risk factor for coronary heart disease (CHD) in women than in men. Diabetes is associated with a three to sevenfold elevation in CHD risk among women

compared with a two to threefold elevation among men (32). Howard et al. reported that the prevalence rate ratio for CHD in individuals with type 2 diabetes was 4.6 in women compared to 1.8 in men (35). According to the American Heart Association, cardiovascular disease was the number one cause of death in women in the year 2000. There were over 945,000 deaths from CVD in the United States. 53% of these deaths due to CVD were in women (12). It is apparent that there is a factor or combination of factors that causes women with diabetes to have more severe cardiovascular disease.

It is known that inflammation mediates the formation of atherosclerotic plaques, which contributes to cardiovascular disease. Inflammation is now considered a cardiovascular risk factor and a recent study of predictors of cardiovascular risk ranks markers of inflammation as comparable to markers of cholesterol (102). The association of inflammation and diabetes is a topic of active research. Our laboratory has demonstrated that neutrophil activation and platelet-neutrophil interactions are increased in type 2 diabetic men (264). Hokoma et al. reported diabetic rat hearts suffer excessive reperfusion injury following ischemia (276). An enhanced inflammatory response may contribute to the greater recurrence of myocardial infarction and ischemic heart failure in diabetes. In this study, we tested the hypothesis that a component of the inflammatory response, pro-inflammatory cytokines, was increased in CVD and further increased in diabetic women with CVD. There appears to be an additive effect of CVD and diabetes on the chronic concentrations of pro-inflammatory cytokines in women. We examined interleukin-6 (IL-6), interleukin-1 (IL-1 β), and tumor necrosis factor (TNF- α) in women

with only CVD, women with only diabetes, and women with both diabetes and CVD, and then compared them to healthy age-matched controls. These pro-inflammatory cytokines appear to play an important role in the pathology of both diabetes and cardiovascular disease. Cytokines aggravate the development of atherosclerosis in CVD, while cytokines potentially cause insulin insensitivity in diabetes. We found trends towards increasing plasma cytokines and the incidence of CVD and diabetes. The association of plasma IL-6 was the most consistent and dramatic finding. Women with diabetes and CVD demonstrated a significant increase in plasma IL-6. This study also revealed an interesting pattern of IL-6 and TNF- α cytokine. Women with both diabetes and CVD had the highest concentration of these cytokines. Lower concentrations were observed in diabetic women without CVD, and women with only CVD, respectively. Finally, healthy age-matched controls had the lowest concentrations of IL-6 and TNF- α . This trend suggests that as women develop diabetes and cardiovascular disease, the inflammatory stimuli in the blood increases, which could then lead to more severe cardiovascular disease, including atherosclerosis. This could possibly be a result of hyperglycemia, but IL-6 concentrations did not correlate with HbA_{1c}. Alternatively, these data suggests the chronic inflammation underlies both CVD and diabetes. In this study, IL-1 β did not differ significantly among any of the groups, suggesting that this cytokine may not be a key component in the cardiovascular disease process observed in diabetic women.

Inflammation in Diabetes and Cardiovascular Disease

The metabolic events that occur during an inflammatory response to a pathogen also occur in certain disease processes, i.e. atherosclerosis and diabetes mellitus. These changes do not appear to have a beneficial purpose but rather contribute to the pathology of the disease. Hayden et al. hypothesized that type 2 diabetes mellitus is a vascular disease (atherosclerosis) and that NOS, NO, and redox stress, components of inflammation, play a causative role in type 2 diabetes, in turn causing atherosclerosis (104). Inflammation, in particular cytokines, plays a key role in atherosclerosis (133). In atherogenesis, endothelial dysfunction is an early step. Injury to the endothelium increases adhesion of leukocytes and platelets. The inflammatory response stimulates migration and proliferation of smooth-muscle cells. This forms an intermediate lesion. Chronic inflammation results in increased numbers of macrophages and lymphocytes. Activation of these cells leads to release of hydrolytic enzymes, cytokines, chemokines, and growth factors that cause more damage and then leads to focal necrosis. The lesion is then covered by a fibrous cap that overlies a core of lipid and necrotic tissue (133,278).

Inflammation was also reported to be increased in type 2 diabetes. Esposito et al. reported that hyperglycemia acutely increased circulating IL-6 and TNF- α (279). Pradham et al. reported baseline IL-6 was increased in women who later developed type 2 diabetes. The relative risk of future type 2 diabetes in women between the highest and lowest quartiles was 7.5 for interleukin-6. The results of this study support a role for inflammation in the development of diabetes (280). These acute phase reactants may

explain the clinical and biochemical features of type 2 diabetes and its complications. The increased cytokines in diabetes appear to originate from non-circulating cells (247) and the likely candidates are adipocytes and endothelial cells. If cytokines chronically circulate in diabetic patients, then there could be continuous aggravation of atherosclerosis causing more sites of atherosclerosis and acceleration of the progression of the lesions. Despite the apparent links between diabetes and cardiovascular disease, there are no reports focusing on the concentrations of cytokines in women with both diabetes and cardiovascular disease.

Cytokines and Cardiovascular Disease

Cytokine activity is implicated in various aspects of the pathogenesis in cardiovascular disease including atherosclerosis (218-220), acute myocardial infarction (221), congestive heart failure (222-226), myocarditis (222,224), and allograft rejection (227). Cardiovascular disease is characterized by a chronic low-level inflammatory process with increased circulating concentrations of pro-inflammatory cytokines (such as IL-6, TNF- α , IL-1 β) (133).

Atherosclerosis and cytokines

Our data demonstrates that patients with diabetes and CVD have increased IL-6. The pathogenesis of atherosclerosis involves many different cytokines, particularly the pro-inflammatory cytokines, IL-6, IL-1 β , and TNF- α . Evidence that IL-6 plays an active role in cardiovascular disease is demonstrated in a study by Huber et al. This investigation

injected Apo-E-deficient mice with IL-6 or saline. Fatty streak lesions were 1.9-5.1 fold larger in the IL-6 treated mice compared to the saline treated mice, demonstrating that exogenously administered IL-6 significantly enhanced fatty lesion development in the atherosclerosis-prone, but not in the atherosclerosis-resistant animals. This suggests that IL-6 likely has an active role in the atherosclerosis disease process (192). It was also determined that there were large quantities of IL-6 found in human atherosclerotic plaques, typically in macrophage rich area (243). IL-1 β was also observed in atherosclerotic lesions (218,219).

In a study by Nicklin et al., mice in which the gene for IL-1 receptor antagonist was knocked out were examined. It was observed that there was a lethal arterial inflammation characterized by transmural infiltration of neutrophils, macrophages, and T lymphocytes. The lesions demonstrated the effects of IL-1 on endothelial cells and smooth-muscle cells. The authors concluded that IL-1 receptor antagonist produced in healthy mice protects them from the effects of interleukin-1 (217).

Interleukin-6

In several studies, IL-6 was increased after acute myocardial infarctions and unstable angina (228-230). Studies by Tsutamoto et al. and Roig et al., demonstrated that IL-6 was increased to 4.1 ± 0.6 pg/mL and 18 ± 19 pg/mL, respectively, in patients with congestive heart failure compared with normal subjects (233,234). In the study by Roig et al., increased IL-6 was associated with lower ejection fraction and worse prognosis

(228). Suzuki et al. reported that patients with stable angina pectoris undergoing elective coronary angioplasty IL-6 was significantly increased one and six hours after the procedure (3.6 and 4.4-fold, respectively). These patients with increased IL-6 later presented with restenosis (241). IL-6 was also reported to be increased preoperatively (196) and after various cardiac surgeries including CABG and valvular surgery (235-240). These studies demonstrate clear evidence that IL-6 is increased in response to various different cardiac diseases. These studies agree with our findings of increased IL-6 in patients with different forms of CVD. In this study, we found that IL-6 was significantly increased in diabetic women with CVD in comparison to healthy age-matched controls. We also found women with CVD only had an increase in IL-6 production compared to healthy age-matched controls, but the data was not significant (Figure 4.2). This increased concentration of IL-6 observed in diabetic women with and without CVD could reflect the diabetogenesis. The even higher IL-6 concentrations observed in diabetic women with CVD could then represent an added risk factor of atherosclerosis.

Tumor Necrosis Factor-alpha

The literature on TNF- α found in various different cardiac disease states is conflicting. Both pro-inflammatory cytokines TNF- α and IL-1 β are implicated in the pathogenesis of myocardial dysfunction in ischemia-reperfusion injury and chronic heart failure. In a study by Cain et al. it was demonstrated that TNF- α and IL-1 β separately and synergistically decreased human myocardial function in a dose-dependent fashion,

affecting systolic more than diastolic function. Concentrations as low as 1.25 pg/mL of TNF- α decreased the force of myocardial development (231). Tsutamoto et al. and Roig et al. observed TNF- α to be increased in patients with congestive heart failure compared to control subjects (233,234). In contrast, Pannitteri et al. observed no significant rise of TNF- α in patients following an acute myocardial infarction and Nomura et al. found TNF- α did not increase preoperatively in a group of patients prior to vascular surgery (196,229). Regarding TNF- α we found this cytokine to be increased in patients with CVD only, diabetes only, and both CVD and diabetes in comparison to normal controls, but not significantly (Figure 4.3). This increase in TNF- α , agrees with some reports in the literature on TNF- α (233,234). The increase in TNF- α observed in diabetic women with CVD could be one of the mediators of atherogenesis. It could also be one of the contributors to the pathogenesis of diabetes itself.

Interleukin-1 β

The literature on IL-1 β found in various cardiac disease states is conflicting. It was demonstrated that IL-1 β decreased the function of human myocardial trabeculae in a dose-dependent fashion, affecting systolic more than diastolic function (231). Nomura et al. found that IL-1 was increased preoperatively in a group of patients prior to vascular surgery (196), but Yazdani et al. found that IL-1 was not significantly different between stable and unstable angina (230). The result of IL-1 β in this study demonstrated that there was no significant difference among the four different groups that were studied;

CVD only, diabetes only, CVD and diabetes, and healthy age-matched controls (Figure 4.4). The data on the role of IL-1 β in diabetes and cardiovascular disease is less convincing. IL-1 β may not be one of the cytokines that is chronically increased in patients with diabetes and CVD, therefore not contributing to the increased severity of disease observed in diabetic women with CVD.

Diabetes and cytokines

It is understood that cytokines play a significant role in diabetes. There is evidence that inflammation is an initiating factor in insulin insensitivity and pro-inflammatory cytokines are the key linkage between inflammation and insulin insensitivity (211,244-246). Our data demonstrated that type 2 diabetic women with CVD had increased concentrations of IL-6. Several studies reported an increase in circulating cytokines in patients with diabetes (247,248). This increase in cytokine production could then lead to inappropriate metabolic effects causing arteriosclerosis. IL-6 and TNF- α were significantly increased in type 2 diabetic patients compared to normal subjects in a study by Pickup et al. The diabetic patients were observed to have higher body mass indices, glycated hemoglobin percentage (HbA_{1C}), and plasma total cholesterol and triglyceride concentration. Five of the twenty diabetic patients in this study had microvascular (including retinopathy and nephropathy) and/or macrovascular disease (including myocardial infarction, angina, peripheral vascular disease, or stroke) and there was no significant difference in comparison to diabetic patients without vascular disease (247).

In contrast, Mooradian et al. reported that diabetes mellitus was not associated with significant changes in serum TNF- α or IL-1 β (249).

Bastard et al. observed obese non-diabetic and diabetic patients with high insulin resistance had increased IL-6 and TNF- α in comparison to lean controls. IL-6, not TNF- α , was found to be proportional to insulin resistance and blood glucose. Thus, severe diabetes is associated with increased IL-6. When the obese patients were placed on a very low-calorie diet (VLCD) for a three-week period, the IL-6 decreased significantly in both adipose tissue and serum. TNF- α however was unchanged. They found serum concentration of IL-6 and TNF- α were significantly correlated with BMI. The authors concluded that the circulating IL-6 possibly reflected adipose tissue production and insulin resistance (248). Vozarova et al. also reported that plasma IL-6 relates positively to adiposity and negatively to insulin sensitivity (250). A study by Kato et al. determined that IL-6 production was positively regulated by hyperglycemia (251).

TNF- α has a complex relationship with diabetes. TNF- α exerts its action on insulin signaling and lipid metabolism by suppressing expression of the insulin sensitive glucose transporter GLUT-4, insulin receptor substrate-1 (IRS-1), and insulin receptor (245,252,253). This causes an excessive free fatty acids (FFA) flux from hypertrophied insulin-resistant adipocytes (245,252,253). This mechanism demonstrates a possible link between TNF- α , obesity, insulin resistance, and type 2 diabetes (211,244-246). It was

also demonstrated that incubating rat skeletal muscle cells with TNF- α results in significant impairment of insulin action (215).

The population of women in this study have different demographics and are on different medications. Demographically, the BMI is significantly increased in diabetic women without cardiovascular disease. Adipose cells are reported to produce IL-6. Since the majority of patients with diabetes and cardiovascular disease are overweight, this is possibly one of the mechanisms by which diabetic patients have an increased production of this cytokine. It was reported that IL-6 correlates with BMI (248). We did not find IL-6 to correlate with BMI. Statins are known to reduce inflammation via decreasing cytokine concentrations (281). We found that the proportions of patient on statins are not significantly different among groups, making statins less likely to influence the result. We did find that when separating the patients on statins from the patients not on statins, diabetic women without CVD not on statins did have significantly higher TNF- α compared to those on statins.

Conclusion

Diabetic patients have more severe atherosclerosis and a higher degree of morbidity/mortality associated with myocardial infarction, congestive heart failure, and other cardiovascular events (1,282). The etiology of this is unclear. Increased concentrations of TNF- α and IL-6 may affect the development of atherosclerosis, creating a more severe lesion. TNF- α and IL-6 may provide the link between

inflammation and insulin insensitivity. We hypothesized that pro-inflammatory conjugates (IL-6, TNF- α , and IL-1 β) are increased in diabetic women with cardiovascular disease compared to non-diabetic women with cardiovascular disease.

In conclusion, the findings of this study indicate that: 1.) diabetic women with cardiovascular disease have significantly increased IL-6 in comparison to age-matched controls, which indicates the potential additive effects of inflammation in these two disease processes, 2.) diabetic women with and without cardiovascular disease have increasing trend of TNF- α in comparison to age-matched controls, which also indicates the potential additive effects of inflammation in these two disease processes, and 3.) there was no correlation between diabetes or cardiovascular disease of IL-1 β . Thus, the higher concentrations of IL-6 and TNF- α in women in the diabetic and cardiovascular disease conditions could work synergistically in comparison to women with cardiovascular disease only or diabetes only and could be a contributor to the more severe cardiovascular disease in this population. The increased circulating concentrations of both TNF- α and IL-6 could also negatively affect myocardial function. Therefore, a patient with these increased cytokines may have a worse prognosis after a myocardial infarction, angina ,or congestive heart failure.

Table 4.1. Demographics of the Women.

<u>Patient Characteristics</u>	<u>ND-CVD (n=24)</u>	<u>ND+CVD (n=7)</u>	<u>D-CVD (n=19)</u>	<u>D+CVD (n=12)</u>
Age (years)	58.5 ± 1.7	63.1 ± 5.0	54.5 ± 2.4	64.4 ± 2.9*
Body Mass Index (kg/m ²)	26.8 ± 1.0*	29.9 ± 2.8*	37.7 ± 1.7	29.5 ± 2.0*
Duration of Diabetes (years)	N/A	N/A	8.9 ± 1.5	11.4 ± 3.6
HbA1c (%)	5.4 ± 0.1*	5.2 ± 0.2*	8.2 ± 0.4	6.9 ± 0.5
Hypertension (%) ^ψ	8%	86%	53%	69%
Hypercholesteremia (%)	25%	57%	56%	54%
Current Smoker (%)	0%	15%	11%	15%
Prior Cardiovascular Events (%)				
Myocardial Infarction ^ψ	0%	29%	0%	62%
Stroke ^ψ	0%	0%	0%	23%
PTCA ^ψ	0%	29%	0%	54%
CABG	0%	14%	0%	15%

*P<0.05 compared to D-CVD group

**P<0.05 compared to ND-CVD group

^ψThe proportions are significantly related (P<0.05)

Table 4.2. Distribution of medications in the women.

<u>Medication (%)</u>	<u>ND-CVD (n=24)</u>	<u>ND+CVD (n=7)</u>	<u>D-CVD (n=19)</u>	<u>D+CVD (n=12)</u>
Beta Blockers ψ	0%	86%	11%	38%
Diuretics ψ	4%	29%	11%	46%
Nitrates	4%	29%	0%	15%
ACE Inhibitors ψ	4%	14%	63%	38%
Positive Inotropes	0%	14%	5%	15%
Ca ²⁺ Channel Blockers	0%	29%	11%	23%
Aspirin ψ	21%	100%	21%	69%
Anti-coagulants	0%	0%	5%	7%
Antioxidants ψ	50%	43%	11%	23%
Hypoglycemic Agents ψ	0%	0%	69%	31%
Insulin	0%	0%	11%	15%
Statins	13%	57%	32%	46%
HRT	50%	71%	58%	46%

ψ The proportions are significantly related (P<0.05)

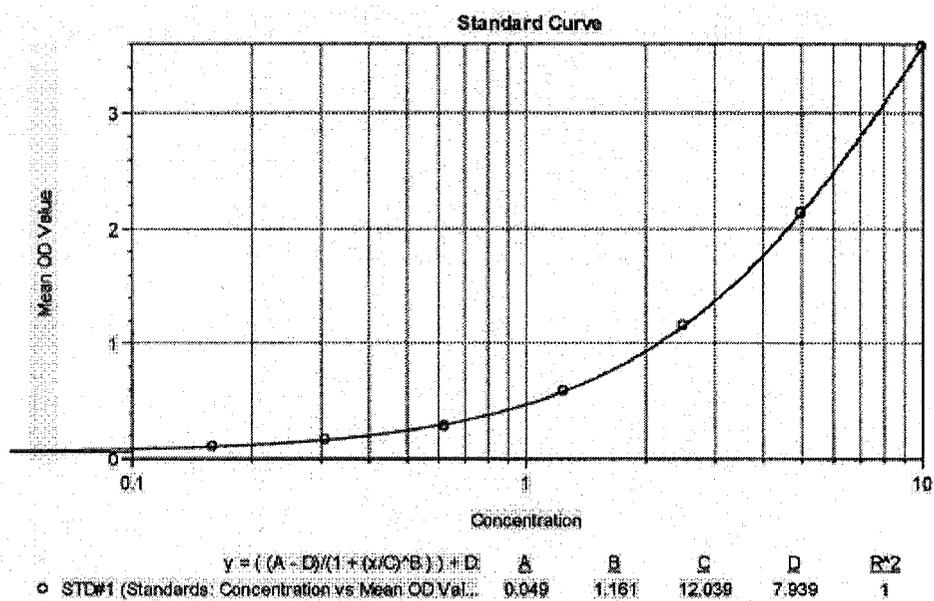
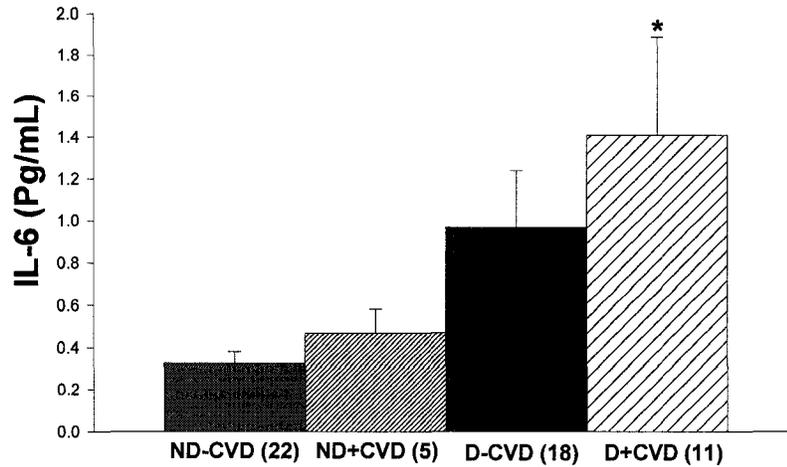


Figure 4.1. Representative standard curve for IL-6 concentration (IL-6 Ultrasensitive kit from Biosource International). The x-axis represents the concentration of IL-6 and the y-axis represents the mean OD value.



* $P < 0.05$ compared to ND-CVD group

Figure 4.2. Summary of ELISA results of IL-6 for all women in the study. Comparison of non-diabetic women without cardiovascular disease (ND-CVD) versus non-diabetic women with cardiovascular disease (ND+CVD) versus diabetic women without cardiovascular disease (D-CVD) versus diabetic women with cardiovascular disease (D+CVD). The IL-6 production was significantly greater in diabetic women with cardiovascular disease in comparison to healthy age-matched controls ($p < 0.05$).

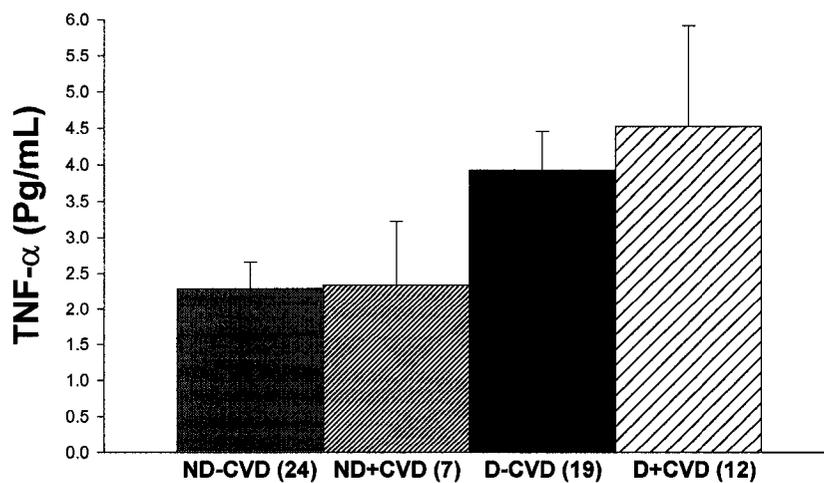


Figure 4.3. Summary of ELISA results of TNF- α for all women in the study. Comparison of non-diabetic women without cardiovascular disease (ND-CVD) versus non-diabetic women with cardiovascular disease (ND+CVD) versus diabetic women without cardiovascular disease (D-CVD) versus diabetic women with cardiovascular disease (D+CVD).

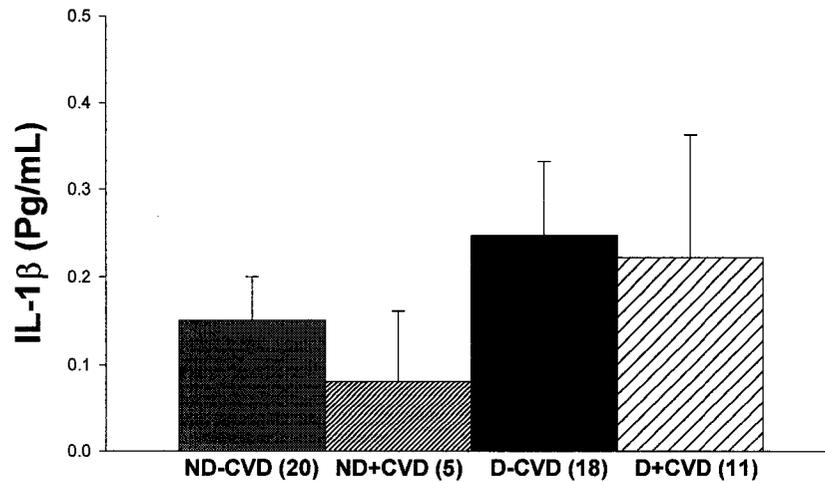
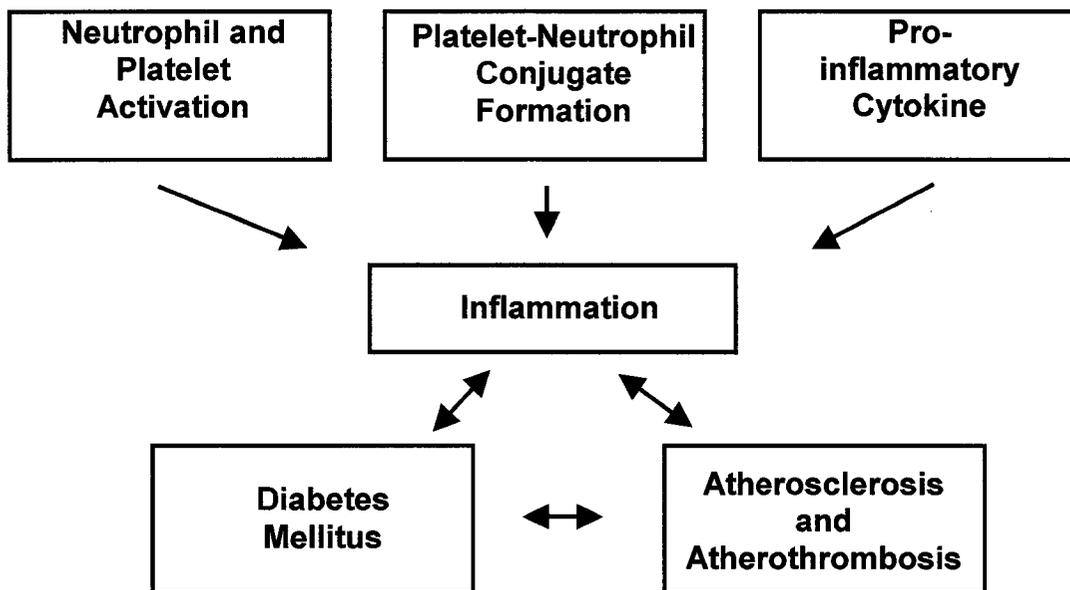


Figure 4.4. Summary of ELISA results of IL-1 β for all women in the study. Comparison of non-diabetic women without cardiovascular disease (ND-CVD) versus non-diabetic women with cardiovascular disease (ND+CVD) versus diabetic women without cardiovascular disease (D-CVD) versus diabetic women with cardiovascular disease (D+CVD).

SUMMARY STATEMENT

Diabetic patients are at an increased risk of cardiovascular disease, in particular diabetic women with cardiovascular disease have 3.8 fold increased risk of coronary heart disease compared to diabetic men. Diabetic patients also have more severe atherosclerosis and a higher degree of morbidity/mortality associated with myocardial infarction, congestive heart failure, and other cardiovascular events (1,282). One link between diabetes and cardiovascular disease is inflammation. Inflammation is now considered a risk factor for cardiovascular disease, comparable to the risk factor of cholesterol (101,102). Hayden et al. hypothesized that type 2 diabetes mellitus is a vascular disease (atherosclerosis) and that NOS, NO, and redox stress, components of inflammation, play a causative role in type 2 diabetes, in turn causing atherosclerosis (104). Biondi-Zoccai et al. reviews the role of inflammation in diabetes and atherothrombosis. They state diabetics have increased dyslipidemic abnormalities, increased oxidative stress, and increased prothrombotic abnormalities, all contributing to increased atherosclerosis and atherothrombosis (103). Vozarova et al. reported that a high white blood cell (WBC) count predicts the development of type 2 diabetes and a worsening of insulin action in Pima Indians (105). Studies by Thorand et al. and Barzilay et al. also found that low-grade inflammation (in particular C-reactive protein) was associated with an increased risk of type 2 diabetes mellitus in middle-aged men and the elderly (106,107). Earl et al. reported that men and women with a higher WBC and sedimentation rate had a greater risk of developing diabetes (108). It is apparent that inflammation plays a significant role in diabetes and cardiovascular disease.



We hypothesize that there is a greater chronic inflammation in diabetic women with cardiovascular disease. Aim one was to test the hypothesis that neutrophil and platelet activation is increased in diabetic women with cardiovascular disease compared to non-diabetic women with cardiovascular disease and diabetic men with cardiovascular disease. An increase in platelet and neutrophil activation in diabetic patients could contribute to the more severe cardiovascular disease in diabetic patients, particularly women. We did not find platelet and neutrophil activation to be increased in diabetic women compared to diabetic men, nor did we find neutrophil activation to be increased in diabetic women compared to non-diabetic women. We did find platelet expression of P-selectin to be increased in diabetic women with cardiovascular disease compared to diabetic women without cardiovascular disease.

Aim two was to test the hypothesis that platelet-neutrophil conjugates are increased in diabetic women with cardiovascular disease compared to non-diabetic women with cardiovascular disease and diabetic men with cardiovascular disease. We found that the baseline concentrations of platelet-neutrophil conjugates were not significantly different among any of the groups studied, however we did find an increased propensity for female blood to form conjugates in response to acute stimulation.

Aim three was to test the hypothesis that pro-inflammatory cytokines (IL-6, TNF- α , and IL-1 β) are increased in diabetic women with cardiovascular disease compared to non-diabetic women with cardiovascular disease. We found diabetic women with cardiovascular disease have significantly increased IL-6 in comparison to age-matched controls, which indicates the potential additive effects of inflammation in these two disease processes. Diabetic women with and without cardiovascular disease also had increasing trend of TNF- α in comparison to age-matched controls, which also indicates the potential additive effects of inflammation in these two disease processes.

The results indicate that excessive platelet activation in diabetic women with cardiovascular disease may contribute to the pathogenesis of thrombosis-induced ischemic events. Platelet-leukocyte adhesion (via P-selectin) may serve to recruit neutrophils and monocytes to sites of cardiovascular damage and thus promote the inflammatory response. Binding could cause neutrophil activation resulting in increased

free radical production. These results also suggest that one of the potential causes of the difference of severity of cardiovascular disease between diabetic men and diabetic women is the diabetic women's propensity to hyper-react to an acute ischemic stimulus and activate platelet-neutrophil conjugates more than that of diabetic men. The increased number of platelet-neutrophil conjugates may localize both cell types in the microcirculation causing obstruction and ROS release. In addition, the complex activation of each of the cells by the other could occur creating a positive feed back loop of increased inflammatory activation. The increased concentrations of IL-6 and TNF- α in women as a result of diabetes and as a result of cardiovascular disease could be a contributor to the more severe cardiovascular disease in this population. This increase in inflammation may then possibly increase the severity of thrombosis and cardiovascular disease. This information would initiate the development of a model of inflammatory activation in diabetic patients and may lead to more effective treatments of the inflammatory response.

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