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Levels of Neutrophils Stimulation and Matrix Metalloprotease in Plasma of Individuals with Elevated Blood Pressure and Acute / Long-term Exercise

A Thesis submitted in partial satisfaction of the
requirements for the degree Master of Science

in

Bioengineering

by

Bryan Sheu

Committee in charge:

Professor Geert W. Schmid-Schönbein, Chair
Professor Paul J. Mills
Professor Marcos Intaglietta

2008

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Chair

University of California, San Diego

2008

Dedication

I want to dedicate this thesis to my parents, Edward Yu-Hwa Sheu and Lilian Chen Sheu, for their full support and faith in me. I would also like to thank my grandma for her love and I want to wish my sister, Jennifer Sheu, success on whatever she decides to do in her future. I love you all!

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ABSTRACT OF THE THESIS

Levels of Neutrophils Stimulation and Matrix Metalloprotease in Plasma of Individuals with Elevated Blood Pressure and Acute / Long-term Exercise

by

Bryan Sheu

Master of Science in Bioengineering

University of California, San Diego, 2007

Professor Geert W. Schmid-Schönbein, Chair

Hypertension is a major risk factor for various cardiovascular diseases and studies on inflammation are extremely important in understanding hypertension. Abnormal Metal Metalloproteases (MMP) content is associated with abnormal extracellular matrix (ECM) metabolism in hypertension and can cause a cascade of inflammatory processes. Long-term exercise is shown to lower inflammation and blood pressure.

The objective of this study is to further examine the link of hypertension with inflammation during acute and long-term exercise. Blood was drawn from untreated hypertensives and normotensives. The neutrophils were viewed, whereas the MMP content in plasma was determined with an ELISA technique. We also stimulated naïve

neutrophils with this plasma and determined pseudopod formation, an indicator of inflammation.

Results show that neutrophils in hypertensives are more susceptible to external damage. However, levels of pseudopod formation generated by the plasma or MMP levels in the plasma did not correlate with blood pressure. Outliers for MMP-9 and MMP-8 in hypertensives suggest that there may be increased risk factors in a subgroup of hypertensives.

The levels of MMP-9, -8, and -2 increased after acute exercise for the normotensives ($p<0.001$ for MMP-9 and -8, $p < 0.05$ for MMP-2) but not the hypertensives. Long-term three month exercise significantly decreased pseudopod formation ($p<0.05$) and MMP-9 and MMP-8 levels (not significant, $p = 0.2$). There is a significant correlation between age and MMP-2 and between MMP-9 and MMP-8. These results suggest that while acute exercise increases markers for inflammation in plasma, long-term exercise reduces selected markers for inflammation in plasma.

Chapter 1: Background

1.1 Hypertension

Hypertension is a major risk factor for a variety of cardiovascular diseases [1], including atherosclerosis and stroke [2-4]. According to the American Heart Association, there are around 73 million people in the US with high blood pressure in the year of 2005. Hypertension is well known as the leading cause of morbidity and mortality in the US and has become the greatest public health risk to date [2, 5]. Proper antihypertensive treatment could potentially reduce these cardiovascular diseases, so studies on hypertension have always been a topic of extreme interest.

Hypertension is defined with a blood pressure of over 140 / 90 mmHg by the National Institute of Health [6], but recently has been associated with many other syndromes. This includes increasing amounts of reactive oxygen species (ROS) and apoptosis, capillary rarefaction, leukocyte membrane adhesion defects, reversed fluid shear responses, Type II diabetes, and recently, chronic inflammation [7-9]. There are an increasing number of studies that are designed to explore more indicators for inflammation in hypertension [1].

1.2 Inflammation

1.2.1 Introduction to inflammation

The inflammatory cascade serves a life-time as the main tissue repair mechanism and consists of a complex series of events to remove damage tissue and to generate new ones. Histologically, it includes dilatation of arterioles, capillaries and venules, with

increased permeability and blood flow, leukocyte migration into an inflammatory tissue region, cell apoptosis, and growth of new tissue and blood vessels [10]. A problem in the ability to properly execute these steps could potentially lead to organ dysfunction and even death.

Despite the importance of inflammation in the human body, recent studies show that excessive forms of inflammation are associated with cell death and can be linked to many diseases. These diseases includes myocardial ischemia [11], Alzheimer's disease [12], hypertension [8], cancer [13-15], and in more severe forms of inflammation, physiological shock and sepsis [16]. As inflammatory markers are relatively inexpensive to detect, there are an increasing number of studies using inflammation as an indicator of certain diseases. In addition, as there is evidence that anti-inflammatory treatments improve conditions of certain diseases, the study of inflammation has become increasingly important [10]. Table 1-1 shows some important inflammatory molecular markers that have relation with hypertension

1.2.2 Pseudopods

While early research on inflammation in hypertension focused on inflammatory cytokines and endothelial activation, studies also show that hypertension is characterized by significant leukocyte activation. Leukocyte activation is known as one of the main reasons for the increase in hemodynamic resistance [17]. There are several forms of leukocyte activation, including oxygen radical formation, expression of adhesion proteins, pseudopod formation, or other form of cell activities [18]. In this study, pseudopod formation is used as an indicator of leukocyte activity because the rigid structure of

pseudopods is one of the sources of the increase of vascular resistance, leading to hypertension.

Pseudopods are temporary projections of F-actin polymerization on the leading edge under an extended membrane. Pseudopods' primary function is for migration and capturing prey in phagocytosis. They are also formed to adhere to places where there is inflammation, and thus pseudopod formation represents an important risk factor in capillary trapping or accumulation of neutrophils at a particular site. If there is too much pseudopod formation, then there is a potential increase in hemodynamic resistance due to too many capillaries being blocked by neutrophils.

Hypertension in the spontaneously hypertensive rat (SHR) form of experimental hypertension is associated with increased pseudopod projection in neutrophils [17]. In addition to capillary trapping, previous animal studies state that neutrophils with pseudopods are known to have an increased expression of CD11a / CD11b and enhanced adherence to the endothelium, that are also both indicators in hypertension [19, 20]. In addition, fluid shear stress on the leukocytes of Wistar Kyoto rats (WKY) leads to retraction of pseudopods, whereas the same shear induces pseudopod projection in spontaneously hypertensive rat (SHR) [17]. Leukocytes are also well known to produce oxygen free radicals and mediators of proteolytic tissue degradation. As oxygen free radicals cause organ injury, there is a possibility that organ injury during chronic hypertension may be associated with activation of leukocytes [3].

1.2.3 Membrane Blebs

Membrane blebs are plasma membrane projections of a cell undergoing apoptosis. Blebs are easily differentiated from pseudopods, as there is little f-actin within the membrane protrusion and they tend to have a membrane with spherical shape (i.e. constant radius of curvature) compatible with the hypothesis that they are generated by a fluid pressure buildup and inflation of either a single or a double leaflet membrane according to Laplace law for membranes [21]. Depending on the cell type, membrane blebs may also form during cell locomotion, cell division, and physical or chemical stresses [22].

During apoptosis, the membrane's attachment to the extracellular matrix is weakened due to a loss of focal adhesions. The blebs form as a result of the breakdown of the actin cytoskeleton and stimulation of actomyosin-based contractility [23]. Rho GTPase targets ROCK-I as a possible cause for bleb formation [24]. Once cleaved by caspases, a protease activated in apoptotic cells, ROCK-I becomes activated and induces phosphorylation of the myosin regulatory light chain (MLC) as well as cell contraction and membrane blebbing [23].

Membrane blebs are important because they are a prominent feature of cell injury, something that is elevated in hypertension. Membrane blebs can rupture or be shed from the membrane that represents irreversible and reversible injury, respectively [25]. Some studies show that blebs contain membrane vesicles that carry oxidative compounds that can amplify inflammation [26]. Apoptosis was shown to play an important role in various cardio-pathological settings, such as cardiomyopathy, acute myocardial infarction,

myocarditis, and atherosclerosis [27]. High levels of apoptotic blebs are especially detected in atherosclerotic plaques [28].

1.3 Hypertension and Inflammation

1.3.1 The link between hypertension and inflammation

Hypertension is also well known to be associated with neuroendocrine and immune activation and cell adhesion molecule activation, including c-reactive protein (cRP), inflammatory cytokines, and endothelial cellular adhesion molecules [2, 29]. The link of hypertension and inflammation is currently in the 'chicken and egg' stage, where insufficient time courses have been studied to determine if inflammation is a pathogenic mechanism or just a secondary effect of hypertension. Nevertheless, the study of chronic low-grade inflammation has been very important in the understanding of hypertension.

There are several hypotheses attempting to explain this association. Some say that inflammation causes an increase in arterial stiffness [30, 31], which changes the mechanical stress in the arterial wall and can lead to an elevation in blood pressure. Studies show that inflammation also causes endothelial dysfunction, which then alters the synthesis and degradation of vasodilatory and vasoconstrictory factors [32]. Other studies show blood platelets activated in SHR, where platelets are known to produce inflammatory mediators that activate leukocytes [3]. ROS serve as signaling molecules to maintain inflammation and contribute to oxidative stress, endothelial dysfunction, and reduced NO availability [33]. A recent study showed that hypertension is associated with this increased oxidative stress [34], which is also associated with an inflammatory state caused by reduced NO availability.

There are many cardiovascular diseases that are linked to both hypertension and inflammation, which promotes studies to research anti-inflammatory effects on these pathologies. End-organ injury caused from the generation ROS are believed to be increased in SHR [35]. Atherosclerosis is a well recognized as inflammatory disease, with hypertension exerting key pathogenic roles on the atherosclerotic processes [2]. Finally, stroke is a significant cause of mortality with hypertension as the most important risk factor. During stroke, the mucosal barrier for pancreatic digestive enzymes is impaired and digestive enzymes initiates self-digestion on extracellular matrix proteins. This auto-digestion is a process that leads to a cascade of inflammatory reactions, thus leading to another possible explanation for the relationship of hypertension and inflammation [36].

1.3.2 Pharmaceutical Effects on Hypertension

Medication is known to reduce blood pressure, but no medication is shown to significantly reduce the risk of atherosclerosis or, with the exception of stroke, other cardiovascular diseases associated with hypertension [37, 38]. Pharmacological monotherapy often does not adequately control BP levels, and patients tend to resist multi-drug regimens [39, 40]. Medication is also costly to a point where it is not fully available to lower income, uninsured patients [41, 42]. In addition, medication has many side effects that reduce the quality of life [43]. We believe that current therapy through medication does not adequately reduce inflammation and cell adhesion activation and thus cannot properly treat hypertension.

1.3.3 Exercise Effects on Hypertension

Exercise is among some of the most effective approaches to reduce inflammation and improve hypertension. Many believe that long-term exercise is a better treatment for primary hypertension, as long-term exercise is well known to reduce inflammation. Studies show that there is acute inflammatory response after acute exercise, suggesting cytokines and/or muscle injury contribute to the inflammatory response [44]. However, long-term exercise training blunts this acute inflammatory response and decreases inflammation [44]. There is evidence that long-term exercise decreases both various inflammatory markers and blood pressure for treated hypertensive patients. For instance, physical activity level and fitness are shown to be inversely related to leukocyte adhesion molecule expression [45]. Another study shows that for healthy men and women, TNF-α and IL-6 are inversely related to physical activity [46].

In fact, constant exercise is a recommended change in lifestyle for all patients diagnosed with hypertension as the physically active tend to have fewer cardiovascular risk factors [47]. The mechanism for the antihypertensive effect through long-term exercise is not fully understood. Some believe that maximum oxygen consumption is claimed to be associated with blood pressure reduction [2]. Another study concluded that the reduction in peripheral vascular resistance by structural adaptations through long-term exercise is the cause for this antihypertensive effect [48]. Long-term exercise is also known to have an effect on the sympathetic and hypothalamic-pituitary-adrenal system (HPA). Hypertension has a direct association with sympathetic and HPA systems, which also has an effect on increased inflammatory cytokines [49, 50]. Thus, further investigation in these systems might tell a lot about the mechanism that reduces

inflammation. The fact is, despite the many possible explanations, this relation may be cause by a multiple, interdependent factors.

There are many studies on exercise and inflammatory effects, but there are few studies that are designed to study untreated hypertensive patients. We are amongst the first to study acute and long-term effects on untreated, sedentary hypertensive patients. As hypertensive patients have more inflammation, there should be an even greater exercise-related reduction in inflammation in hypertensive populations than that in normotensive populations.

1.4 Proteases

1.4.1 Metal Metalloproteinase (MMP)

Metal Metalloproteinases are zinc-based enzymes that degrade many proteins in the extracellular matrix [51, 52]. MMP is a family of 28 different enzymes, 24 of which are found in humans to this date [53]. This proteolytic effect for MMPs can play a role in vascular remodeling, cellular migration, and activation or inactivation of various ECM proteins and adhesion molecules. In addition, MMPs and other pancreatic digestive enzymes are believed to be the cause of severe inflammatory cascade accompanying shock [35]. MMPs have been widely researched because of their biological significance in a number of pathological conditions that include cancer, inflammation, and cardiovascular diseases [54]. Still, the substrates are not completely characterized. Known substrates of MMPs include fibronectin, laminin, collagen, adhesion molecules, apoptotic mediators, cytokines, receptors, intercellular junctional proteins, growth factors, and even other MMPs [54].

Metal Metalloproteinase generally consists of 4 different structures. There is the prodomain, which is the part of the enzyme that, when attached, alters the configuration of the active and zinc site in a form that will not bind properly to the substrates. The prodomain needs to be cleaved from the enzyme before MMP are active. The catalytic domain directly connects to the prodomain and contains the active site of the enzyme and a zinc-binding motif. For the gelatinases, MMP 2 and 9, there is fibronectin type II repeats in-between active site and zinc motif for in aide to degrade gelatin, another term for degraded collagens. Then there is the hinge that links catalytic domain to C-terminal domain. The Hemopexin-like C-terminal domain consists of four β-propeller structures and is involved in protein-protein interactions. This includes the interaction of tissue inhibitor of metalloproteinases (TIMP), which inhibits MMP activity.

DeLano and Schmid-Schönbein have recently shown the first time that the insulin receptors are greatly cleaved in SHR [36]. With the addition of doxycycline, a drug used to inhibit MMP activity, the insulin receptors are restored on the endothelium and restores normal glucose and glycosylated hemoglobin levels [36]. Although MMP is not the only enzyme that doxycycline inhibits, independent measurements of the MMP levels indicate that the inhibition of MMP is what is restoring these insulin receptors [36]. MMPs are implicated in the pathology of many cardiovascular diseases, including aneurysm, varicose veins, and atherosclerosis [53]. As many of these diseases are risk factors of hypertension, it is believed that increased MMP activity is associated with the multiple symptoms associated with hypertension.

1.4.2 MMP-2 and MMP-9

MMP-2 (Gelatinase A) and MMP-9 (Gelatinase B) are the two gelatinases. They both are secreted with many connective tissue cells as well as monocytes, macrophages, neutrophils, keratinocytes, and fibroblasts [55]. MMP-2 is approximately 72 kDa, where MMP-9 is slightly larger at the size of 92 kDa. The primary functions of the gelatinases are both the degradation of various types of proteins in the extracellular matrix, including decorin, elastin, fibrillin, laminin, gelatin, and various types of collagen [54]. In addition, MMP-2 plays a role in tissue remodeling events, cell migration, inflammation, and metastasis [56]. MMP-9 also plays additional roles in inflammation, tissue remodeling, wound healing, mobilization of matrix-bound growth factors and processing of cytokines.

There are many studies on the association of MMP-2 and MMP-9 with hypertension. The abnormal ECM metabolism in hypertension is believed to be associated with abnormal MMP content. However, there are contradicting views on the role of MMPs in hypertensive patients. Some studies show elevation in levels and activity of MMP -2 and -9 for plasma in hypertension with the explanation that increased deposition of collagen in ECM causes vascular fibrosis with increased arterial stiffness [57-59]. Other studies showed depression in activity of MMP -2 and -9 for plasma in hypertension with the explanation that diminished MMP activity may contribute to remodeling of resistance vessels and facilitate collagen accumulation in vascular wall [60]. MMP-9 activation in similar studies was suggested to be beneficial for hypertension by preserving vessel compliance and alleviating blood pressure increase [61]. The most current evidence, however, suggests that activation of MMP-2 and MMP-

9 is not beneficial for hypertension and affects not only the blood pressure but also other cell dysfunctions encountered in hypertension [36].

1.4.3 MMP-8

MMP-8 (Neutrophil Collagenase) is an enzyme that, like the other collagenases, possesses high substrate specificity for fibrillar collagens. However, unlike most MMPs that are secreted in its pro-form and then activated in the extracellular matrix (ECM), MMP-8 is stored in secondary granules within neutrophils and is activated by autolytic cleavage before being secreted [62]. Various inflammatory agents, such as IL-1 and IL-8, TNF-α, and GM-CSF, stimulate the release of MMP-8 from neutrophils [63]. It is 75 kDa large, and its primary function is to initiate breakdown of collagens in the ECM during pathological processes such as inflammation and arthritis.

The association between hypertension and MMP-8 has not been studied. However, there are studies that associate MMP-8 concentration in serum as an indicator for predicting acute coronary syndrome and acute myocardial infarction [64]. In addition, MMP-8 has been associated with various inflammatory diseases [53], including cancer and acute liver failure. MMP-8 also has similar functions to MMP-1, which shows that the serum levels of MMP-1 are decreased in patients with essential hypertension, leading to depressed extracellular degradation [65].

1.5 Goals of the Investigation

1.5.1 Overall objective

The **objective** of this study is to further examine the link between hypertension and inflammation during acute and long-term exercise. Although there have been many studies that have linked hypertension with inflammation [1], there are few studies that have used untreated hypertensive patients, especially within the US. Many studies look into more severe cases of hypertension and patients with congestive heart failure or other serious cardiovascular diseases. In addition, there are few studies that control the lifestyle of the patients with physical activity. Our study wants to view simple blood pressure effects on hypertension for untreated human patients. To do this, we screen our normotensive controls so that they are also obese (BMI ≈ 30) and sedentary. This is the first study that tests the effects of blood pressure and acute / long-term exercise on inflammation and protease levels in untreated, relatively sedentary human subjects.

1.5.2 Objective and Hypotheses

This thesis is part of a project in the laboratory of Professor Paul Mills. The objective of the project is to determine the effects of a 12-week long-term exercise and a 12-week long-term exercise + diet interventions on inflammation and cell adhesion in 150 patients with hypertension.

In addition to looking into only hypertensive patients before and after long-term exercise, we also look into patients with normal blood pressure. Normal blood pressure according to the National Institute of Health is defined as a blood pressure of 120 mmHg / 80 mmHg or less. Any patient in-between 120 mmHg / 80 mmHg and 140 mmHg / 90 mmHg are considered pre-hypertensive. Blood pressures of > 140 mmHg / 90 mmHg are

considered hypertensive. Keeping all other factors constant, the study determines the difference between those with hypertension and those without, including both the normotensive and the pre-hypertensive patients.

We **hypothesize** that hypertension is associated with neutrophil activation. We also hypothesize that hypertension is associated with elevated MMP levels. Last, we hypothesize that although acute exercise will temporarily increase inflammation, the inflammation will decrease after 3-month long-term exercise. This includes pseudopod formation of naïve neutrophils after exposure to plasma from hypertensives and MMP-2, -8, and -9 level in plasma as a way to demonstrate the presence of inflammatory mediators in hypertensive plasma.

1.5.3 Specific Aims

1. ***Determine and compare the levels of inflammation in hypertensive and normotensive subjects***
    a. Compare autologous neutrophil susceptibility to damage for hypertensive and normotensive subjects
    b. Compare pseudopod formation on naïve neutrophils resulting from patient plasma stimulation
    c. Compare the patient plasma levels of MMP-9, MMP-8, and MMP-2
2. ***Determine the effects of acute and long-term exercise on inflammation in hypertensive subjects***
    a. Determine effects of acute and long-term exercise on pseudopod formation on naïve neutrophils resulting from patient plasma stimulation

b. Determine effects of acute and long-term exercise on the patient plasma levels of MMP-9, MMP-8, and MMP-2

3. Determine the correlation between demographic features, neutrophil activation, MMP levels, and various inflammatory cytokines

Table 1-1. Inflammatory Markers.

Inflammatory Marker	Source	Function	Relation with Hypertension
C-Reactive Protein (CRP)	Liver, production stimulated by cytokines, notably IL-6	Acute phase response protein. Assists in complement and innate immune responses. Stimulates monocyte release of nitric oxide synthase, leading to impaired endothelium-dependent relaxation	Elevated with higher blood pressure [66]
Interleukin-6 (IL-6)	Secreted by leukocytes, endothelial cells, adipocytes	Proinflammatory cytokine, promotes expression of cellular adhesion molecules	Elevated with increasing blood pressure [67]
Tumor Necrosis Factor alpha (TNF-α)	Secreted by leukocytes, endothelial cells, adipocytes	Proinflammatory cytokine, promotes expression of cellular adhesion molecules	Elevated with increasing blood pressure [67]
CD11a	Expressed on leukocytes	Integrin, secure tethering of leukocytes and migration through endothelium	Might be upregulated in hypertension [68]
CD11b	Expressed on leukocytes	Integrin, secure tethering of leukocytes and migration through endothelium	Elevated by experimental hypertension in animal studies [69]

[2]

Chapter 2: Experimental Study

2.1 Materials and Methods

2.1.1 Patient Selection

Patients were screened before participating in the study. "Hypertensive patients" were defined with a blood pressure over 140 / 90 mmHg but can be no higher than 175 / 104 mmHg. The patients were between 25 and 60 years old and had a BMI between 23.5 and 34 kg/m^2. All patients were untreated and sedentary as defined by a Leisure Time Exercise Questionnaire. The patients must have had a normal echocardiogram without any significant cardiovascular pathology. Subjects with diabetes, congestive heart failure, bronchospastic pulmonary disease, history of myocardial infarction, known secondary hypertension, angina, history of life-threatening arrhythmia's, or kidney damage were excluded from the study. Patients that were on any diet were also be excluded from the study, along with any pregnant women. Finally, any patient with arthritis or any other disorder that may be made worse with exercise was excluded from the study. The normotensive and pre-hypertensive controls follow the same criteria, but have a blood pressure that was less than 140 / 90 mmHg. Table 2-1 shows the distribution of patients used in this procedure.

2.1.2 Acute exercise and long-term exercise regimens

Long-term exercise was defined as a 12-week exercise long-term exercise (YMCA) applied to all the hypertensive patients, but none of the normotensive patients. In the study, some of the hypertensive patients went through only long-term exercise while others went through long-term exercise with diet changes. In this thesis, we

included both of these groups in the long-term exercise group due to the low number of subjects. As no normotensive patient go through long-term exercise, a before long-term exercise measurement was applied for all normotensive groups. Acute exercise, in contrast, referred to a protocol of short-term exercise the patient underwent on the day of the blood draw. Specifically, the patients exercise on a treadmill for 20 minutes at 65% – 70% of their VO_2 max (determined at an earlier date). This test was repeated for the hypertensives after the 12-week long-term exercise.

Venous blood from the forearm (5 mL) was collected into vacutainers with sodium heparin as anticoagulant. This blood is drawn from the hypertensive patients before and after acute exercise, both before and after long-term exercise, leading to a total of four blood draws for each hypertensive patient. For the normotensive patients, blood was only drawn before and after acute exercise, leading to a total of two blood draws for each normotensive patient. These tubes were centrifuged for 10 minutes under 400 g (Sorvall® Instruments RC5C), the plasma was collected and immediately frozen at -80°C for no more than 2 years. This plasma was used throughout this study.

Upon thawing the plasma, we tended to see a "protein clot" (see Figure 2-1). It was common for these protein clots to appear despite use of heparin as an anticoagulant [70]. We avoided the protein clot because it is not possible to aspirate into a pipette.

2.1.3 Bleb Formation on Neutrophils

Upon collection of blood form the patients, 500 μL of blood was taken out and directly fixed with 500 μL of 1X formaldehyde. Then, 5 mL of blood was taken in the same matter after acute exercise, with 500 μL of it fixed with 500 μL of 1X formaldehyde.

After fixation for at least 10 minutes, 300 μL of blood for both samples were diluted in a 1:2 ratio with 1X hypotonic lysing solution for 7 minutes in a 37°C water bath for the purpose of red cell lysing. All samples were centrifuged in a microcentrifuge (MiniSpin®, Eppendorf) for 2 minutes at 400 g. The supernatant was discarded. Then 600 μL more lysing solution was added and placed in a 37°C water bath for one minute. Once again the samples were centrifuged and the supernatant was discarded. The remaining pellets were washed twice with 1 mL of 1X PBS, the samples were centrifuged at the same settings, and the supernatants were discarded. Finally, the pellet was resuspended in 25 μL of PBS and stained with 3 μL of crystal violet in 1X PBS and formalin for enhanced recognition of cell features under the microscope.

Cells were viewed under a light microscope (Seiler H5232, Helmut Hund, Wetzlar, Germany) with a 100x oil-immersion objective, and a 20x camera. The total magnification was 2000X the normal size. Pictures were taken with MiniVID (LW Scientific, Inc.) of the first 70 cells seen. Neutrophils without blebs appear in an almost spherical shape (see Figure 2-2). Blebs were detected as a protrusion of the cell membrane (see Figure 2-3). All studies were carried out blind by mixing the pictures to prevent any possible bias.

2.1.4 Neutrophil Pseudopod Formation after Stimulation

30 mL of whole blood was taken from a healthy donor in sodium heparin as the anticoagulant. The whole blood was mixed with 3 mL in 6% dextran (Sigma-Aldrich Corp., St. Louis, MO) and then was allowed to settle for 45 minutes to sediment the RBCs. The top plasma player was withdrawn and placed gently on top of 7.5 mL of

Histopaque-1077 (Sigma-Aldrich Corp.) and centrifuged for 20 minutes at 600 g (RC5C, Sorvall® Instruments) to separate the plasma and most of the lymphocytes and monocytes. The supernatant was discarded and the pellet of granulocytes was suspended with 5 mL of PBS at 7.4 pH. In 15 mL tube, a full transfer pipette (~2 to 3 mL) of 74% isotonic Percoll was placed carefully below 3 mL of 55% isotonic Percoll (Sigma-Aldrich Corp.), and the 5 mL of pellet suspended in PBS was placed carefully of top of the two Percoll layers. With the brake of the centrifuge turned off, this tube is centrifuged for 15 minutes at 600 g. The purified neutrophils were drawn from approximately the 3 mL mark in-between the two Percoll layers into another tube and washed with 12 mL of PBS. Finally, this tube was centrifuged for 10 minutes at 100 g, yielding a pellet with purified neutrophils.

The cells were counted to determine the concentration of cells, and then diluted accordingly with PBS. 30 μL of neutrophils from a concentration of $2*10^6$ neutrophils / mL was stimulated with 30 μL of plasma, PBS as the negative control, or the neutrophil activator, fMLP, as the positive control for 10 minutes, and then fixed with 30 μL of 3% glutaraldehyde in PBS. Before viewing with a light microscope (Helmut Hund), the cells were stained with 90 μL of crystal violet diluted in PBS, and 10 μL of the stained cells were placed into a hemocytometer for viewing (Bright-Line, Fisher Scientific). The magnification of the light microscope was 40X on the objective and 10X on the lens, for a total magnification of 400X. The fraction of cells with pseudopod (Figure 2-4), defined as a membrane protrusion greater than 1 μm from the undeformed (spherical) neutrophil (Figure 2-5) surface, were counted for 100 neutrophils.

Glutaraldehyde is known to form gels in undiluted whole blood, making it impossible to extract cells from the well for view under the microscope. Because of this, there are some samples that cannot be analyzed. As a result, we determined as a separate measurement the percentage of samples in the hypertensive patients and the normotensive controls that formed this gel.

2.1.5 Sandwich ELISA

An Enzyme-Linked ImmunoSorbent Assay (ELISA) (Quantikine®, R&D Systems, Inc.) was used to detect concentrations of MMP-9 and MMP-8, their pro or active forms, in heparin plasma. The ELISA is a technique to determine the amount of protein (like MMPs) in a particular sample. A 96-well plate coated with antibodies against the protein of interest (MMPs) is used. After addition of the sample, the protein to that specific antibody in the well will attach, whereas the rest of the sample is washed away. A detectable signal is then generated by use of a secondary antibody to the protein of interest conjugated to horseradish peroxidase (HRP) in addition to tetramethylbenzene (TMB) that can generate a colorimetric reaction that visible under light.

In more detail, standards, plasma samples, and assay diluents (buffered protein base) were prepared and placed with the assay diluents into the antigen-coated wells. The plasma, standards, or controls were added into each plate and incubated for 2 hours. Afterwards, the plates were washed and an antibodies conjugated with HRP were added to the wells that now have the enzyme attached to the antibody for 1-2 hours, depending on the assay. After another wash, TMB was added and incubated for 30 minutes. Finally, stop solution was added and the optical density was measured with a spectrophotometer

(Spectramax 340PC384, Molecular Devices). All samples were analyzed in duplicate, and controls (R&D Systems, Inc.) were made, also in duplicate, to confirm the quality of the ELISA kit.

2.1.6 Sandwich Electrochemiluminescence Detection Method

An electrochemiluminescence detection method was used to detect MMP2 concentration levels in plasma (Multi-Array®, MSD, Gaithersburg, Maryland). The concept is similar to an ELISA kit, but it was used instead due to lack of stock chemicals for the ELISA from the supplier (R&D Systems). The basic procedure was as follows. First, the assay diluents was incubated on the carbon wells provided in the kit (MSD) for 30 minutes, and then the sample, standard, or control were added into the wells and incubated for 2 hours. After a wash, the detection antibody was added and incubated for another two hours. Finally, the read buffer, consisted of 2X sulfuric acid, was added and the light electrochemiluminescence emitted from a SULFO-TAG label read by the SECTOR imager (MSD).

In more detail, the SULFO-TAG label contains Ruthenium (II) tris-bipyridine-(4-methylsulfonate) NHS ester ($Ru(bpy)_3$). When a voltage was ran through the carbon wells, this SULFO-TAG label gains an electron and emits light, which was detected by light sensor (SECTOR Imager, MSD). All samples were analyzed in duplicate to confirm the data, and quality controls were made, also in duplicate, to confirm the quality of the electrochemiluminescence kit.

2.1.7 Statistical Analysis

All measurements were summarized as a mean ± standard deviation. Three different kinds of analysis were carried out. After checking the measurements for outliers, an individual sample t-test, which is equivalent to one-way ANOVA, was performed to determine any significant differences between the hypertensive and the normotensive subjects. In addition, the effects of hypertension and acute and long-term exercise effects were evaluated. This was done by using repeated measures ANOVA with acute exercise as the repeated factor. Any covariant that needs to be controlled for is also done by using a repeated ANOVA. Finally, a linear correlation was performed to detect potential correlations between groups of measurements. Statistical analysis of the data was performed with SPSS 15.0 software (SPSS Inc, Chicago, Illinois).

Table 2-1. Patient Demographics. Age still has a significant difference between the groups, so all results will need to be adjusted with age.

	Normotensive	Hypertensive Before Long-term exercise	Hypertensive After Long-term exercise
n	14	16	
Age	37.4 ± 8.4	48.2 ± 9.0*	
Sex (male/female)	8 / 6	8 / 8	
SBP (mmHg) [a]	124.6 ± 10.9	149.0 ± 6.7**	137.1 ± 9.2
DBP (mmHg) [a]	75.4 ± 5.8	87.1 ± 9.2**	78.9 ± 10.4
BMI (kg/m^2)	29.9 ± 4.7	30.5 ± 3.6	29.8 ± 3.2
VO_2 max (mL/kg/min)	32.5 ± 10.3	26.9 ± 6.6	29.5 ± 6.5

*$p < 0.05$ compared to the corresponding normotensive group

** $p < 0.001$ compared to the corresponding normotensive group

[a] – blood pressures are measurements before acute exercise

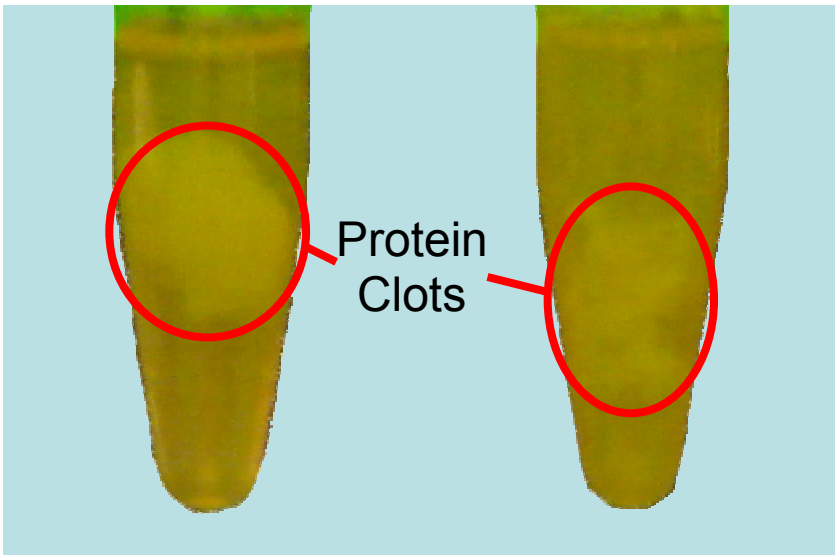

Figure 2-1. Examples of two protein clots as seen in blood samples after a freeze-thaw of heparin plasma.

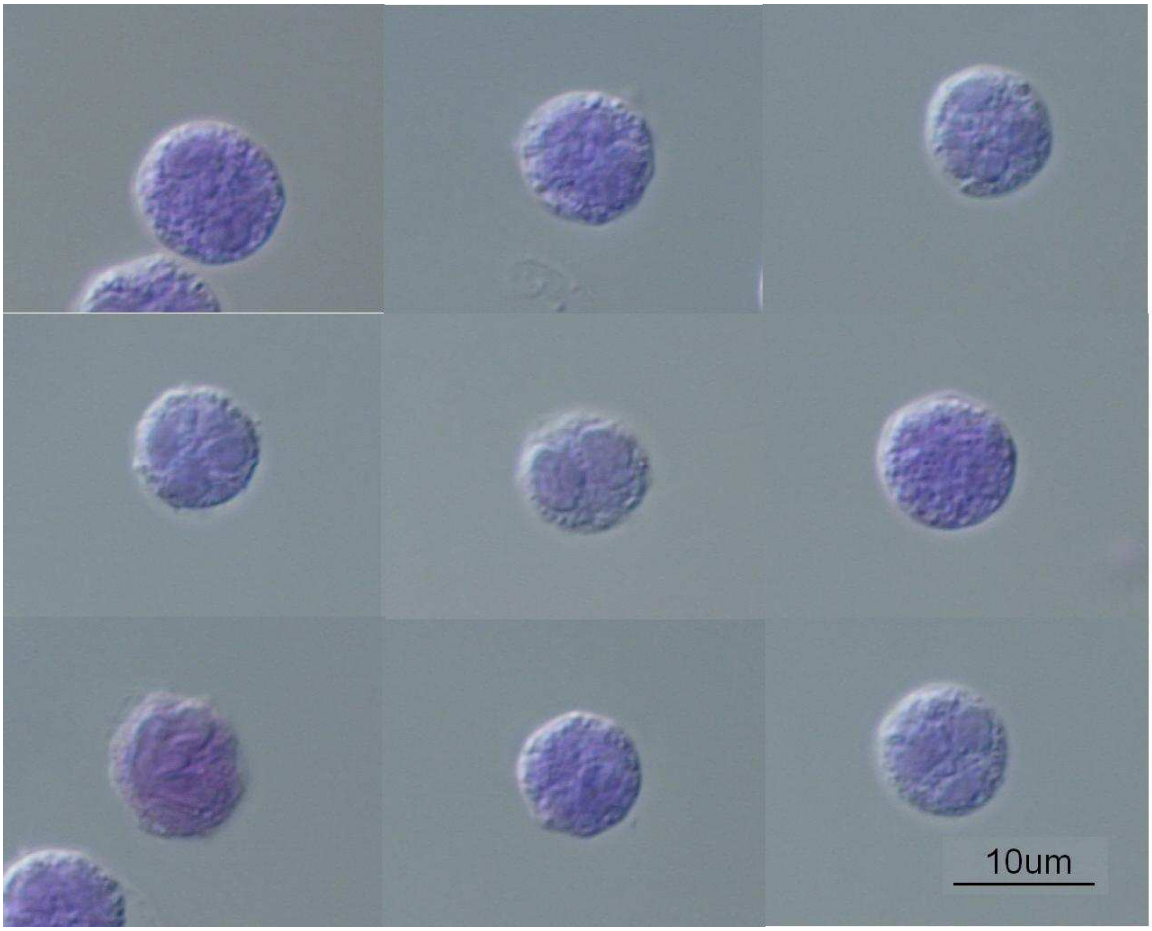

Figure 2-2. Examples of human neutrophils without blebs suspended in PBS.

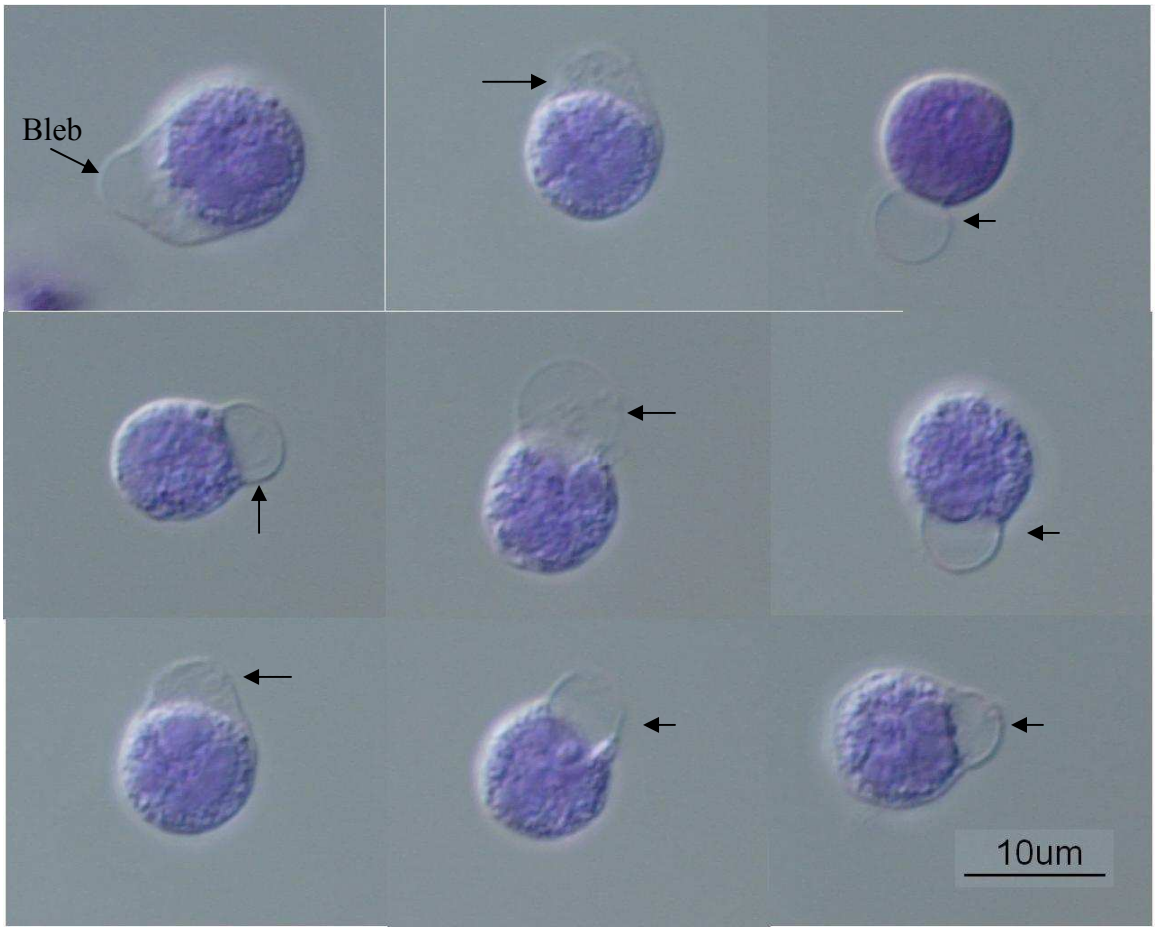

Figure 2-3. Examples of human neutrophils with blebs suspended in PBS. Arrows indicate the location of the blebs.

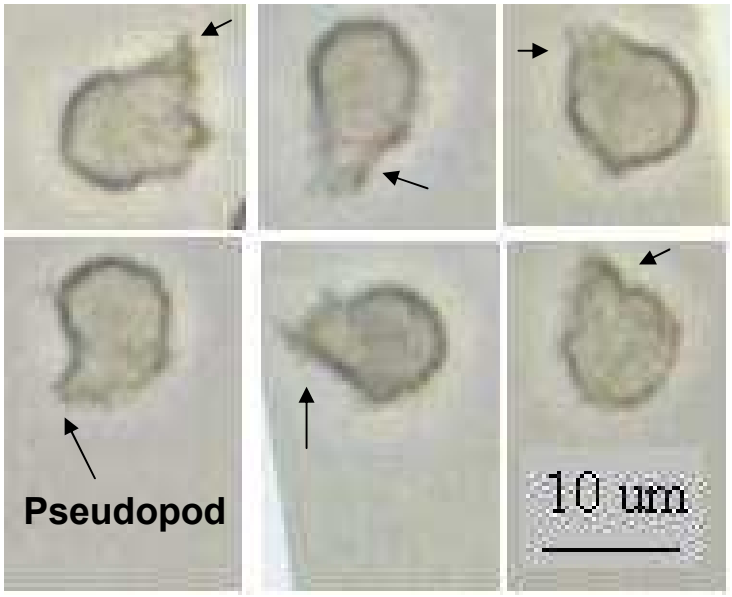

Figure 2-4. Examples of human neutrophils with pseudopods suspended in PBS. Arrows indicate the location of the pseudopods.

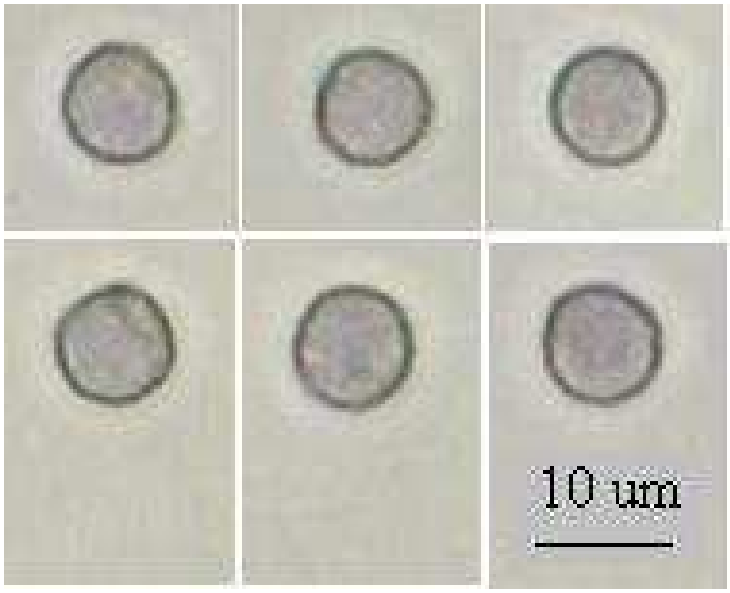

Figure 2-5. Examples of human neutrophils without pseudopods suspended in PBS.

2.2 Results

2.2.1 Bleb Formation on Autologous Neutrophils

The bleb count after formaldehyde fixation is significantly higher in hypertensive patient's neutrophils than in normotensive control's neutrophils (Figure 2-6). This means that neutrophils of hypertensive patients are more susceptible to damage by the formaldehyde fixation procedure when compared with their normotensive controls. Acute exercise has no significant effect on the neutrophils bleb formation as seen after formaldehyde formation.

2.2.2 Naïve Neutrophil Pseudopod Formation after Stimulation

The results in shows that there is no significant difference in pseudopod formation between the hypertensive patients (n = 4), and the normotensive patients (n = 4) (Table 2-2). Acute exercise seems to have little effect on pseudopod formation, and the same applies to blood pressure. However, long-term exercise seems to create significant anti-inflammatory effects. These results are in line with our hypothesis that plasma after long-term exercise has lower levels of inflammatory mediators.

We found a great deal more gel formation in the plasma samples of the hypertensive patients (Table 2-3) when compared with the normotensive controls, as well as an increase in gel formation for all three groups after acute exercise. There is a possibility that the samples that cannot be viewed are the ones that cause the most inflammatory stimulation for the neutrophils.

2.2.3 MMP -2, -8, -9 Levels

Blood pressure does not affect MMP levels in the plasma. Acute exercise, on the other hand, has a significant effect on the normotensive patients in all 3 of the MMP levels (Figures 2-7, 2-9, 2-11). An outlier is present for both the MMP-8 and MMP-9 levels in the hypertensive group (Figures 2-8, 2-10), suggesting that with hypertension, there may be another collaborating factor (e.g. low fitness levels, gender issues, or neutrophil activity) that is significantly increasing the MMP levels in plasma. With the exception of MMP-8, however, the same acute exercise shows no significant increase in MMP levels in hypertensive patients.

Although not significant, long-term exercise tends to reduce the levels of MMP-9 and MMP-8, but not MMP-2 ($p = 0.2$). This suggests that long-term exercise may be beneficial by decreasing MMP-9 and MMP-8 levels in plasma, and thus reducing potential cell dysfunctions caused by these MMPs. Intra-assay and inter-assay coefficient of variation (CV's) are shown in Table 2-4. Quality controls for each plate fall within their predicted range.

2.2.4 Correlations

Table 2-5 shows the linear correlations I examined that reached statistical significance. MMP levels, especially MMP-2, are correlated with age (Figure 2-12). There is a trend to indicate that, rather than the blood pressure, age will increase the MMP levels in plasma of our hypertensive patients. Looking at correlations between MMPs and various inflammatory markers (Table 2-6), the only statistically significant correlation found is between MMP-9 and MMP-8 (Figure 2-13). Since both are secreted

by activated neutrophils, there is a chance that MMP-9 and MMP-8 could be associated with neutrophil activation and degranulation.

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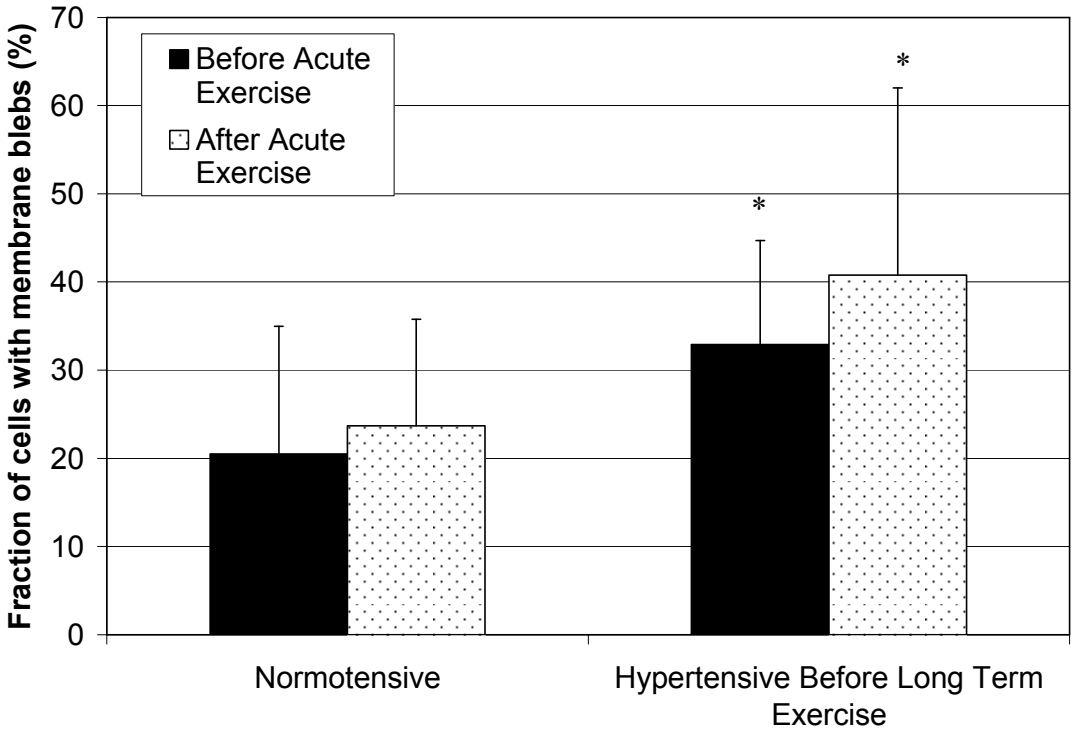

Figure 2-6. Fraction of autologous neutrophils with bleb formation. There is a significant increase in bleb formation for the hypertensive patients when compared with that of the normotensive patients.

*$p<0.05$ compared with the corresponding normotensive group. mean ± SD.

Table 2-2. Percentage of naïve neutrophils that contains pseudopods after stimulation. After long-term exercise, there is a significant decrease in pseudopod formation. As the negative control shows high pseudopod formation, there is a chance of anti-inflammatory effects in plasma. mean ± SD.

	Before Acute Exercise	After Acute Exercise
Normotensive (n = 4)	17.3 ± 3.8 %	15 ± 3.0 %
Hypertensive Before Long-term Exercise (n = 4)	19.8 ± 3.9 %	17.3 ± 5.4 %
Hypertension After Long-term Exercise (n = 4)	11.8 ± 3.6 %*	13.9 ± 4.5 %
Negative Control (PBS)	23.2 ± 5.5 %	
Positive Control (fMLP)	59.9 ± 5.9 %	

*$p < 0.05$ compared to the corresponding before long-term exercise group

Table 2-3. The percentage of samples that has gel formed from glutaraldehyde fixation. The negative and positive controls formed no gel, so it is the contents of the plasma that causes the gel formation.

	Before Acute Exercise	After Acute Exercise
Gel formed in normotensive samples (%)	6	25
Gel formed in hypertensive samples before long-term exercise (%)	58	75
Gel formed in hypertensive samples after long-term exercise (%)	38	66

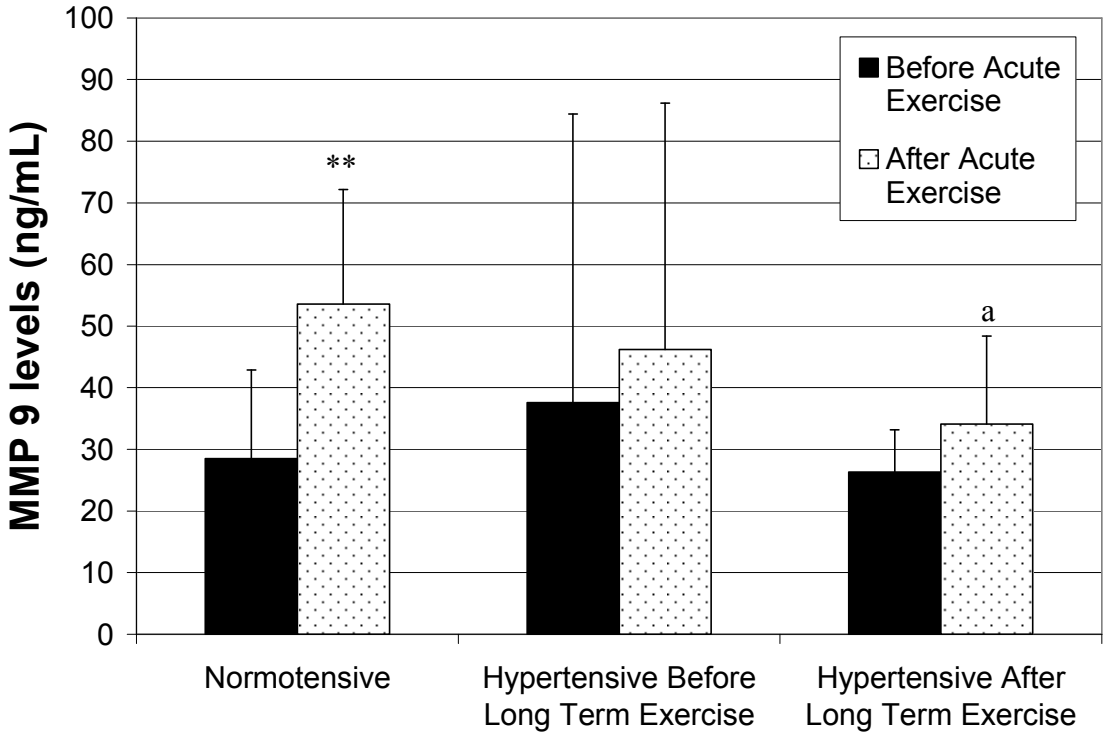

Figure 2-7. MMP-9 levels in heparin plasma. There is a significant acute exercise increase in MMP-9 for only the normotensive group. In addition, there is an approaching significant effect (p=0.2) for long-term exercise. **p<0.001 compared with its respective before acute exercise group. [a]p=0.2 long-term exercise decrease in MMP-9 levels. mean ± SD.

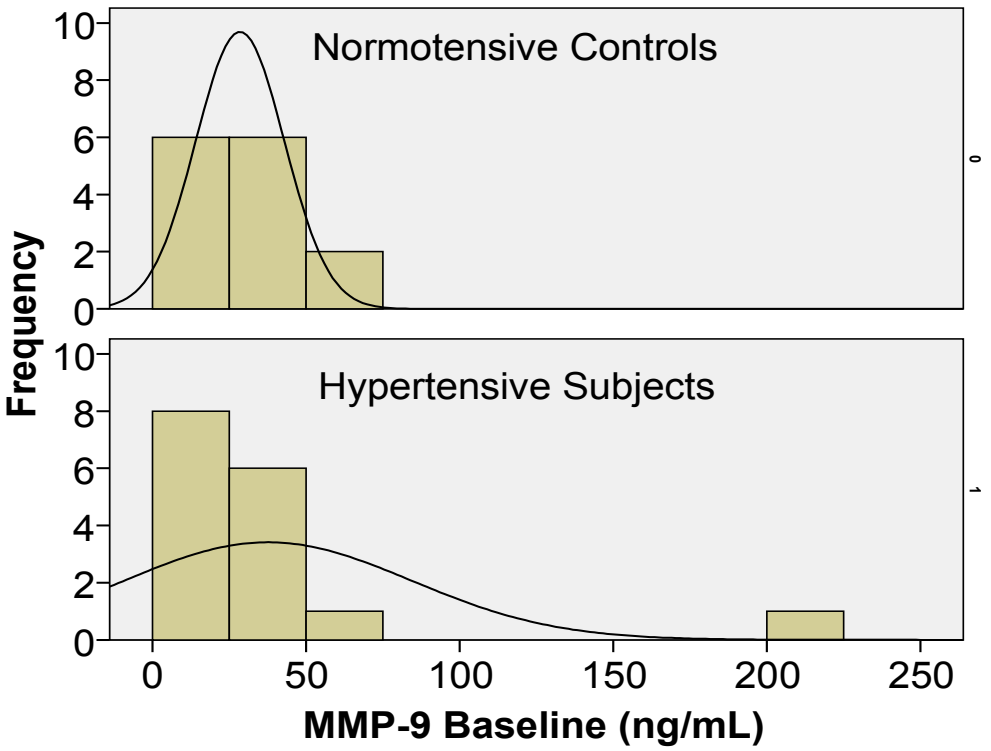

Figure 2-8. Frequency distribution of MMP-9 levels. The outlier disrupts the normal distribution curve for the hypertensive subjects.

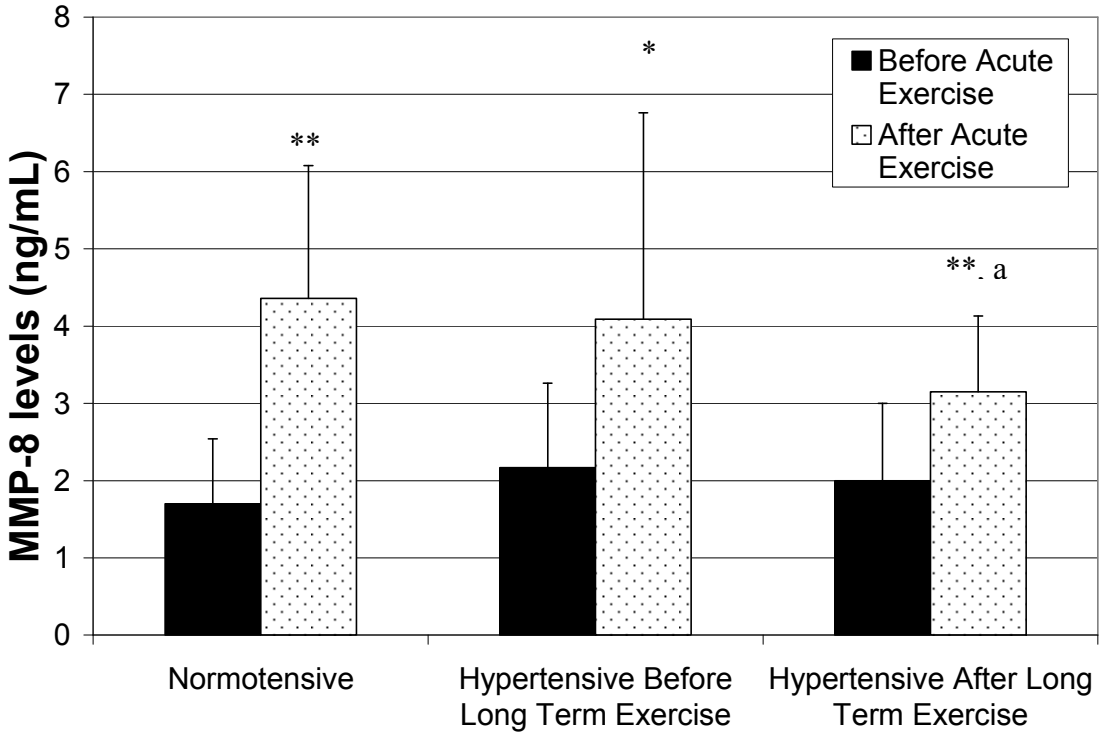

Figure 2-9. MMP-8 levels in heparin plasma. There is a significant acute exercise increase in MMP-8 for all three groups. In addition, there is an approaching significant effect (p=0.2) for long-term exercise. *p<0.05 compared with its respective before acute exercise group. ** p<0.001 compared with its respective before acute exercise group. [a]p=0.2 long-term exercise decrease in MMP-8 levels. mean ± SD.

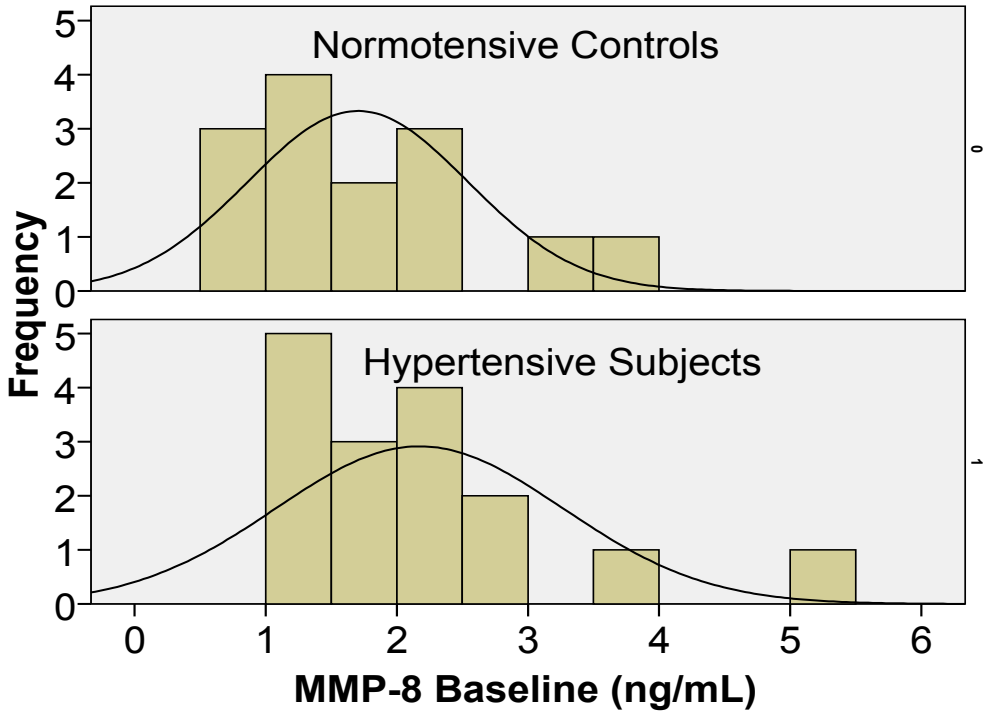

Figure 2-10. Frequency distribution of MMP-8 levels. The outlier disrupts the normal distribution curve for the hypertensive subjects.

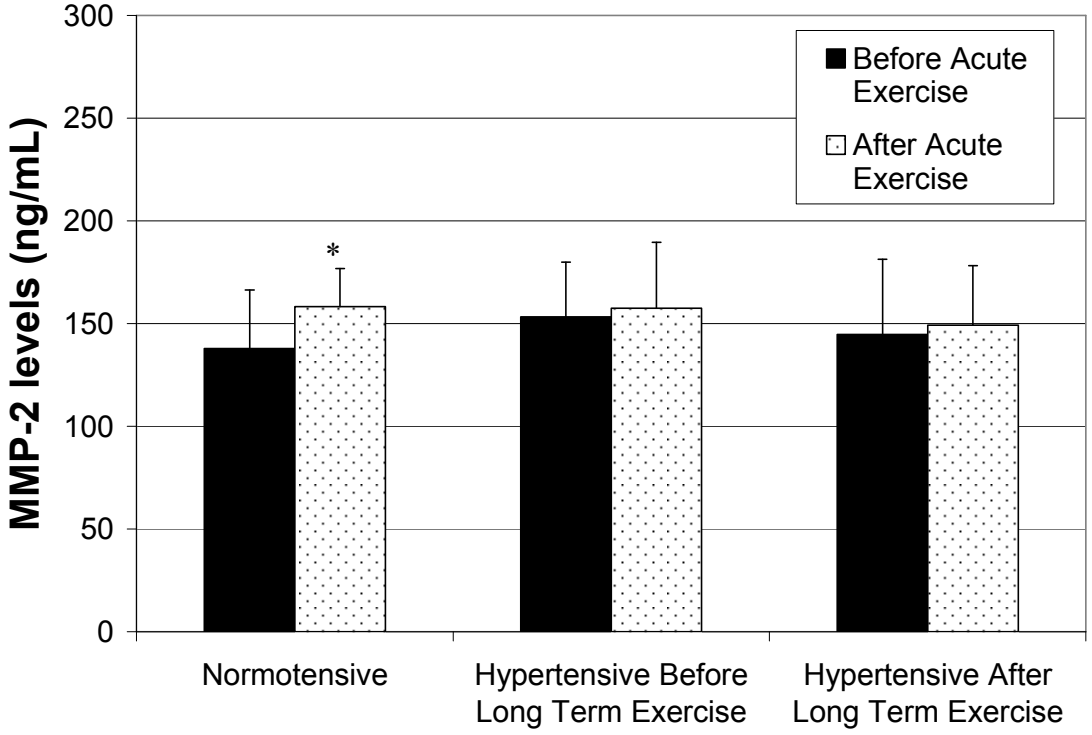

Figure 2-11. MMP-2 levels in heparin plasma. Only the acute exercise effects of normotensive patients are significant. *$p<0.05$ compared with its respective before acute exercise group. mean ± SD.

Table 2-4. Intra-assay and inter-assay CVs for MMP detection levels in plasma. With an average of around 8%, the CVs are within reasonable range. mean ± SD.

	Intra-assay CV Plate 1 (%)	Intra-assay CV Plate 2 (%)	Intra-assay CV Plate 3 (%)	Inter-assay CV (%)
MMP-2	8.6 ± 8.5	4.9 ± 3.3	7.9 ± 7.5	7.1 ± 2.0
MMP-8	12.5 ± 9.5	8.4 ± 9.4	8.6 ± 9.7	9.8 ± 2.3
MMP-9	12.3 ± 13.4	6.8 ± 6.4	4.2 ± 5.2	7.8 ± 4.1

Table 2-5. Listing of significant linear correlations for both the normotensive and hypertensive populations. From the table, we can determine that age correlates with MMP-2 levels, where MMP-9 correlates with the MMP-8 levels.

		age	MMP9 Baseline (ng/mL)	MMP8 Baseline (ng/mL)	MMP2 Baseline (ng/mL)
age	Pearson Correlation Sig. (2-tailed) N	1 31	.317 .082 31	.302 .099 31	***.366(*)*** ***.043*** ***31***
MMP-9 Baseline (ng/mL)	Pearson Correlation Sig. (2-tailed) N		1 31	***.597(**)*** ***<.001*** ***31***	.069 .712 31
MMP-8 Baseline (ng/mL)	Pearson Correlation Sig. (2-tailed) N			1 31	.267 .147 31
MMP-2 Baseline (ng/mL)	Pearson Correlation Sig. (2-tailed) N				1 31

* significant at the p=0.05 level (2-tailed).
** significant at the p=0.01 level (2-tailed).

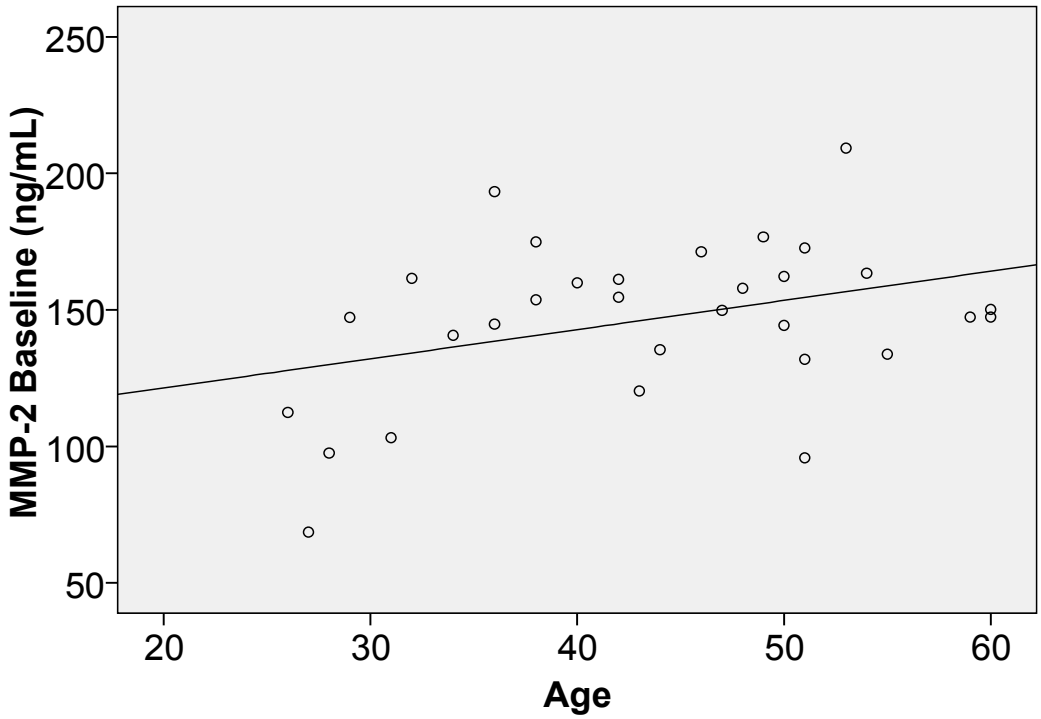

Figure 2-12. Correlation between age and MMP-2 Levels for both the normotensive and hypertensive populations. $r = 0.366$, $p < 0.05$.

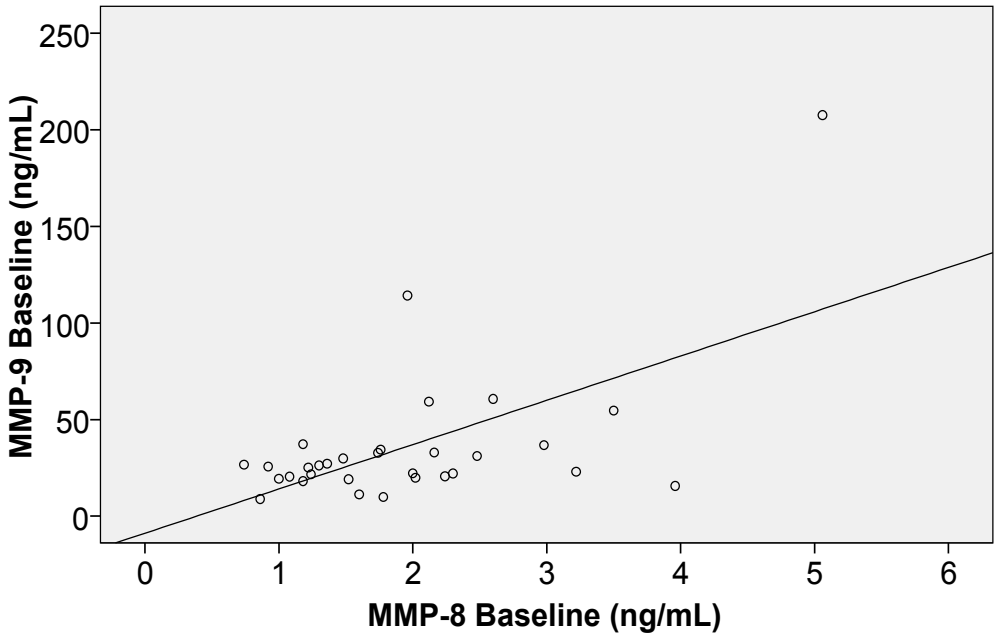

Figure 2-13. Correlation between MMP-9 and MMP-8 Levels for both the normotensive and the hypertensive populations. $r = 0.597$, $p < 0.001$

Table 2-6. Listing of correlations between various MMPs and inflammatory cytokines. From the table, we can determine that other than the correlation between MMP-9 and MMP-8 levels, there is no other significant correlation.

		CRP Baseline (ng/mL)	TNF-a Baseline (pg/mL)	IL-6 Baseline (pg/mL)	MMP2 Baseline (ng/mL)	MMP8 Baseline (ng/mL)	MMP9 Baseline (ng/mL)
CRP Baseline (ng/mL)	Pearson Correlation	1	.149	.185	.124	.102	-.030
	Sig. (2-tailed)		.442	.354	.523	.600	.877
	N	29	29	27	29	29	29
TNF-a Baseline (pg/mL)	Pearson Correlation		1	-.120	-.098	.055	.139
	Sig. (2-tailed)			.534	.601	.769	.454
	N		31	29	31	31	31
IL-6 Baseline (pg/mL)	Pearson Correlation			1	.172	.141	.101
	Sig. (2-tailed)				.372	.466	.602
	N			29	29	29	29
MMP2 Baseline (ng/mL)	Pearson Correlation				1	.267	.069
	Sig. (2-tailed)					.147	.712
	N				31	31	31
MMP8 Baseline (ng/mL)	Pearson Correlation					1	**.597(**)**
	Sig. (2-tailed)						**<.001**
	N					31	31
MMP9 Baseline (ng/mL)	Pearson Correlation						1
	Sig. (2-tailed)						
	N						31

** Correlation is significant at the 0.01 level (2-tailed).

2.3 Discussion

2.3.1 Patient Selection

Although we went through an extensive effort, it is harder to enroll in our experiment younger patients that are hypertensive and older normotensive patients than older hypertensives. Thus, there is a significant difference between the ages of the patients with hypertension and that of the patients with normotension. As age correlates with MMP-2 levels, we controlled the MMP-2 statistics for age.

2.3.2 Bleb Formation on Neutrophils

The membrane bleb count was initially intended as analysis of a pseudopod formation. However, after several control studies, I found out that the formaldehyde I used causes abnormal bleb formation during cell isolation. The cell surface projections caused by blebs can be similar in size and shape to pseudopod although they are not filled with actin. Consequently I could not carry out a pure pseudopod count.

The exact mechanism for this bleb formation by formaldehyde is unknown, but several hypotheses can be postulated. Even though the bleb formation is caused by formaldehyde, hypertensive patients have more bleb formation. Bleb formation leads to vesiculation, which may lead to release of various digestive enzymes (i.e. MMP-8 or MMP-9) to further breakdown the ECM [26]. As digestive enzymes are believed to be a cause of injury mechanism, a greater bleb count in hypertensive patients could potentially explain how they are more prone to injury mechanisms than normotensive controls.

2.3.3 Neutrophil Pseudopod Formation after Stimulation

One of our measurements to explore the presence of inflammatory mediators' measurements consists of stimulation of naïve neutrophils from a healthy volunteer by plasma from the hypertensive patients and normotensive controls and detection of pseudopods. For a limited number of wells that did not form clots, the pseudopod formation in neutrophils was analyzed. Lower levels of inflammation reduce the risk factors for various cardiovascular diseases [1]. Our results show that long-term exercise is indeed anti-inflammatory and beneficial for the patients.

Since formaldehyde causes abnormal bleb formation, glutaraldehyde was used as a fixation in this experiment. Glutaraldehyde crosslinks proteins so that the plasma samples that form gels may be the ones with more enzymes [71]. Thus, there is a chance that the hypertensive patients do cause more inflammation in the neutrophils, but the ones that do cause more inflammation may be the ones that formed the gels.

When looking at the negative controls, in which we suspended the neutrophils with PBS, we see pseudopod levels that are higher than if we stimulate with plasma (Table 2-2). This may suggest that, plasma may contain anti-inflammatory activity that decreases the incidence for pseudopod formation. There is also a chance that the neutrophil isolation protocol may do something to the cells that activates pseudopods. In other words, this study shows that plasma does indeed has an effect on pseudopod formation in neutrophils, but to find the exact mechanism needs further investigation.

2.3.4 MMP -2, -8, -9 Levels in Heparin Plasma

The findings in this study did not confirm our hypothesis that there may be different levels of MMPs in normotensive and hypertensive plasma samples. The patients used in this study are all sedentary, obese patients with a BMI of approximately 30 kg/m^2, which means that even the normotensive patients are not completely healthy and could also have high levels of circulating MMP. This could possibly explain why the MMP levels are similar between the hypertensive and the normotensive groups.

There are outliers in the hypertensive group, but not the normotensive group, for both MMP-9 and MMP-8 that fall outside a normal Gaussian distribution. This suggests a subgroup of hypertensives that may be potentially more susceptible to shock and other cardiovascular diseases. I repeated the analysis after exclusion of these outliers. The results indicate that there is a reduction of MMP-9 levels caused by hypertension (Appendix A), with the MMP-8 results similar to that when including the outliers.

For the first time, the effects of acute exercise were tested for MMP levels in heparin plasma. Acute exercise induces potential cytokine increase or muscle injury which in turn increases inflammation [44]. Our findings suggest that MMP levels for both gelatinases do not increase with acute exercise as much for hypertensive patients as they do normotensive patients. MMPs are digestive enzymes involved in the inflammatory cascade, so it seems to be a normal response that MMP levels will increase after exercise. MMP-9 levels did not show the acute exercise effect for the hypertensive group because high standard deviation. MMP-2 levels also did not show this acute exercise effect, suggesting a weaker response for acute exercise on circulating MMP-2 levels in hypertensive patients. After the 12-week long-term exercise period, the

circulating MMP-8 and MMP-9 levels drop, suggesting anti-inflammatory effects after long-term exercise.

Many other studies [57, 60] in the past have shown a significant difference for MMP levels in hypertension. However, no studies associate MMP levels with physical activity. After long-term exercise, the hypertensive group shows lower MMP-9 and MMP-8 levels. Since long-term exercise is well known to decrease inflammation and lower blood pressure, there is a good chance that the hypertensive patients are mostly sedentary, where the normotensive controls could have sufficient amounts of exercise in other studies. In addition, diabetes also should be taken into consideration. As MMP-9 may cleave the insulin receptors [36], thus leading towards diabetes, it is important that all studies take diabetes into consideration.

Another concern is associated with the generation of protein clots I saw in the hypertensive plasma. There is a possibility that such protein clot contain the proteins of interest [70], and that in particular MMPs may be trapped. The hypertensive patients' plasma has more protein clots than the normotensive patients' plasma. In the presence of a protein clot the pipette tip becomes obstructed. Even though we see no difference in the plasma levels between normotensive and hypertensive patients, this protein clot needs to be studied further before making any final conclusions.

2.3.5 Correlations

Since the effects of blood pressure on various MMP levels are shown not to be significant in this study, there might be some other factors that may show a correlation with MMP levels. We correlated various demographic features like age, VO_2 max,

gender and BMI as well as inflammatory markers such as TNF-α, IL-6, and CRP with the MMP levels. Results for the various inflammatory cytokines (Appendix B) indicate that except for IL-6, there is little difference in inflammation between the hypertension group and the normotension group.

MMP participates in many pathological processes such as arthritis, cancer and cardiovascular disease [54]. The correlation between MMP-2 and age could be a potential reason why older people are more prone to these pathological processes. The correlation between MMP-9 and MMP-8 may be due to the fact that after acute exercise, there is an increase in neutrophils account that in turn increases the MMP-9 and MMP-8 concentration in plasma. After the 12 week long-term exercise regimen, there is less of an increase in MMP-9 and MMP-8 concentration because there is less inflammation following exercise. MMP-2 has no correlation with MMP-9 and MMP-8, indicating that MMP-2 does not have the same acute and long-term exercise response as MMP-9 and MMP-8.

2.3.6 Limitations

There are several limitations to this study. Standard deviations are generally higher in human populations compared to animal models, so around 15 patients in each group might not be sufficient to reach levels of sufficient statistical power. The outliers with the hypertensive group could also cause inaccurate statistical evaluations. Another limitation lies within the plasma. Since not all of the patient's plasma is drawn simultaneously, it is required to freeze the plasma in order to sustain enzyme activity.

This freeze-thaw cycle could cause potential deformation in the proteins, leading to inaccurate measurements.

It is important to note that MMP protein level is not equivalent to MMP activity. Pilot studies show that even one freeze thaw cycle has a near 20% decrease in MMP activity. We believe that this freeze-thaw cycle will affect the MMP activity even more than the MMP levels in plasma. MMP-8 is known to be activated within the neutrophils before being secreted in the plasma. Therefore, it is likely that all the MMP-8 detected in the plasma is already in its active form, explaining the relatively low levels of MMP-8 in human plasma. Until testing of protocol plasma samples that do not involve a freeze-thaw cycle, all studies that focus on MMP activity levels may therefore be subject to an in-vitro modification of the activity due to the freeze-thaw cycle.

In addition, protein clots occurring after the thawing of plasma might contain most of the proteases we want to measure. Trying to avoid the transfer of this protein clot might very well be the reason why we saw no significant increase in MMP levels between hypertensive and normotensive subjects. Last, there is no perfect fixative known to date for our studies. In order to preserve the neutrophils at a certain state, it is necessary to use a fixation solution. However, there are setbacks for these fixatives. When using formaldehyde, we see abnormal bleb formation on the neutrophils. Glutaraldehyde stops this abnormal bleb formation, but due to its strong cross-linking nature, it tends to form gels when mixed with plasma.

2.4 Conclusion

The hypertensive group is not associated with naïve neutrophil pseudopod formation or MMP-2, MMP-8, and MMP-9 levels, which did not confirm our hypothesis. The outliers present in the hypertensives but not the normotensives suggest some increased risks with a subgroup of hypertensive patients. Meanwhile, in align with our hypothesis, acute exercise temporarily increases MMP-2, MMP-8, and MMP-9 levels, but long-term exercise decreases these MMP levels and is shown to have anti-inflammatory effects. When calculating correlations, we see a possibility that MMP levels are more related to age rather than blood pressure.

Appendix A

A.1 MMP-9

If the outliers were eliminated from the analysis, there is a significant decrease in MMP-9 levels in hypertensives during the acute exercise (Figure A-1). Also, acute exercise causes an almost ($p=0.06$) reduction of the MMP-9 levels in both before and afterlong-term exercise for hypertensive patients. The after long-term exercise MMP-9 levels are the same as they are before long-term exercise. This suggests that, excluding an outlier subpopulation in the hypertensive group, long-term exercise has little effect when it comes to circulating MMP-9 levels.

A.2 MMP-8

Even with the outliers removed, there is no change of MMP-8 levels compared to the results before the outliers are removed. The increase of MMP-8 during acute exercise is still relevant, and long-term exercise significantly decreases in the hypertensive patients ($p=0.02$).

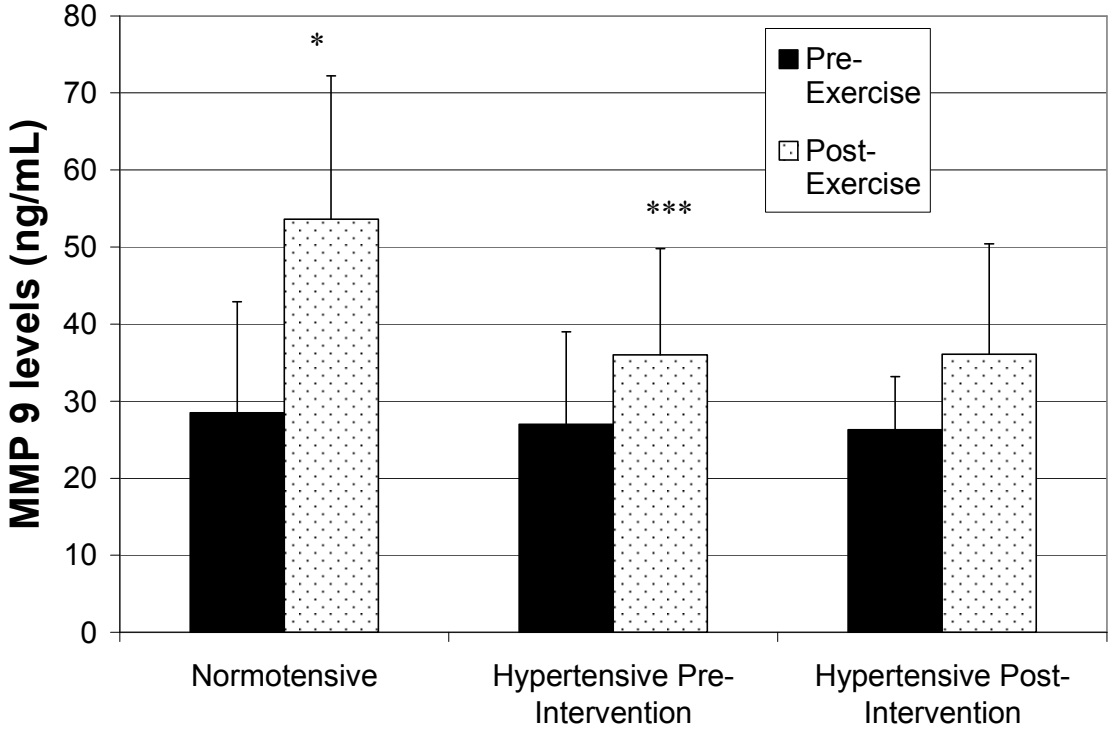

Figure A-1. MMP-9 levels in plasma without the outliers. The normotensive acute exercise effect is significant ($p<0.05$), where the hypertensive before and after long-term exercise groups have an acute exercise effect that is approaching significance ($p=0.06$). What is interesting is that hypertension has a significant effect on the acute exercise effect. *$p < 0.001$ in correspondence to the before acute exercise group. ***$p<0.05$ in correspondence to the normotensive group. mean ± SD.

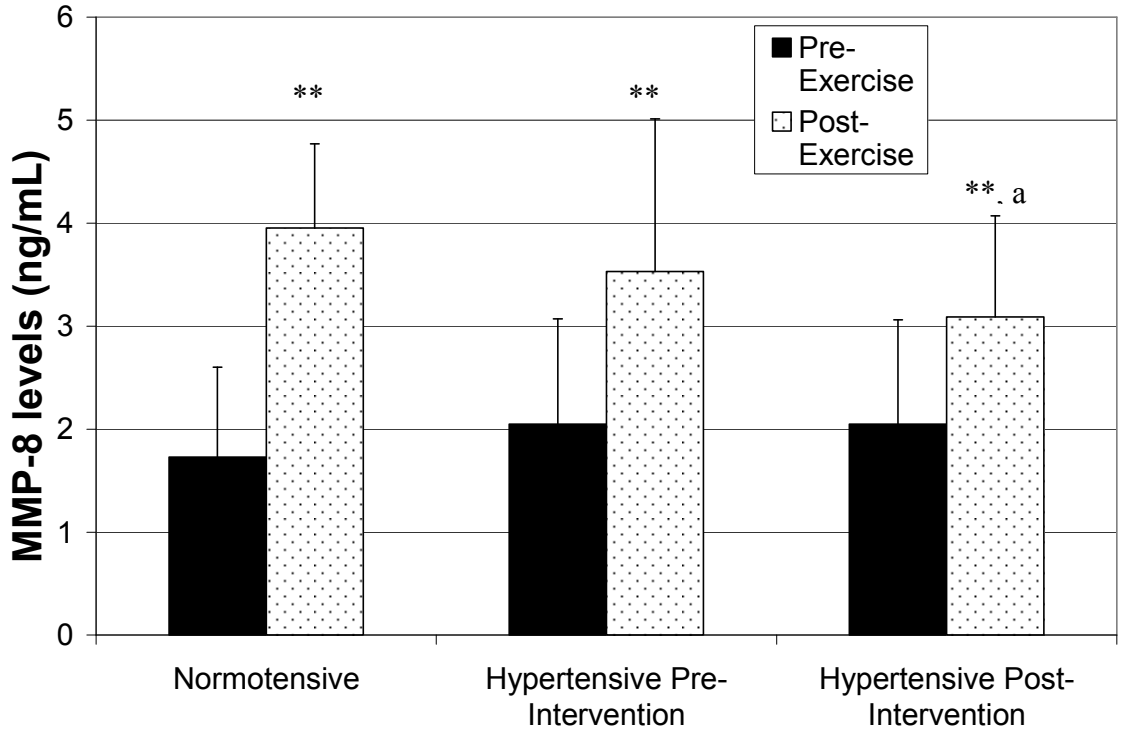

Figure A-2. MMP-8 levels in plasma without the outliers. All three groups have a significant acute exercise effect. What is interesting is that hypertension has a significant effect on the acute exercise effect. The long-term exercise effect, like the samples without outliers, is approaching significance (p=0.2). **p<0.001 in correspondence to the before acute exercise group. [a]p=0.2 long-term exercise decrease in MMP-8 levels. mean ± SD.

Appendix B

B.1 Protocol

The results for the inflammatory markers are completed by collaborators using the ELISA technique (R&D Systems) with the same plasma. We interpreted the data for potential correlations with our neutrophil activation / protease level results. All statistics are done with SSPS 15.0 for windows (SSPS).

B.2 IL-6

The results show an approaching significant ($p=0.1$) increase in the hypertensive population when compared with the normotensive population for IL-6 levels, both before and after acute exercise. This suggests higher inflammation levels in the hypertensive population. There is no acute or long-term exercise effects, suggesting that exercise might have no effect on IL-6 levels.

B.3 TNF-α

TNF-α levels in plasma decreases after acute exercise for the hypertensive group before long-term exercise only. To understand the true acute exercise effect needs further investigation with more subjects. Meanwhile, long-term exercise has an approaching significant ($p=0.1$) decrease in TNF-α levels in plasma at least in the hypertensive population. This suggest anti-inflammatory effects for long-term exercise. There is no difference between the hypertensive and normotensive populations in TNF-α levels, suggesting that blood pressure has little correlation for this inflammatory cytokine.

B.4 CRP

There is currently no significant difference between the CRP levels of hypertensive and normotensive patients. There is also no acute or long-term exercise effect on the CRP levels at least in the hypertensive population. This suggests little difference in CRP levels for the measurements of our study.

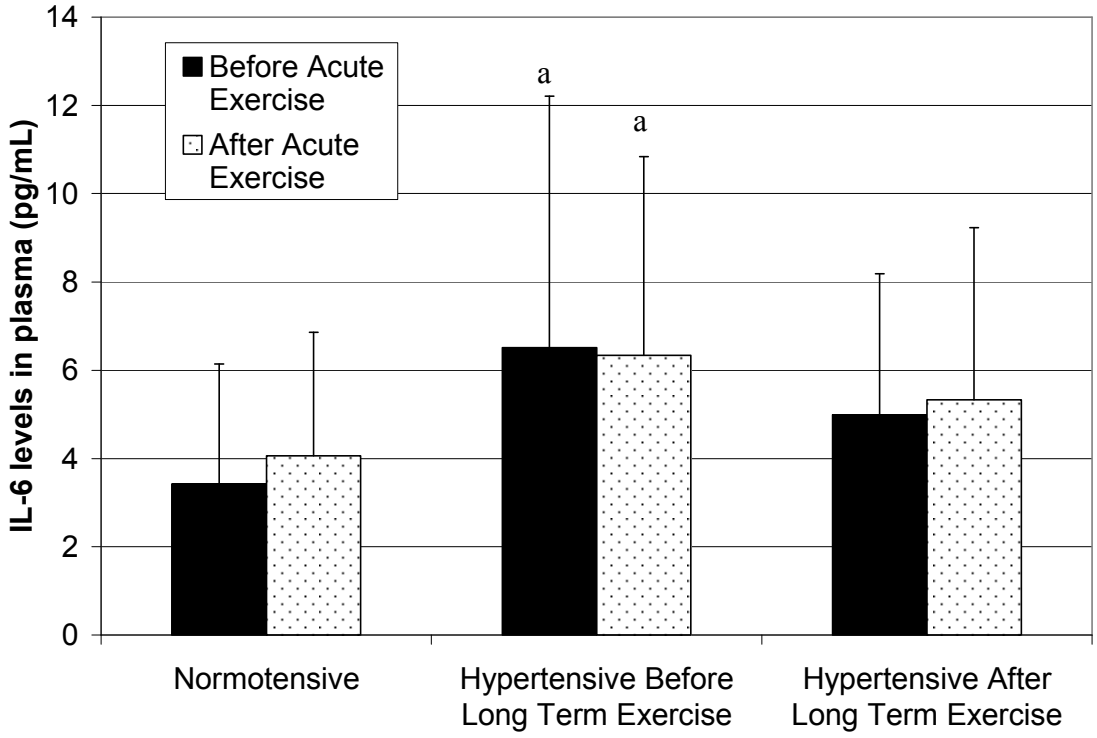

Figure B-1. IL-6 levels in the plasma. There is little acute or long-term exercise effect on the IL-6 levels. There is an approaching significant (p=0.1) increase in IL-6 levels in the hypertensive group. [a]p=0.1 hypertension group increase in IL-6 levels over normotensive controls. mean ± SD.

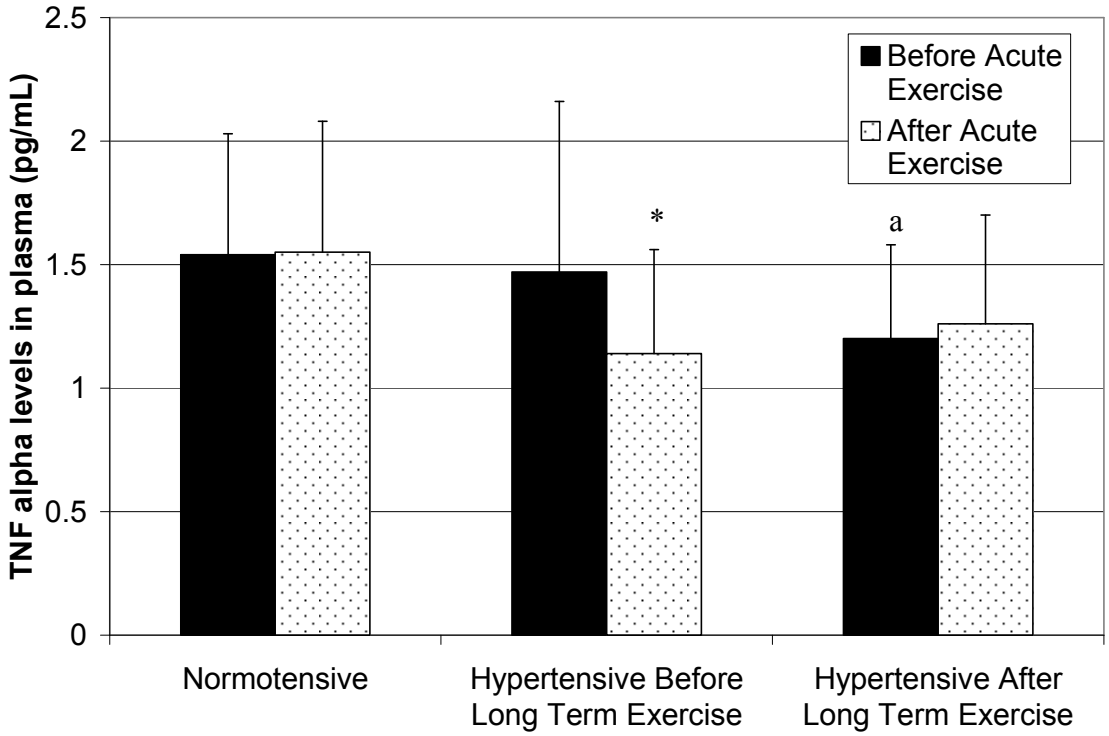

Figure B-2. TNF-α levels in plasma. There is an acute exercise decrease and an approaching significant long-term exercise decrease ($p=0.1$) in TNF-α levels for the hypertensive group. *$p<0.05$ in correspondence to the before acute exercise group. [a]$p=0.1$ long-term exercise decrease in in TNF-α levels. mean ± SD.

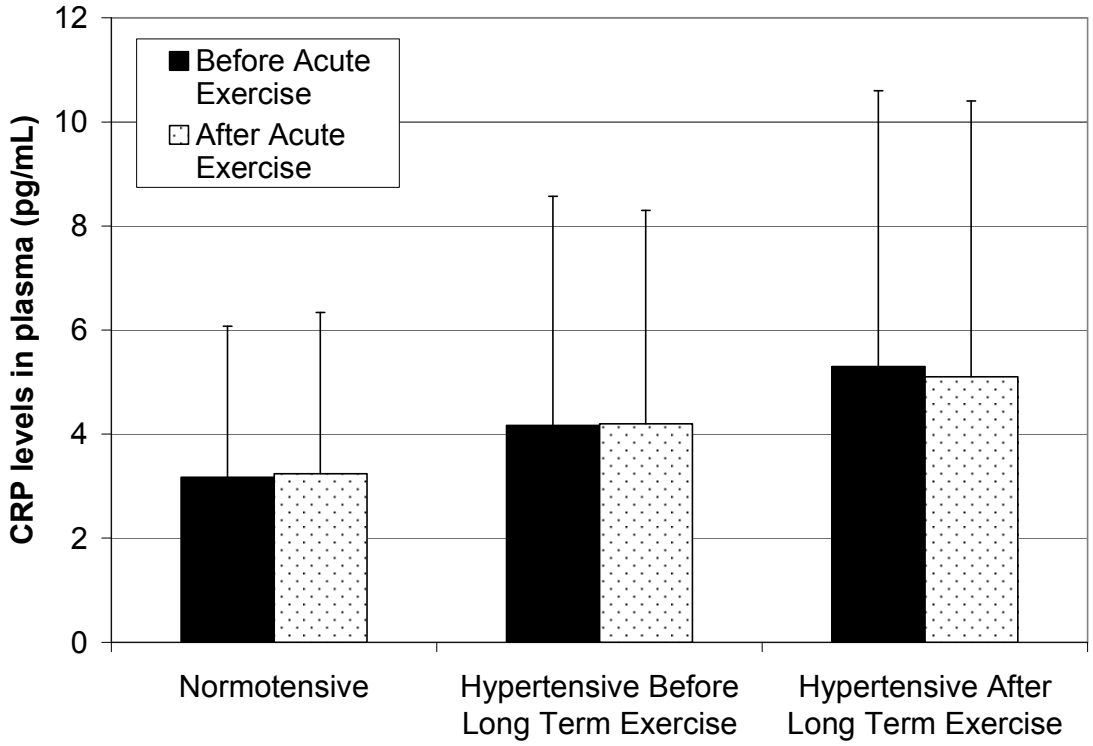

Figure B-3. CRP levels in plasma. Due to the high amounts of standard deviation, there is no significant difference in any of the results. mean ± SD.

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