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Does heart rate variability predict endothelial dysfunction? (A study in smokers and atherosclerosis patients)

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DOES HEART RATE VARIABILITY PREDICT ENDOTHELIAL DYSFUNCTION?

(A STUDY IN SMOKERS AND ATHEROSCLEROSIS PATIENTS)

by

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CERTIFICATE OF APPROVAL

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MASTER'S THESIS

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This is to certify that the Master's thesis of

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To Mom and Dad

For the LORD gives wisdom; from his mouth come knowledge and understanding. He holds success in store for the upright, he is a shield to those whose walk is blameless, for he guards the course of the just and protects the way of his faithful ones

Proverbs 2:6

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CHAPTER I

INTRODUCTION

Epidemiology

Cardiovascular diseases are the cause of many deaths and morbidities in the United States. Overall mortality rate has declined by about 44% since 1968 (1). However, cardiovascular diseases still remain to be the leading cause of death with 831,000 deaths (34.2% of all deaths) in the year 2006 (1). The decline of mortality since the 1960's is believed to be attributed to improvements in treatment of cardiovascular diseases and reduction in risk factors such as hypertension, high cholesterol and smoking. In 2006, it was estimated that about 81.1 million people in the US had some form of cardiovascular diseases and about 74.5 million people had hypertension (1). Cardiovascular diseases are not only a burden to the patients and their families, but also to the US economy as it is estimated to cost 503 billion dollars in the year 2010 (1, 78).

Heart Rate Variability: Overview

Heart rate variability is defined by the variation of the time intervals between two heartbeats (2). In 1965, Schneider and Costiloe reported that the period of time between two consecutive heartbeats is not constant throughout a person's heartbeats (4). In the same year this idea was further explored by Hon and Lee (3) who showed a clinical relevance of heart rate variability. They studied the fetal heart rate patterns preceding the death of fetuses. Hon and Lee found that there were characteristic changes in consecutive heart beat intervals before fetal distress (3).

Since those early studies, knowledge on heart rate variability has progressed and it is now believed that heart rate variability can indicate changes in autonomic nervous system and/or change in the heart's ability to respond to changes in cardiac autonomic activity. The heart is able to rapidly respond to changes in autonomic nervous system activity (2). The autonomic nervous system, an output from the central nervous system, has three distinct branches:

sympathetic, parasympathetic, and enteric. As the name suggests, the autonomic nervous system is mostly governed independently from self-will. The heart is innervated by both sympathetic and parasympathetic branches of the autonomic nervous system (6). The heart also contains an intrinsic cardiac nervous system that includes interneurons and ganglia to form a complex plexus at the base of the heart (24). This intrinsic cardiac nervous system is not only important for relaying signals from the sympathetic and parasympathetic nervous system to the heart, but also for integrating signals from interneurons and myocardial sensory neurons (24). The heart rate is determined from the balance of the activities of these two branches. The sympathetic branch, once activated, releases norepinephrine to the sinoatrial (SA) node, atria and ventricles to increase both heart rate and contractility. The parasympathetic branch releases acetylcholine to the SA node, AV node and atria to decrease the heart rate. Parasympathetic activation arises from vagal nerves that have a strong tonic inhibitory input to the heart (6). Heart rate variability can provide information on autonomic influences on cardiac function. Therefore, heart rate variability can be used to study the interplay between autonomic nervous system activity directed to the heart and cardiac autonomic responsiveness (2, 90).

Heart Rate Variability Analyses

The analysis of heart rate variability to diagnose patients with possible cardiovascular diseases has advantages in that the test is simple, economical and non-invasive. The initial data can be obtained from a single lead chest electrocardiogram recording. Chest electrocardiogram is simple, inexpensive and can be done fast as it is widely used in a variety of departments throughout hospitals (5, 7). Heart rate variability is a measure of electrical activity rather than mechanical activity of the heart because an electrocardiogram measures the electrical activities of the heart (5).

There are two types of heart rate variability analyses: short- and long-term. Short-term heart rate variability, derived from 5-minute EKG recordings (7), may have limited predictive value for assessment of cardiovascular diseases and risk factors such as myocardial infarction or

smoking (23). The predictive power of short-term recordings of heart rate variability is significantly lower than that of long-term recordings of heart rate variability (23). Long-term heart rate variability, derived from 24-hour EKG recordings (7), may provide higher power of prediction of cardiovascular events. However, the technical difficulties and the cost of such methods are significantly higher. Both methods of analyses are widely used. Due to their advantages and disadvantages, either method can be used, but the method that is best suited for the purpose of the study should be used (21, 23).

In both long-term and short-term recordings of heart rate variability, time domain and frequency domain analysis can be performed (2). The time domain analysis utilizes continuous data of an electrocardiogram for a given period of time. Each physiological QRS complex is labeled as normal (N). From this, normal to normal intervals (NN) and instantaneous heart rate can be obtained. For the purpose of this thesis, standard deviation of NN intervals (SDNN), the square root of the mean squared differences of successive NN intervals (RMSSD), and the standard deviation of differences between adjacent NN intervals (SDSD) were used. SDNN measures both high frequency variations and low frequency variations. RMSSD and SDSD measure mainly of high frequency variations in heart rate (7). With frequency domain analysis, sympathetic and parasympathetic modulation of cardiac function are assessed in the low frequency range (0.04 – 0.15 Hz) while parasympathetic modulation is assessed in the higher frequency range (0.15 – 0.4 Hz) of heart rate variability (2, 9, 90). The Task Force of the European Society of Cardiology and The North American Society of Pacing and Electrophysiology suggested five minutes of recording should be used to evaluate short-term heart rate variability (7). Frequency domain analysis provides a powerful tool to distinguish between sympathetic and parasympathetic contribution to heart rate variability in a subject (8, 9).

Risk Factors that Affect Heart Rate Variability

Previous studies have shown that there are relationships between heart rate variability and common risk factors for heart disease. These risk factors include alcohol, depression, smoking, stress, and obesity (2).

Alcohol

In 1981, Melgaard and Somnier showed in their study that chronic alcoholics had peripheral autonomic neuropathy that led to cardiomyopathy (13). Alcohol, during acute consumption, can decrease heart rate variability. The mechanism for this reduction was proposed to be an increase in sympathetic nervous system activity and/or decrease in parasympathetic nervous system activity directed to the heart (14, 15). Alcoholics, therefore, are more likely to have sudden cardiovascular related deaths (16). Heavy alcohol drinkers, defined as 100 oz of alcohol or more per month (16) tend to have higher blood pressure than the norm. With this regard, hypertensive patients, due to a variety of etiologies including autonomic dysregulation, are reported to have lower heart rate variability (20).

Depression

According to the National institute of Mental Health, about 7% of Americans over the age of 18 suffer from clinical major depression (37). For patients with a history of myocardial infarction, depression has been shown to be a risk factor for increase in cardiac morbidity and mortality (38, 39). “Major depression is associated with a four-fold increase in the risk of mortality during the first six months after acute myocardial infarction” (38). Patients with depression had a significant decrease in heart rate variability compared to non-depressed patients with a history of acute myocardial infarction. Although the exact pathophysiology is unclear (37, 38), it does raise the question if heart rate variability in patients that are treated for depression will increase and if cardiovascular morbidity and mortality will improve.

Smoking

Acute and/or chronic cigarette smoking has adverse affects on the human body. Bartecchi et al. (31) stated in a review article that in the United States over 179,000 patients died in the year 1990 from cardiovascular diseases that were caused by cigarette smoking. Cigarette smoking is a risk factor for a variety of cardiovascular diseases including coronary artery diseases, myocardial infarction, atherosclerosis and sudden death (32, 33, 34, 35, 36). Acute cigarette smoking causes an increase in release of norepinephrine. The increase in release of norepinephrine has been shown to increase the risk for ventricular fibrillation (32, 33). Development of atherosclerosis from chronic smoking is evident from vascular endothelium damage (32, 34). Chronic heavy smokers, defined by anywhere between 20 pack a year to 20 cigarettes per day depending on the studies, have been shown to have unstable (increase in blood pressure and sympathetic nervous activity) autonomic nervous system control of the heart compared to matched non-smokers (32, 33, 34, 35). Chronic heavy smokers have higher sympathetic nervous system activity and decreased cardiac vagal tone. This increase in sympathetic nervous system activity may explain the reduction in baroreflex gain seen in these subjects (32, 33). Collectively, these mechanisms lead to reduced heart rate variability (32, 33, 34, 35). It is worth noting that even second hand smoke has been shown to decrease heart rate variability (36).

Obesity

Obesity is one of many independent risk factors for cardiovascular diseases including coronary heart disease, hypertension and atherosclerosis. Although the pathophysiology of obesity leading to cardiovascular diseases is not yet well understood, there is a strong correlation between the two. Obesity has been shown to cause autonomic nervous system dysfunction indicated by decreased parasympathetic nervous system modulation and increased sympathetic nervous system activity. Obesity can also lead to endothelial dysfunction with impaired endothelium-dependent vasodilation (40, 64, 65)

Myocardial Infarction

Previous studies have shown a relationship between heart rate variability and cardiovascular diseases such as myocardial infarction and hypertension, and other diseases including diabetes and renal failure (2, 21). Heart rate variability can provide important information about a patient's cardiovascular system. Therefore, heart rate variability is widely used as an independent prognostic parameter in post-myocardial infarction patients. Post-myocardial infarction patients with reduced 24-hour (long-term) heart rate variability have 3 – 4 times greater risk for cardiovascular related mortality (5, 20, 21, 22, 23). There is insufficient parasympathetic nervous system modulation of heart rate after a patient has suffered from acute myocardial infarction, as indicated by reduced heart rate variability. The decreased parasympathetic modulation of cardiac function often is associated with an increased sympathetic modulation of cardiac function. This phenomenon can contribute to development of ventricular fibrillation, leading to higher risk of sudden cardiac death or cardiovascular related mortalities. This decrease in parasympathetic cardiac modulation is persistent even 6 months after an acute-myocardial infarction. However, parasympathetic nervous system function directed to other organ systems do not seem to be impaired, for example as measured by pupillometry (2, 3, 22, 23, 24).

Diabetes

Almost 24 million people in the US had some form of diabetes in the year 2007. Diabetes leads to many complications resulting in cardiovascular related deaths and renal failure (28). Furthermore, diabetes mellitus can cause autonomic neuropathy (2). Although the pathophysiology of autonomic neuropathy is not yet well understood, hyperglycemia is thought to be one of the main reasons. Hyperglycemia causes an increase in intracellular glucose level in nerve cells. This leads to a reduction in Na^+/K^+-ATP pump activity, impaired axonal transport, and structural breakdown of nerves. These structural and functional abnormalities in the autonomic nervous system hinder action potential propagation leading to neuropathy (29, 30).

The problem of autonomic neuropathy in diabetes mellitus patients is that the symptoms of the disease have a very late onset. Therefore, patients can stay relatively asymptomatic and have no forewarning of sudden cardiac death (25). Heart rate variability has been introduced to possibly diagnose autonomic neuropathy in diabetes mellitus patients that are asymptomatic. Wheeler et al. (26) reported that autonomic neuropathy causes cardiac vagal denervation as indicated by a decrease in heart rate variability in patients with autonomic neuropathy (26). Singh et al. (27) also reported that "Heart rate variability is inversely associated with plasma glucose levels and is reduced in diabetics as well as in subjects with impaired fasting glucose levels" (27).

Blood Vessels and Endothelium

Blood vessel structure varies depending on function and location. All arteries and most veins have four components that make up the vessel structure: endothelium, elastic fibers, smooth muscles and collagen fibers. Capillaries and venules may lack in elastic fibers, smooth muscle and collagen fibers. For the purpose of this study, arteries and their endothelium were the main focus. Endothelium is a group of cells that form a single layer in the inner part of the cardiovascular system including arteries, veins, and the heart. These endothelial cells produce cytokines that affect local platelet activation, leukocyte adhesion, inflammation, thrombosis and vasomotor activities. Endothelial function can be assessed by endothelial-dependent vasodilation (6, 10, 41, 47).

Endothelial Function

Arteries can constrict or dilate depending on different hemodynamic conditions. During physiologic conditions, endothelial cells maintain vascular homeostasis through complex interactions that involve nitric oxide (42, 47). Endothelial function can be assessed by several different methods including brachial artery flow-mediated dilatation and forearm vascular resistance. Flow-mediated dilatation measures endothelial function by vascular response to reactive hyperemia. Doppler ultrasound technology is used to observe the change in vessel diameter elicited by a sudden change in blood flow through a conduit vessel such as the response

to opening a previously occluded cuff around the upper arm (Figure B1) (88). Forearm vascular resistance can be determined as the ratio of change in blood pressure and blood flow. This method is based on plethysmography rather than Dopper ultrasound technology, because plethysmography not only assesses flows through conduit arteries but also assesses flow through resistance arteries (41, 45, 46, 8).

Endothelial Dysfunction in Atherosclerosis

The pathophysiology of many cardiovascular diseases including atherosclerosis has been attributed in part to endothelial dysfunction (45, 47, 48, 66). Endothelial dysfunction is broadly defined by "an imbalance between vasodilating and vasoconstricting substances produced by (or acting on) endothelial cells" (45) and is associated with some cardiovascular disease risk factors such as smoking, dyslipidemia, hypertension and diabetes mellitus (47). Atherogenesis, a process in which plaques are formed in the inner lining of arteries, leads to the pathologic condition of atherosclerosis. There are several factors that contribute to atherogenesis including immune system activation, chronic inflammation, reactive oxygen species, C-reactive protein and nitric oxide/endothelin balance (44, 45, 46). These factors may act in synergy with each other or in a "vicious circle" to intensify endothelial dysfunction. During atherogenesis there is an up-regulation of adhesion molecules such as selectins and integrins. These adhesion molecules promote leukocyte and monocyte adhesion and chemotaxis into the sub-endothelial layer. Presence of these immune cells promotes the release of cytokines such as tumor necrosis factor-α and interleukin-6. Interleukin-6 further stimulates hepatic release of C-reactive protein. These cytokines and proteins enhance atherogenesis and vasculitis (46, 48, 50). Reactive oxygen species play an important role in the endothelium to cause endothelial dysfunction. Reactive oxygen species disrupt physiological bioactivity of nitric oxide by decreasing the activity of nitric oxide synthase and by shortening the half life of nitric oxide. Nitric oxide not only causes vasodilation, but also has anti-inflammatory and antithrombotic effects. Furthermore, a decrease in nitric oxide bioavailability causes an imbalance between nitric oxide and endothelin

concentrations. Nitric oxide and endothelin have opposite effect on the vasculature: vasodilation and vasoconstriction, respectively. Imbalance of these two compounds in the vasculature can cause endothelial dysfunction via upregulation of adhesion molecules, inflammation and vasoconstriction. The reactive oxygen species superoxide anion reacts with nitric oxide to form peroxynitrite which damages endothelial cells (45, 46, 47, 50, 66). In summary, endothelial dysfunction eventually leads to atherosclerotic lesion formation and progression, plaque activation/rupture, and decreased blood flow due to thrombosis and vasospasm. Furthermore, there are some risk factors that have been associated with increased risk of endothelial dysfunction such as smoking, dyslipidemia and diabetes (48, 66) that will be discussed next.

Endothelial Dysfunction Risk Factors

Cigarette Smoking

Cigarette smoking is considered a major risk factor that can lead to many cardiovascular diseases including atherosclerosis and coronary heart disease. There have been numerous studies done that link smoking with endothelial dysfunction, (53 – 56) demonstrating impairment of endothelium-dependent vasodilation in cigarette smokers. It has been found that cigarette smoking increases activation and adhesion of platelets and leukocyte in the endothelium. As discussed previously, this can lead to endothelial dysfunction. Cigarette smoking also seems to affect plasma lipid levels, insulin resistance and thrombotic agents like tissue plasminogen activators (tPA) (46, 51, 53, 55). Newby et al. (51, 52) demonstrated in their studies that "despite higher basal plasma tPA antigen concentrations, cigarette smokers have a markedly impaired capacity of the endothelium to release tPA acutely" (51). This finding illustrated that "cigarette smoking can lead to arterial thrombosis and myocardial infarction"(51). Newby et al. further demonstrated that changes in the endothelium such as a decrease in fibrinolytic capacity precede endothelial dysfunction, atherothrombosis and myocardial infarction in cigarette smokers (52). Cigarette smoke contains high concentrations of reactive oxygen species. As previously discussed, this can lead to endothelial dysfunction via peroxynitrite formation and a decrease in

bioavailability of nitric oxide (46, 55). Furthermore, exposure to a smoking environment (second hand smoke) also causes endothelial dysfunction and accelerated atherosclerosis. The pathophysiology of second hand smoke seems to be similar to direct cigarette smoking, but the endothelial changes seem to be reversible (53, 56).

Diabetes Mellitus

Diabetes mellitus leads to many complications and health problems including cardiovascular diseases. Type I (insulin-dependent) and type II (non-insulin dependent) diabetes mellitus both seem to have an effect on endothelial function. Previous studies have reported that there is a decreased endothelium-dependent vasodilation in arteries from diabetic patients. Patients with type II diabetes seem to be more sedentary and have a higher prevalence of obesity and metabolic syndrome. These patients also suffer from higher plasma levels of adhesion molecules, tPA inhibitors, pro-inflammatory cytokines and pro-thrombotic precursors. Furthermore, in type II diabetes mellitus, there is an inverse relationship between endothelial function and insulin resistance. Consequently, patients with hyperinsulinemia are likely to have more severe endothelial dysfunction than patients without hyperinsulinemia (57, 58, 59).

Dyslipidemia

The Center of Disease Control has estimated that 34% of adult Americans had obesity in 2008 (1). Obesity is one of many risk factors for cardiovascular diseases and has a strong association with dyslipidemia (60). Dyslipidemia is a condition when a patient has abnormal amounts of lipid in the blood circulation. Most of dyslipidemia cases in the US are hyperlipidemia, which include hypercholesterolemia. The causes of these conditions are both hereditary and environmental, i.e. diet. Endothelium-dependent vasodilatation in both animal models of hyperlipidemia and clinical studies of adult patients with hypercholesterolemia was reduced compared to healthy controls. Clinical studies of pediatric patients with a family history of hypercholesterolemia showed decrease in endothelium-dependent vasodilatation by nearly 90% when compared to healthy controls. The major pathophysiologic finding in the pediatric

population was insufficient production of local vasodilators such as nitric oxide. These findings indicate that both environmentally- and/or genetically-induced hypercholesterolemia can lead to endothelial dysfunction (60, 61, 63).

Atherosclerosis and Cardiovascular Diseases

Atherosclerosis is one of the major cardiovascular disease which can lead to myocardial infarction and stroke. Historically, atherogenesis was believed to be the accumulation of fatty deposits on arterial walls that increase in size large enough to block blood flow. However, as previously discussed, formation of atherosclerosis is far more complex involving many different types of cells and soluble factors, such as cytokines. The endothelium also plays a crucial role in atherogenesis through inflammatory factors and reactive oxygen species. Furthermore, the location of atherogenesis favors conduit vessels that lacks in laminar blood flow. Within the endothelium, atherogenesis starts with recruitment of monocytes. Once monocytes reach the inner lining (intima), they can mature into macrophages that release more inflammatory factors and engulf modified lipoproteins such as cholesterol esters. As more macrophages are recruited and more lipoproteins are engulfed, the beginning stages of atherosclerosis have started. These lesions are known as fatty streaks. As the lesions get bigger, the vessel lumen becomes narrower. However, the major cardiovascular disease risk arises when the fibrous cap breaks off from the vasculature and causes thrombosis. The pathophysiology of this event involves inflammation of the area. The lesion is covered by the fibrous cap. Fibrous cap is a layer of fibrous connective tissue that contains macrophages, smooth muscle cells, lymphocytes and collagen. The inflammation interferes with collagen formation and weakens the layer. Lesions with weaker fibrous caps are now more prone to rupture and cause thrombosis and embolism. Emboli can travel to a variety of different organs (brain and heart) to cause stroke or myocardial infarction (67 - 71).

Flow-mediated Dilatation and Its Prognostic Value for Cardiovascular Diseases

Previous studies have shown that endothelial dysfunction can lead to many different cardiovascular events. Therefore, it is necessary to have proper means to assess a patient's endothelial function. Flow-mediated dilatation is a non-invasive method to measure a patient's endothelial function using Doppler ultrasound. This technique measures the increase in the diameter of a blood vessel (brachial artery in this study) in response to an increase in blood flow. Physiologically blood vessels dilate upon an increase in blood flow and a subsequent increase in shear stress. Because this flow-mediated dilatation is mediated mainly through endothelial mechanisms, patients with dysfunctional endothelium have diminished blood vessel dilation upon an increase in blood flow. Experimentally, the increase in blood flow is induced via reactive hyperemia. Reactive hyperemia is a physiologic phenomenon, characterized by a transient increase in blood flow secondary to a brief period of ischemia (41, 45, 74, 75, 76, 77). Previous studies (41, 75, 76) have shown that flow-mediated dilatation is a reliable indicator to predict future cardiovascular events. Karatiz et al. found that "impaired FMD [Flow-mediated Dilatation] in [males with] acute coronary syndromes without ST-segment elevation is an independent predictor of future cardiovascular events" (76). Anderson stated in his review that endothelial dysfunction assessed by flow-mediated dilatation can predict atherosclerosis and "restenosis after percutaneous coronary intervention and complications after vascular surgery" (41). Muiesan et al. demonstrated that endothelial dysfunction evaluated through flow-mediated dilatation can be used for estimation of cardiovascular prognosis in hypertensive and normotensive patients (75). Thus, an overwhelming number of studies demonstrated that flow-mediated dilatation can reveal endothelial dysfunction, which can initiate future cardiovascular events.

Predicting a Binary Outcome of a Test: Receiver Operating Characteristics Curve (ROC) Analysis

Receiver Operating Characteristics (ROC) curve analysis is a statistical tool used in variety of research fields, including medicine to assess the accuracy of predictions. In medical situations where binary outcomes (e.g. development of myocardial infarction or not) are possible, a threshold value for a numeric parameter can be defined to predict the binary outcome. Based on the threshold value of the numeric parameters (e.g. number of cigarettes smoked per day) a table is produced listing the number of cases with positive outcomes versus number of cases with negative outcomes. An example of such a table is shown in Figure B2. This table can demonstrate True Positive (TP) cases, False Positive (FP, or type 1 error) cases, False Negative (FN, or type 2 error) cases, and True Negative (TN) cases. From tables such as the one shown in Figure B2, sensitivity (probability that a test result will be positive when the disease is present) and specificity (probability that a test result will be negative when the disease is not present) can be calculated using equations 1 and 2.

$$\text{Sensitivity} = \frac{(\text{True Positive})}{(\text{True Positive} + \text{False Negative})}$$

Equation 1

$$\text{Specificity} = \frac{(\text{True Negative})}{\text{False Positive} + \text{True Negative}}$$

Equation 2

A ROC curve is essentially a graph with 1 – specificity (false positive rate, α) on the x-axis and sensitivity on the y-axis. The graph is plotted using different threshold values for the numeric parameters as they produce different values for sensitivity and for specificity. Once the graph is plotted, the area under the curve (AUC) of the ROC curve is calculated to predict the outcome of the binary parameter. An AUC of 0.5 represents randomness (no predictive power),

while an AUC of 1 represents perfect prediction. In medicine, an AUC value of 0.75 is often considered satisfactory for predictive power (82 - 84).

Hypotheses and Study Aims

This study has multiple aims. First, the study will evaluate heart rate variability differences between smokers and age-matched controls. We hypothesize that different heart rate variability parameters (SNDD, RMSSD, SDNN) will be lower in a group of smokers compared to an age-matched control group. Second, the study will investigate heart rate variability differences between subjects with atherosclerosis and age-matched controls. We hypothesize that different heart rate variability parameters (SNDD, RMSSD, SDNN) will be lower in a group of patients with atherosclerosis compared to an age-matched control group. Third, the study will assess differences in endothelial function between smokers and age-matched controls using flow-mediated dilation to assess endothelial function. We hypothesize that smokers will have diminished endothelial function compared to age-matched controls. Fourth, the study will examine differences in endothelial function between patients with atherosclerosis and age-matched controls using flow-mediated dilation. We hypothesize that atherosclerotic patients will have diminished endothelial function compared to age-matched control subjects. Lastly, this study will investigate if heart rate variability can be used to predict endothelial dysfunction in smokers and patients with atherosclerosis. We hypothesized that lower heart rate variability can predict endothelial dysfunction. This part of the study will be conducted by constructing receiver operating characteristics curves for the prediction of flow-mediated dilatation by heart rate variability. The importance of this part of the study is that flow-mediated dilatation, although non-invasive, is costly, time consuming, and requires trained technicians, whereas heart rate variability can be obtained quickly, with minimal cost, and may even be utilized in emergency medicine settings.

CHAPTER II

METHODS

Smokers Study

The following is an excerpt from a grant application from Dr. William Haynes who kindly provided the EKG recordings and preliminary flow-mediated dilatation data for this study (44).

Subject Recruitment

Several groups of subjects were recruited for the original study, but only two groups (A and B) of subjects were used in this current study. Groups encompass healthy volunteers and those with risk factors for atherosclerosis: cigarette smoking, hypertension, hypercholesterolemia and diabetes mellitus. For the current study only the data from groups of healthy volunteers and smokers were utilized. Subjects were recruited by advertisement in the local newspapers and university and community groups. Written, witnessed consent was obtained from all subjects using IRB approved information forms. Subjects attended a screening session for clinical history and physical examination before admission. Blood was drawn for routine chemical analyses and electrocardiography was performed. Average age of the subjects was 38.6 ± 2.3 years. The following inclusion and exclusion criteria were used for the different groups:

Box 1 Inclusion and exclusion criteria

Inclusion	Exclusion
• 18-85 yr • Male or female • Fasting Blood Glucose (FBG) < 125 • Fasting LDL cholesterol < 130 • Fasting triglyceride < 200 • Systolic Blood Pressure < 130 mm Hg • Diastolic Blood Pressure < 80mm Hg	• BMI > 30 kg/m^2 • Clinical evidence of atherosclerosis • Vasoactive or lipid-lowering medications in past 2 wk • Vitamins A, C or E in past 4 wk • Aspirin or NSAID in past 7 days • Estrogen use in past 4 wk • Tobacco use in past 1 yr • Hyperuricemia • Hypertension

	• Hypercholesterolemia • Diabetes mellitus • Peripheral vascular disease • Coagulopathy • Vasculitis/ Raynauds phenomenon

Note: Group A: This group comprises healthy volunteers who fulfill the criteria above.
Group B: All the above criteria except for cigarette smoking (1 pack/day for more than one year)

Data Collection

Arterial pressure and heart rate

Arterial pressure and heart rate were measured in the sitting position using a mercury sphygmomanometer after the subject had been sitting for 5 min. Each measurement was taken twice. Additional measurements were taken if the readings differed by more than 10%. Supine arterial pressure was measured in duplicate at intervals during the experimental protocols using a non-invasive automated oscillometric sphygmomanometer (Lifestat 200, Physio-Control, Redmond, WA). Heart rate was measured continuously using a lead II electrocardiogram.

Forearm blood flow measurement

Subjects were asked to lie supine while both arms were supported. This allowed for venous emptying without impediment. Forearm blood flow was measured by venous occlusion plethysmography using indium/gallium-in-silastic strain gauges that were securely applied to the widest part of each forearm and attached to a plethysmographic unit. The hand circulation was occluded during measurements by inflation of a wrist cuff to 220 mmHg. Upper arm cuffs were intermittently inflated to 40 mmHg for 12 sec period separated by 8 sec to temporarily prevent venous outflow from the forearm and thus obtain plethysmographic recordings. Forearm blood flow was recorded repeatedly over 3 min periods.

Endothelial function of resistance vessel

Blood flow in the resistance vessels in both arms were measured using venous occlusion plethysmography. Measurements were taken before and after infusion of vasoactive to test resistance vessel endothelial function. Drugs were dissolved in 0.9% saline. Drugs used include nitroprusside (1-10 ~g/min), acetylcholine (3-30 ~g/min), bradykinin 0.03-0.3 ~g/min) and L-NMMA (1-4 ~mol/min). Sufficient time was allowed between drugs so that forearm blood flow returned to baseline. Arterial pressure was measured twice at baseline, and after each dose. The timeline for this protocol is shown in Figure B3.

Conduit vessel endothelial function

Ultrasound measurement of brachial artery diameter during changes in brachial artery flow was used as a non-invasive assessment of conduit vessel endothelial function. This technique used a 7.5 MHz linear array transducer ultrasound system (Sonos 2000, Hewlett-Packard, Andover, MA). A 5 cm length of the brachial artery was imaged in longitudinal section above the antecubital fossa and the optimal probe site on the skin marked. Baseline images of brachial artery diameter and Doppler velocities from the center of the vessel were recorded on videotape. While images for brachial artery diameter were being continuously recorded, an occluding forearm cuff placed just below the antecubital fossa was inflated to 50 mmHg above systolic pressure for 5 min. Brachial artery diameter and Doppler velocities were continuously recorded before, during and after cuff deflation. After 10 min, once basal diameter and flow were restored, nitroglycerin (400 μg) was administered sublingually and measurements made for an additional 6 min. The ultrasound images were digitized and were then analyzed using computer software. The diameter of the brachial artery was measured at a fixed distance from an anatomical marker using electronic calipers. Measurements were performed at end-diastole, coincident with the R wave on the ECG. Arterial blood flow was calculated using the Doppler flow velocity and the vessel diameter.

EKG Readings

During the experimental procedure for assessment of forearm vascular resistance, a 3-lead EKG recording was received using the PowerLab software running on a Macintosh computer. (ADInstruments) The data sampling rate was 100 Hz. These recordings were then converted to text files and imported into the freely available Hemolab software (www.haraldstauss.com/HemoLab/HemoLab.php) for heart rate variability analysis.

Aging Study (Atherosclerosis)

The following is an excerpt from a grant application by Dr. David Moser who kindly provided the EKG recordings and preliminary flow-mediated dilatation data for this study (91).

Subject Recruitment

Patient lists were received from the cardiology department of the University of Iowa Hospitals and Clinics (UIHC), and from those lists they determined which patients were between the ages of 55 and 90. Then, they conducted a chart review of the potential eligible patients to see if they met the basic inclusion and exclusion criteria in Box 2 and 3. A patient qualified for our study, they mailed them a contact letter explaining our study. Patients were instructed to return the letter indicating whether or not they were interested in learning more about the study.

Healthy subjects were reviewed and recruited in two ways. A list of healthy elderly subjects who have previously participated in Dr. Haynes' research in the Human Cardiovascular Physiology Laboratory and who had expressed an interest in being contacted for future studies was reviewed. The Seniors Together in Aging Research (STAR) registry was also reviewed.

The main study population of atherosclerotic participants was recruited through a variety of ways. First, patients through the cardiology clinic at UIHC were reviewed. Second, research registries of elderly individuals interested in participating in research (the Seniors Together in Aging Research (STAR) registry) were reviewed. Third, advertisements were placed in local newspapers, the UIHC newsletter, flyers in clinic waiting rooms, local senior centers, churches, libraries, community centers, retirement homes, nursing homes, senior centers, assisted living

centers, community recreational centers, various organizational meeting places such as a Elks Lodge, American Legion, and on community boards at various commercial businesses such as a grocery store or bowling alley. Average age of the subjects was 66.4 ± 1.2 years.

Box 2 Atherosclerotic participants Inclusion and Exclusion Criteria

Inclusions	Exclusion
• Age 55-90 at enrollment • Unequivocal diagnosis of Atherosclerotic Vascular Disease (AVD) • Angina pectoris • Past myocardial infarction • Percutaneous transluminal coronary angioplasty • Placement of a coronary artery stent • Peripheral vascular disease (claudication)	• Open-heart surgery involving heart/lung bypass machine (i.e. CABG, valve replacement) • History of carotid endarterectomy • History of stroke • History of head injury with loss of consciousness over 30 minutes • Diagnosis of dementia • History of major psychiatric illness (i.e. schizophrenia, bipolar affective disorder, psychosis) • History of developmental cognitive dysfunction (i.e. mental retardation, severe learning disability) • History of focal neurological sign • History of systemic illness or neurological disorder potentially affecting cognition (i.e. seizure disorder, demyelinating disorder).

Box 3 Healthy control participants Inclusion and Exclusion Criteria

Inclusions	Exclusion
• Age 55-90 at enrollment	• Clinical evidence of Atherosclerotic Vascular Disease (AVD) • Any history of angina pectoris • Myocardial infarction • Percutaneous transluminal coronary angioplasty • Placement of a coronary artery stent • History of peripheral vascular disease (claudication) • History of coagulopathy • History of vasculitis/Raynaud's phenomenon • History of coronary artery bypass grafting, valve replacement • Carotid endarterectomy

	• History of stroke or TIA • History of head injury with loss of consciousness > 30 minutes • Diagnosis of dementia • History of major psychiatric illness (i.e. schizophrenia, bipolar affective disorder, psychosis) • History of developmental cognitive dysfunction (i.e. mental retardation, severe learning disability) • History of focal neurological sign • History of systemic illness or neurological disorder potentially affecting cognition (i.e. seizure disorder, demyelinating disorder)

Data Collection

Forearm blood flow measurement

Subjects were asked to lie supine while both arms were supported. This allowed for venous emptying without impediment. Forearm blood flow was measured by venous occlusion plethysmography using indium/gallium-in-silastic strain gauges that were securely applied to the widest part of each forearm and attached to a plethysmographic unit. The hand circulation was occluded during measurements by inflation of a wrist cuff to 220 mmHg. Upper arm cuffs were intermittently inflated to 40 mmHg for 12 sec in every 8 sec to temporarily prevent venous outflow from the forearm and thus obtain plethysmographic recordings. Forearm blood flow was recorded repeatedly over 3 min periods.

Endothelial function of resistance vessel

Blood flow in the resistance vessels in both arms were measured using venous occlusion plethysmography. Measurements were taken before and after infusion of vasoactive to test resistance vessel endothelial function. Baseline forearm blood flows were obtained during infusion of 0.9% saline (1 mL/min) for 30 minutes. Acetylcholine (3 to 30 μg/min), nitroprusside (1 to 10 μg/min), and verapamil (10 to 100 μg/min) were infused separately into the left arm, with each dose infused for 6 minutes. The order of administration was randomized for each drug; however, verapamil was always administered last due to longer half-life than the other 2 drugs.

In all cases, blood flow was allowed to return to baseline level before infusion of the next drug. The timeline for this protocol is shown in Figure B4.

Conduit vessel function

A high resolution ultrasound measurement of brachial artery diameter was used during changes in brachial artery flow as a non-invasive assessment of conduit vessel endothelial function. The diameter of the brachial artery was measured from 2-dimentional, b-mode ultrasound images using a 10-13 MHz linear array transducer (Biosound ESAOTE, Indianapolis, IN). Conduit vessel endothelial function was assessed using flow-mediated dilatation of the brachial artery using reactive hyperemia as a stimulus. In each study, images were obtained at rest, during reactive hyperemia, again at rest, and after sublingual nitroglycerine (400 μg). The brachial artery was measured 5-15 cm above the antecubital fossa, scanned in longitudinal section. The focus zone was set to the depth of the near wall. Depth and gain settings were set to optimize the images of the lumen/arterial wall interface and the images are magnified.

Subjects rested for at least 10 minutes before the exam was started. Arterial flow velocity was measured using a pulsed wave Doppler signal at a 70° angle to the vessel and the range gate in the center of the artery. A baseline scan was performed for 30 seconds before cuff inflation. Reactive hyperemia was induced by release of a a pneumatic tourniquet inflated to a pressure of 240 mm Hg for 5 minutes. With reactive hyperemia, the diameter measurement was performed for 2 minutes after cuff deflation. Flow velocity was also recorded for the first 15 seconds after the cuff was released and again at 1 minute and 2 minutes post deflation. With each Doppler scan, peak flow velocity was analyzed for three cardiac cycles and the average of these values was used to calculate average peak flow velocity. A 3 minute resting period was then allowed for the vessel to return to baseline and a repeat resting baseline scan was performed. One spray of nitroglycerin was then administered sublingually (400 mcg/spray), and images were obtained for 4 minutes post nitroglycerin. Flow velocities were recorded at 1 minute, 2 minutes and 4 minutes post nitroglycerin. A continuous electrocardiogram (ECG) recording was obtained, and

non-invasive arterial pressure (NIBP) was measured at baseline and after each intervention. NIBP's were taken in the contralateral arm pre-exam, during the resting period between the reactive hyperemia and the nitroglycerin and post-exam. The ultrasound images were digitized and were then analyzed using computer software. The diameter of the brachial artery was measured at a fixed distance from an anatomical marker using electronic calipers. Measurements were performed at end-diastole, coincident with the R wave on the ECG. Arterial blood flow was calculated using the Doppler flow velocity and the vessel diameter.

EKG Readings

During the experimental procedure for assessment of forearm vascular resistance, a 3-lead EKG was recorded using the PowerLab software running on a Macintosh computer (ADInstruments). The data sampling rate was 100 Hz. These recordings were then converted to text files and imported into the Hemolab software (www.haraldstauss.com/HemoLab/HemoLab.php) for heart rate variability analysis.

Heart Rate Variability Analysis

Heart rate variability was analyzed from the text files obtained from EKG recordings obtained during forearm vascular resistance assessment in both studies (smokers and atherosclerosis). The HemoLab software (freely available for download from www.haraldstauss.com/HemoLab/HemoLab.php) was used. First, the text files had to be converted from the Macintosh into the PC file format. This process was done using a program called Batch Processor, a module of the HemoLab software. Once the text files were converted to PC format, each subject's EKG was reviewed using a software called Analyzer, which is another module of the HemoLab software. Each subject had different amplitudes of R-waves. Each subject's P, QRS and ST waves and its peak intensity had to be identified. Some subjects' QRS complexes were more difficult to identify; therefore, a butterworth filter was applied before a beat-by-beat heart rate time series was derived from EKG recordings. Most subjects had some episodes of arrhythmia including: extrasystole, premature ventricular contractions and/or

premature atrial contractions. Each of those episodes were identified and removed prior to heart rate variability analysis that was done using the Batch Processor module of the Hemolab software. Three minute recordings of the EKG were used for the xanthine oxidase study (smokers) and five minute recordings of the EKG were used for the aging study (atherosclerosis). Standard deviation of normal to normal intervals (SDNN), the square root of the mean squared difference of successive normal to normal intervals (RMSSD) and the deviation of the successive differences between normal to normal intervals (SDSD) were determined as time-domain heart rate variability parameters.

Statistical Analysis

Unpaired Student's t tests were used to test for differences between SDNN, RMSSD and SDSD in control subjects and smokers or atherosclerotic subjects. Statistical power analysis was done using the PS software (Power and Sample Size Calculations version 3.0 copyright © 1997-2009 by William D. Dupont and Walton D. Plummer). Receiver Operating Characteristics (ROC) Curve analysis was used to study predictability of endothelial function by heart rate variability. The ROC curves were generated using software provided by the "R Project for Statistical Computing" (www.r-project.org)

ROC curves were generated to test the predictability of endothelial dysfunction in each study (smokers and atherosclerotic patients) by three parameters of heart rate variability (SDNN, RMSSD and SDSD). For each study, a threshold value of flow-mediated dilatation was used to classify a subject as having either endothelial dysfunction or normal endothelial function (binary parameter). Once subjects were classified, ROC curves were generated using the three heart rate variability parameters. For each study (smokers and atherosclerotic patients), the threshold value for flow-mediated dilatation to classify a subject as having endothelial dysfunction or not was identified as the threshold that resulted in the larger AUC of the ROC curve. This was necessary, since there is no consensus from the literature, as to which flow-mediated dilatation value classifies as subject as having endothelial dysfunction or not.

CHAPTER III

RESULTS

Heart Rate Variability in Smokers and Control Subjects

Three time domain heart rate variability parameters were examined: Standard deviation of NN intervals (SDNN), the square root of the mean squared differences of successive NN intervals (RMSSD) and standard deviation of differences between adjacent NN intervals (SDSD). There were 14 control and 11 smoking subjects in this study. The average values of SDNN, RMSSD and SDSD in control subjects and smokers are shown in Graph C1 and in Table A1. The control group had a trend towards higher SDNN compared to the smoker group (30.84 ± 7.79 ms vs. 18.87 ± 3.79 ms, $p = 0.22$). The control group also had a trend towards higher RMSSD and SDSD compared to the smoker group. As pointed out by Bhatia et al. (89), RMSSD and SDSD often reveal identical results. In this study, RMSSD and SDSD also revealed exactly the same numeric results of 5.69 ± 1.51 ms vs 3.08 ± 0.62 ms, $p = 0.16$. The statistical power of this data set was relative low with ~26% for SDNN and ~33% for RMSSD and SDSD. ROC curves were generated for each heart rate variability parameter to determine if heart rate variability can predict if a subject is a smoker or not. The ROC curves are shown in graphs 2, 3 and 4. The area under the curve (AUC) for SDNN was 0.604 and for RMSSD and SDSD it was 0.649. These values indicate that heart rate variability is a weak predictor for determining if a subject is a smoker or not.

Heart Rate Variability in Atherosclerotic Patients and Control Subjects

Heart rate variability was analyzed during the baseline conditions and acetylcholine infusion. From baseline conditions data, 11 control subjects and 15 atherosclerotic subjects were analyzed. The average values of SDNN, RMSSD and SDSD for control and atherosclerotic subjects are shown in Graph C5 and Table A2. The control group tended to have a higher average SDNN compared to the atherosclerotic group (52.09 ± 26.67 ms vs. 25.78 ± 9.25 ms, p =

0.31). The control group also had a tendency for higher average RMSSD and SDSD values compared to the atherosclerotic group (7.45 ± 3.84 ms vs. 2.66 ± 1.19 ms, p = 0.19 and 7.45 ± 3.83 ms vs. 2.63 ± 1.20 ms, p = 0.19, respectively). Again, RMSSD and SDSD revealed almost identical values. Again, the statistical power of this data set was relative low with ~20% for SDNN and ~29% for RMSSD and SDSD. ROC curves were generated for each of the three heart rate variability parameters to determine if heart rate variability can predict if a patient is atherosclerotic or not. These ROC curves are shown in graphs 6, 7 and 8. The area under the curve (AUC) for SDNN was 0.509, for RMSSD and SDSD it was 0.685. The AUC for the SDNN values indicate that heart rate variability is a poor predictor for determining if a patient is atherosclerotic or not. The AUC for the RMSSD and SDSD values, however, indicate that heart rate variability may be a weak predictor for determining if a patient is atherosclerotic or not.

A second EKG recording was obtained during a five minute local acetylcholine infusion into the brachial artery and heart rate variability parameters were derived from these recordings in 12 control subjects and 24 atherosclerotic patients. The average values for SDNN, RMSSD and SDSD for control and atherosclerotic subjects are shown in Graph C9 and Table A3. The control group had higher average SDNN values of 48.09 ± 14.97 ms when compared to 23.25 ± 4.41 ms in the atherosclerotic group (p = 0.049). The control group also had a tendency towards higher average RMSSD and SDSD values of 6.24 ± 1.8 ms and 6.23 ± 1.8 ms, respectively, compared to 3.04 ± 0.74 ms and 3.03 ± 0.73 ms, respectively, in the atherosclerotic group (p = 0.059). The statistical power of this data set was in the range of 50% for all three parameters of heart rate variability. ROC curves were generated for each of the three heart rate variability parameters to determine if heart rate can predict atherosclerosis under conditions of acetylcholine infusion. These ROC curves are shown in graphs 10, 11 and 12. The AUC for SDNN was 0.609, for RMSSD and SDSD it was 0.661. The AUC for the SDNN values indicate that heart rate variability cannot predict if a patient is atherosclerotic or not. The AUC for RMSSD and SDSD, however, indicate that heart rate variability may be a weak predictor for determining if a patient is atherosclerotic or not.

Flow-mediated Dilatation in Smokers and Controls

The change in blood vessel diameter was examined before and after a blood cuff occlusion for the group of smokers and an age-matched control group. The values for mean percent change of blood vessel diameter for each group are reported in Table A4 and Graph C13. Percent change of blood vessel diameter was calculated using equation 3:

$$\text{Percent Change} = \left\{\frac{[(60 \text{ seconds post deflation}) - (\text{Base})]}{(\text{Base})}\right\}(100\%)$$

Equation 3

A small percent change in artery diameter is indicative for endothelial dysfunction. The mean percent change in artery diameter in the control group was 8.21 ± 1.96%, while the percent change in artery diameter in the smoker group was 5.43 ± 0.98%. The P value for this difference was 0.24 indicating no statistical significant difference in flow-mediated dilatation between smokers and controls. The ROC curve, shown in Graph C14, was generated to study if flow-mediated dilatation can predict if a subject is a smoker or not. The AUC was 0.538, indicating no predictability.

Flow-mediated Dilatation in Atherosclerotic Patients and Control Subjects

The percent change in brachial artery diameter in response to reactive hyperemia was examined in the atherosclerotic group and in age-matched controls. The values for the percent changes in artery diameter for both groups are reported in Table A5 and Graph C15. The percent change in brachial artery diameter was calculated using equation 3. The percent change in artery diameter in the control group was 5.01 ± 0.59%. The percent change in artery diameter in the patients with atherosclerosis was 5.68 ± 0.60%. The P value for this difference was 0.48 indicating no significant difference between atherosclerotic patients and controls. A ROC curve for the predictability of whether a subject has atherosclerosis or not by flow-mediated dilatation

was generated (Graph C16). The AUC for this ROC curve was 0.563, indicating that flow-mediated dilatation cannot predict if a subject has atherosclerosis or not.

Predictability of Endothelial Dysfunction Using Heart Rate Variability Parameters

ROC curves were generated to investigate the predictability of endothelial dysfunction in each study (smokers, atherosclerotic patients) by the three heart rate variability parameters (SDNN, RMSSD and SDSD). For each study, threshold value for flow-mediated dilatation were determined to classify a subject as having either endothelial dysfunction or normal endothelial function based on maximizing the AUC of the ROC curves. For the study in smokers this threshold was determined as an increase in brachial artery diameter of 11.00%. For the study in atherosclerotic patients, the threshold was determined as 9.25%. At these threshold values, 19 subjects were classified as having endothelial dysfunction and 4 subjects were classified as having normal endothelial function in the study of smokers, and 26 subjects were classified as having endothelial dysfunction and 3 subjects were classified as having normal endothelial function in the study of atherosclerotic patients.

The histograms of the flow-mediated dilatation data in the study in smokers and atherosclerotic patients are shown in graphs 17 and 18, respectively. These graphs also indicate the threshold values used to classify a subject as having endothelial dysfunction or normal endothelial function. From the histograms it becomes apparent that the values for flow-mediated dilatation response are distributed in an unimodal distribution. Thus, there are no distinct subgroups of subjects with endothelial dysfunction and normal endothelial function.

The ROC curves for the predictability of endothelial dysfunction by heart rate variability in smokers and atherosclerotic patients are shown in graphs 19 and 20, respectively. It turned out that the AUC for the ROC curves determined from the study in smokers was identical for all three heart rate variability parameters (0.789) and revealed a strong predictive power. Similarly,

the ROC curves for the study in atherosclerotic patients revealed a strong predictive power with identical AUC values of 0.821 for all three heart rate variability parameters.

CHAPTER IV

DISCUSSION

The most important finding of this study was that parameters derived from heart rate variability may be used to predict endothelial dysfunction in smokers or atherosclerotic patients. However, parameters derived from heart rate variability cannot predict if a patient is a smoker or not and if a patient has atherosclerosis or not. Furthermore, flow-mediated dilatation cannot predict if a patient is a smoker or not and if a patient has atherosclerosis or not.

Heart Rate Variability: Smokers

Heart rate variability was investigated in a group of smokers and an age-matched control group. In a sample size of 25 subjects (14 controls and 11 smokers) there were no significant differences in heart rate variability. SDNN is a measure of short-term high and low frequency heart rate variability. The mean SDNN tended to be lower in the smoker group compared to the control group ($p = 0.22$). RMSSD and SDSD mainly reflect short-term high frequency variability of heart rate. RMSSD and SDSD also tended to be lower in the smoker group than in the control group ($p = 0.16$ for both RMSSD and SDSD). Although these values are not statistically significant, the trend may suggests that smokers may have less parasympathetic modulation of heart rate than non-smokers. These findings are consistent with previous studies that have reported that chronic heavy smokers tend to have decreased vagal tone to the heart as indicated by higher resting heart rate values and lower heart rate variability (32, 33, 34, 35). Furthermore, previous studies showed a reduction in baroreflex sensitivity in chronic heavy smokers related to an elevated sympathetic nervous system activity (32). Although the exact pathophysiologic mechanisms for decreased vagal tone and decreased baroreflex sensitivity induced by cigarette smoking is yet unknown, there are numerous studies suggesting that cigarette smoking causes greater release of norepinephrine (32, 33). This greater release of norepinephrine has been suggested to increase the risk for ventricular fibrillation (32, 33). Furthermore, previous studies

have found that chronic cigarette smoking can cause damage to the vascular endothelium (32, 34), which can promote to development of atherosclerosis.

The effects of cigarette smoking on the endothelium and on the heart may have separate and independent pathophysiologies. However, it is possible that endothelial dysfunction, caused by cigarette smoking, may be indirectly related to cardiac dysfunction as assessed by heart rate variability. This study attempted to explore this relationship by studying if heart rate variability can predict endothelial dysfunction assessed by flow-mediated dilatation in groups of smokers and non-smokers.

Heart Rate Variability: Atherosclerosis

In a second part of the study, heart rate variability was investigated in a group of atherosclerotic patients and age-matched control subjects. In this part of the study, heart rate variability was assessed during baseline condition and during local infusion of acetylcholine into the brachial artery.

During baseline conditions heart rate variability tended to be lower in atherosclerotic patients compared to control subjects. (SDNN: p=0.31, RMSSD: p= 0.19, SDSD: p=0.19). Although this trend was not statistically significant, a reduction in heart rate variability in the atherosclerosis group may suggest less parasympathetic modulation of heart rate compared to control subjects. This suggestion is consistent with previous studies (20, 78). Huikuri et al. (78) reported lower heart rate variability in atherosclerotic patients than in control subjects. Base on their finding, Huikuri et al. (78) proposed that heart rate variability may serve as a marker for disease progression in atherosclerosis.

ROC curve analysis revealed that RMSSD and SDSD have a weak prediction power (AUC = 0.69) in identifying atherosclerosis subjects. In contrast to SDNN that could not predict if a subject is atherosclerotic or not (AUC = 0.51).

Interestingly, SDNN was significantly reduced in atherosclerotic patients compared to control subjects during local acetylcholine infusion. For RMSSD and SDSD, the comparison

between atherosclerotic patients and controls just barely failed statistical significance (p = 0.059). Local acetylcholine infusion is assumed to have no systematic effects, because its half-life is only 60 – 90 seconds. Furthermore, heart rate and blood pressure did not change during the local acetylcholine infusion. Therefore, the different findings during baseline conditions and acetylcholine infusion are surprising, but may be explained by the experimental protocol. Baseline measurements were taken during the first five minutes of the experimental protocol, where measurements during the local infusion of acetylcholine were obtained in the middle of the experimental protocol, approximately one or two hours after the baseline recording. It is possible that a time effect (e.g. subjects are more relaxed later in the protocol) may explain this difference in heart rate variability at baseline and during acetylcholine infusion.

Similar to baseline conditions, RMSSD and SDSD (graphs 10 and 11) had a modest predictability of whether a subject is atherosclerotic or not (AUC = 0.66), while SDNN (Graph C9) had no predictive power (AUC = 0.609). These results further suggest that parasympathetic nervous system has a modest influence on the progression of atherosclerosis.

Endothelial Function: Smokers

The flow-mediated dilatation technique was used to quantify endothelial function. In a sample size of 24 subjects (13 controls and 11 smokers) there was no significant difference (p=0.24) in the percent change of brachial artery diameter between the two groups in response to reactive hyperemia. However, reduced endothelium-dependent vasodilation during reactive hyperemia in smokers had been observed in other studies (46, 51, 53, 55). Impaired endothelial function in cigarette smokers can be attributed to various mechanisms including: increased insulin resistance, increased reactive oxygen species, and increased activation and adhesion of platelets and leukocytes in the endothelium (46). Cigarette smoking can also affect plasma lipid levels and tPA that can lead to endothelial dysfunction (46, 51).

Because there was no significant difference in flow-mediated dilatation in smokers and non-smokers, it is not surprising that ROC curve analysis (Graph C14) demonstrated that the

flow-mediated dilatation technique cannot accurately predict if a subject is a smoker or a non-smoker.

Endothelial Function: Atherosclerosis

In this part of the study a total number of 36 subjects were investigated which included 24 atherosclerotic subjects and 12 age-matched control subjects. The results were quite unexpected. Endothelial function assessed by flow-mediated dilatation was not significantly different in atherosclerotic patients and control subjects, which is in striking difference to the expected outcome based on the pathophysiology of atherosclerosis and its impact on endothelial function (45, 47, 48, 66). The lack of impaired flow-mediated dilatation in atherosclerotic patients compared to control subjects may be related to the age of the subjects. The mean age for the control subjects was 68.0 ± 19.6 years, ranging from 56 to 82 years. The mean age for the atherosclerotic patients was 65.7 ± 13.4 years, ranging from 54 to 77 years ($p=0.3756$). A previous study by Noto et al. (85) suggested that older populations have a greater tendency of free radical production in the vasculature. They are also more likely to have higher oxidative stress and a higher rate of catabolism of endothelium-derived regulators that can modify vasomotor tone (85). These alterations in older populations may lead to reduced flow-mediated dilatation. Furthermore, aging causes vessel modification including "luminal enlargement with wall thickening (remodeling) and a reduction of elastic properties (stiffening) at the level of large elastic arteries" (86). Al-Shaer et al. (87) found that the endothelium of older populations produce less nitric oxide compared to younger populations which can lead to endothelial dysfunction. These findings suggest that aging is an important risk factor that can lead to endothelial dysfunction. Furthermore, it is conceivable that age is a stronger underlying factor for endothelial dysfunction than atherosclerosis. As a result, flow-mediated dilatation may depend on age to a greater extent than atherosclerosis. Thus, flow-mediated dilatation was similar in both control subjects and atherosclerotic patients.

Because there was no significant difference in flow-mediated dilatation between atherosclerotic patients and control subjects, it is not surprising that ROC curve analysis (Graph C16) demonstrated that the flow-mediated dilatation technique cannot accurately predict if a patient has atherosclerosis or not.

Prediction of Endothelial Dysfunction Using Heart Rate Variability

A flow-mediated dilatation threshold of 11% (change in brachial artery diameter during first minute of reactive hyperemia) was used for ROC curve analysis in smokers and controls. This threshold value was determined by maximizing the AUC of the ROC curve. Previous studies reported that control subjects (normal endothelial function) have flow-mediated dilatation values of 8 – 10% compared the 2 – 5 % endothelium-dependent dilation in smokers (53 – 56). These findings support the validity of the threshold values used in this study. However, at the threshold of 11%, 19 subjects were classified as having endothelial dysfunction and 4 subjects were classified as having a normal endothelial function. This was an interesting finding because the mean age from this study (38.6 ± 2.3 years) and mean ages from previous studies (~30 years) did not deviate much from each other (53 – 56).This result suggest that endothelial dysfunction may be due to reasons other than age. However, those reasons remain unclear as each of the control patient's past medical history was unremarkable for diseases and risk factors that may have attributed to endothelial dysfunction such as diabetes mellitus, ischemic heart disease, peripheral vascular disease, renal disease, hypertension and obesity. Previous studies (53, 56) have reported that environmental exposure to cigarette smoke can cause endothelial dysfunction. It is a possibility that many of the control subjects were regularly exposed environmental cigarette smoke. However, this study did not have access to that information.

A flow-mediated dilatation threshold of 9.25% was used for ROC curve analysis in atherosclerotic patients and control subjects. This threshold value was determined in same way as for the smoker study. Again, a high number of subjects were classified as having endothelial

dysfunction (26 endothelial dysfunction and 3 normal endothelial function). However, this may be due to age-mediated deterioration of endothelial function (66.4 ± 1.2 years). Ogino et al. (70) studied flow-mediated dilatation in a younger population (13.1 ± 2.1 years). They reported a mean flow-mediated dilatation of 18.8 ± 2.8% in control subjects, 8.9 ± 2.8% in subjects with moderate coronary artery lesion and 4.2 ± 1.5% in subject with severe coronary artery lesion suggesting that the threshold values used in our study are reasonable.

The high AUC values for the ROC curves for prediction of endothelial dysfunction by time-domain heart rate variability parameters indicate that heart rate variability is a good predictor for determining if a subject has endothelial dysfunction or not (75).

CHAPTER V

CONCLUSION

In conclusion, heart rate variability tended to be lower in smokers and atherosclerotic patients compared to control subjects. This lower heart rate variability trend in the experimental groups may suggest impaired parasympathetic modulation of cardiac function in smokers and atherosclerotic patients because RMSSD and SDSD, which reflect cardiac parasympathetic modulation, were reduced more than SDNN, which reflects cardiac sympathetic and parasympathetic modulation. Consistent with previous studies, a trend towards a lower flow-mediated dilatation in the smoker group may suggest that smoking causes endothelial dysfunction. Finally, the results of this study support the conclusion that heart rate variability can predict if a patient has endothelial dysfunction or not.

CHAPTER VI

LIMITATIONS

In the group of smokers and age-matched controls, heart rate variability was derived from the first three minutes of the EKG recording during baseline conditions. The Task Force (7) indicated that five minute-long recordings should be used for short-term analysis of heart rate variability. However, five minute-long continuous baseline recordings were not available and, therefore, only three minutes could be analyzed. Furthermore, a low statistical power (0.264 for SDNN and 0.327 for RMSSD and SDSD) for the heart rate variability data suggests that there is a high probably of a type 2 error (false negative). Low statistical power may be due to a small sample size and the short length of the EKG recordings. Low statistical power and insignificant heart rate variability differences between smokers and age-matched control lessens the support for the conclusion that heart rate variability can predict if a patient has endothelial dysfunction.

Although heart rate variability in atherosclerotic patients and control subjects derived during local acetylcholine infusion showed a significant difference, low statistical power (0.523 for SDNN and 0.493 for RMSSD and SDSD) suggest that half of the results may contain type 2 errors (false negative). An acceptable statistical power of a study is around 0.8.

A higher statistical power may be achieved with a higher number of subjects (n = 300 – 400) and analyses of longer duration of EKG recordings. On the other hand, it is possible that heart rate variability and flow-mediated dilatation are simply unrelated. Heart rate variability depends on autonomic innervation of the heart whereas flow-mediated dilatation depends on the functionality of the endothelium, which is independent of autonomic innervation of the vasculature. Blood pressure variability may be a better predictor for endothelial function than heart rate variability. However, blood pressure recordings that allow assessment of blood pressure variability were not available from these studies.

Flow-mediated dilatation could not distinguish smokers and atherosclerotic patients from control subjects. This was due to a lack of a bimodal distribution as shown in graphs 17 and 18.

The lack of bimodal distributions for flow-mediated dilatation made an accurate prediction of endothelial dysfunction from heart rate variability parameters slightly more complicated.

APPENDIX A

TABLES

Table A1 Heart rate variability parameters in smokers and control subjects

Heart Rate Variability Parameters	**Control n = 14**	**Smokers n = 14**	**P**	**Statistical Power**
SDNN (ms)	30.84 ± 7.79	18.87 ± 3.79	0.22	0.264
RMSSD (ms)	5.69 ± 1.51	3.08 ± 0.62	0.16	0.327
SDSD (ms)	5.69 ± 1.51	3.08 ± 0.62	0.16	0.327

Table A2 Heart rate variability parameters in atherosclerotic patients and control subjects (baseline)

Heart Rate Variability Parameters	**Control n = 12**	**Atherosclerosis n = 24**	**P**	**Statistical Power**
SDNN (ms)	52.09 ± 26.67	25.78 ± 9.25	0.31	0.195
RMSSD (ms)	7.45 ± 3.84	2.66 ± 1.19	0.19	0.288
SDSD (ms)	7.45 ± 3.83	2.63 ± 1.20	0.19	0.291

Note: These parameters were derived from EKG recording during baseline recording.

Table A3 Heart rate variability parameters in atherosclerotic patients and control subjects

Heart Rate Variability Parameters	**Control n = 12**	**Atherosclerosis n = 24**	**P**	**Statistical Power**
SDNN (ms)	48.09 ± 14.97	23.25 ± 4.41	0.049	0.523
RMSSD (ms)	6.24 ± 1.80	3.04 ± 0.74	0.059	0.493
SDSD (ms)	6.23 ± 1.80	3.03 ±0.73	0.059	0.493

Note: These parameters were derived from EKG recording during local acetylcholine infusion.

Table A4 Flow mediated dilatation in smokers and control subjects

	Control n = 13	**Smoker n = 11**	**P**
Change in vessel diameter (%)	8.21 ± 1.96	5.43 ± 0.98	0.24

Table A5 Flow mediated dilatation in atherosclerotic patients and control subjects

	Control n = 12	**Atherosclerosis n = 24**	**P**
Change in vessel diameter (%)	5.01 ± 0.59	5.68 ± 0.60	0.48

Table A6 Predictability of endothelial dysfunction using heart rate variability parameters derived from smokers and control subjects

Heart Rate Variability Parameters	AUC
SDNN	0.789
RMSSD	0.789
SDSD	0.789

Note: Predictability of endothelial dysfunction using heart rate variability parameters derived from smokers and control subjects. Threshold value for flow mediated dilatation was set at 11% (change in brachial artery diameter during first minute of reactive hyperemia). With flow mediated dilatation threshold set at 11% there were 19 subjects with endothelial dysfunction and 4 subjects with normal endothelial function. ROC curve is shown in Graph C8.

Table A7 Predictability of endothelial dysfunction using heart rate variability parameters derived from atherosclerotic patients and control subjects

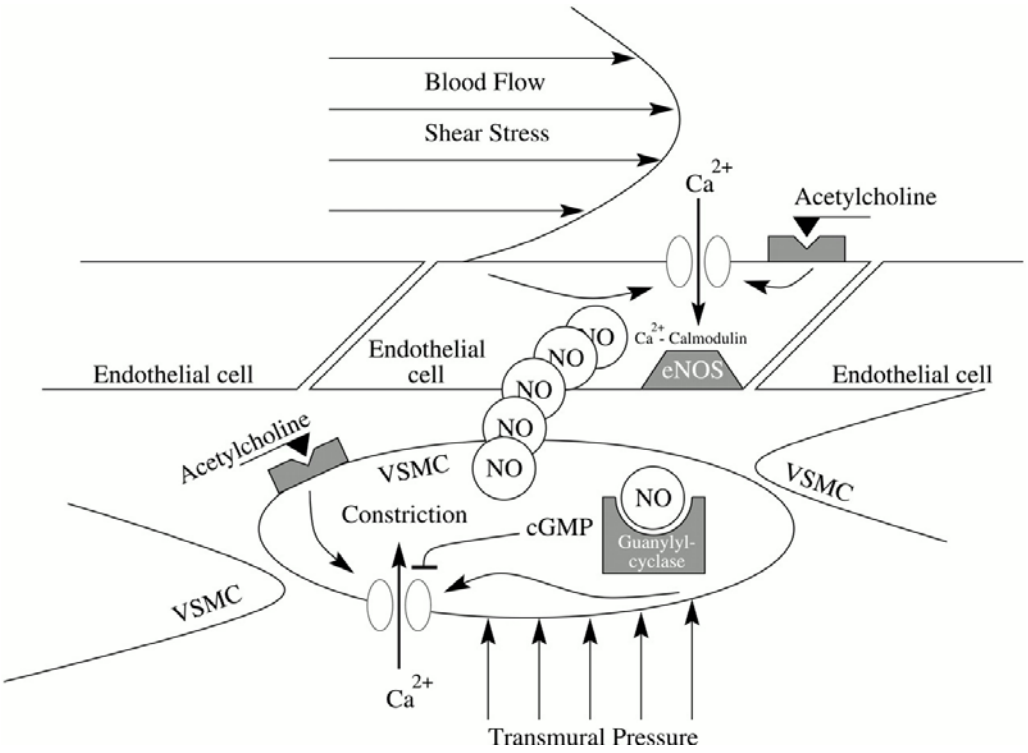
Heart Rate Variability Parameters	AUC
SDNN	0.821
RMSSD	0.821
SDSD	0.821

Note: Predictability of endothelial dysfunction using heart rate variability parameters derived from atherosclerotic patients and control subjects. Threshold value for flow mediated dilatation was set at 9.25% (change in brachial artery diameter during first minute of reactive hyperemia). With flow mediated dilatation threshold set at 9.25% there were 26 subjects with endothelial dysfunction and 3 subjects with normal endothelial function. ROC curve is shown in Graph C9

APPENDIX B

FIGURES

Figure B1 Mechanism of flow mediated vasodilation



Note:"Vascular shear stress promotes Ca^{2+} influx into endothelial cells. Subsequently, the Ca^{2+}-calmodulin complex activates endothelial nitric oxide (NO) synthase (eNOS). NO diffuses to the adjacent vascular smooth muscle cells (VSMC), where it activates the guanylyl cyclase. cyclic Guanine monophosphate causes a reduction in intracellular Ca^{2+} concentration in VSMC and, therefore, leads to vasodilation."

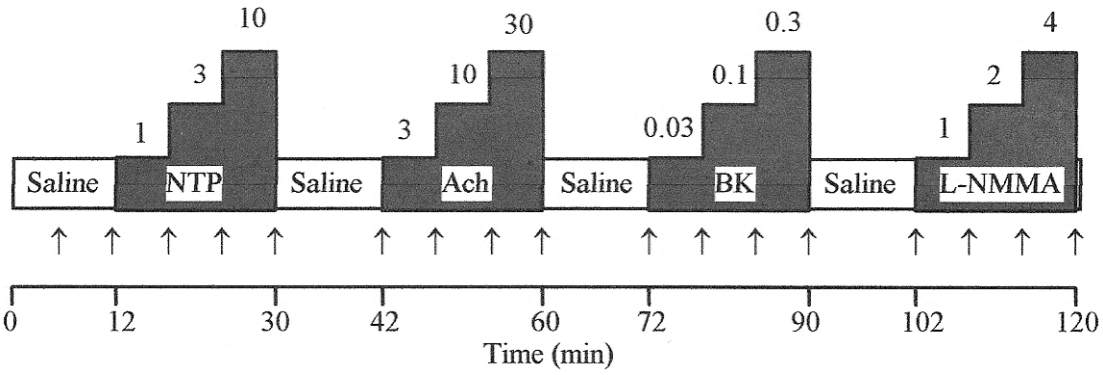
Source: Stauss, H., and P. Persson. "Role of Nitric Oxide in Buffering Short-Term Blood Pressure Fluctuations." *News in Physiological Sciences* 15.5 (2000): 229-33.

Figure B2 Receiver Operating Characteristic Curve Table

Prediction	Observed		
	Positive	Negative	Total
Positive	True Positive (TP)	False Positive (FP)	TP+FP
Negative	False Negative (FN)	True Negative (TN)	FN+TN
Total	TP+FN	FP+TN	TP+FP+FN+TN

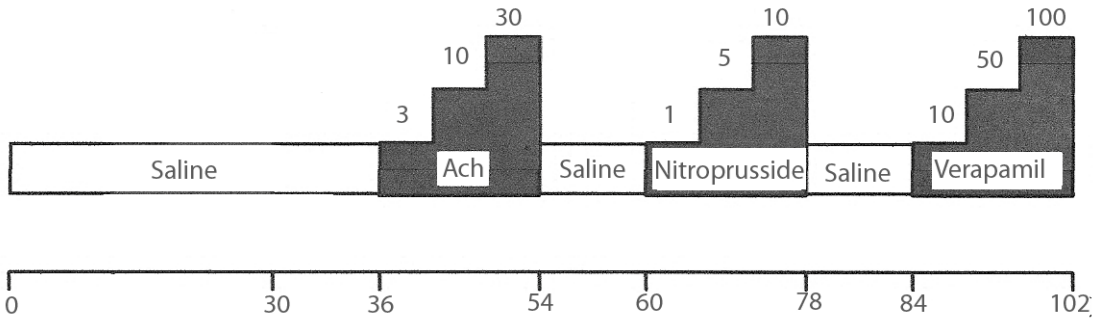
Note: (TP), False Positive (FP, or type 1 error), False Negative (FN, or type 2 error), and True Negative (TN) can be found. From this table the sensitivity (probability that a test result will be positive when the disease is present) and the specificity (probability that a test result will be negative when the disease is not present) can be calculated using equations 1 and 2 (shown in the text).

Figure B3 Forearm Vascular Resistance protocol in smokers and controls



Source: Haynes WG. Guthikonda S, Sinkey C, Knapp H, Sivitz W, Schrott H. "Role of Xanthine Oxidase Products in Modulation of Endothelial Fucntion." NIH grant: NHLBI, HL-55006; NHLBI, HL-58972; NCRR General Clinical Research

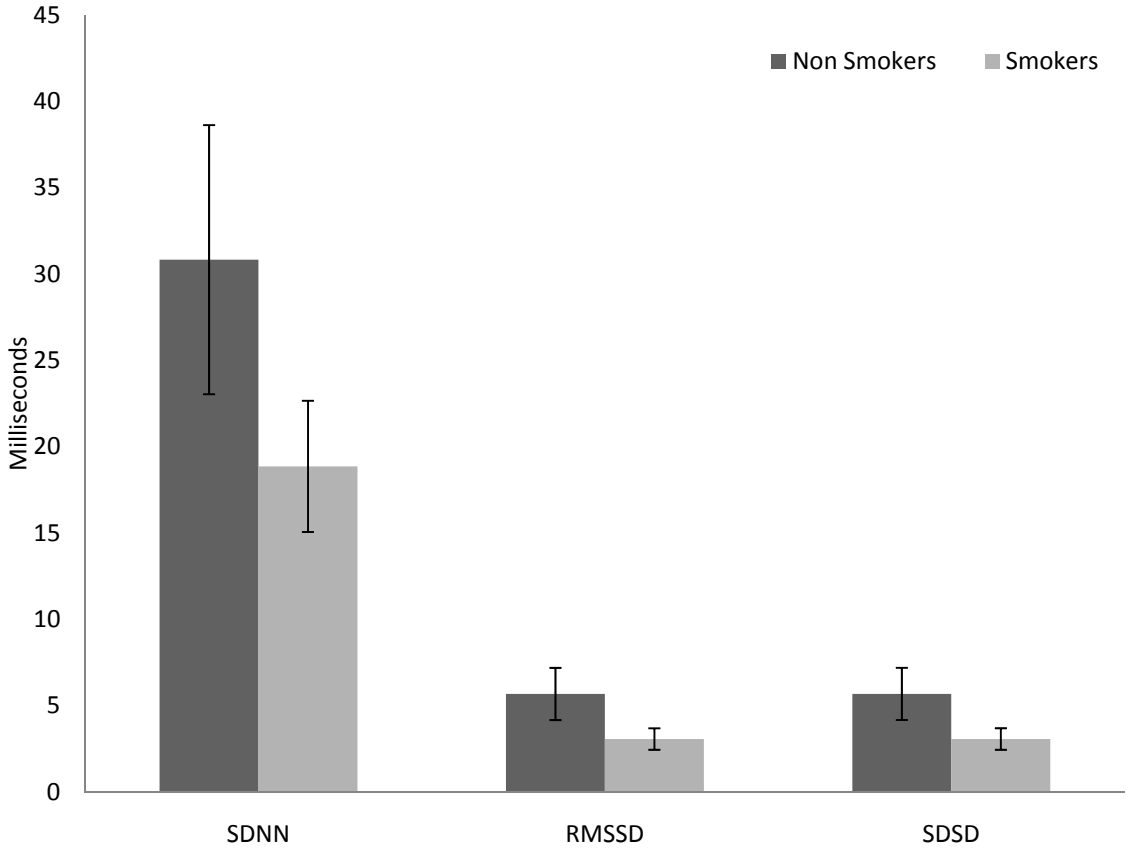
Figure B4 Forearm Vascular Resistance protocol in atherosclerotic patients and control subjects



APPENDIX C

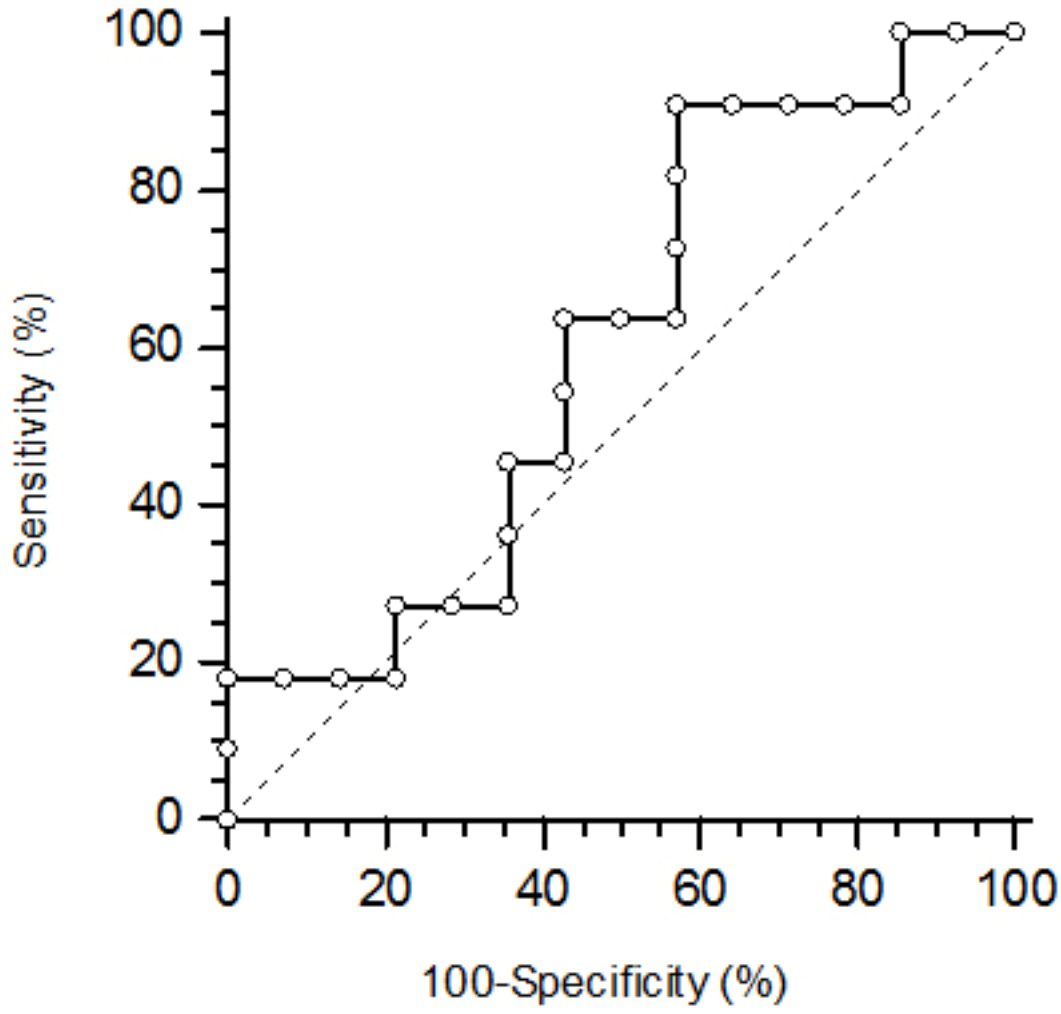
GRAPHS

Graph C1 Heart rate variability in controls and smokers



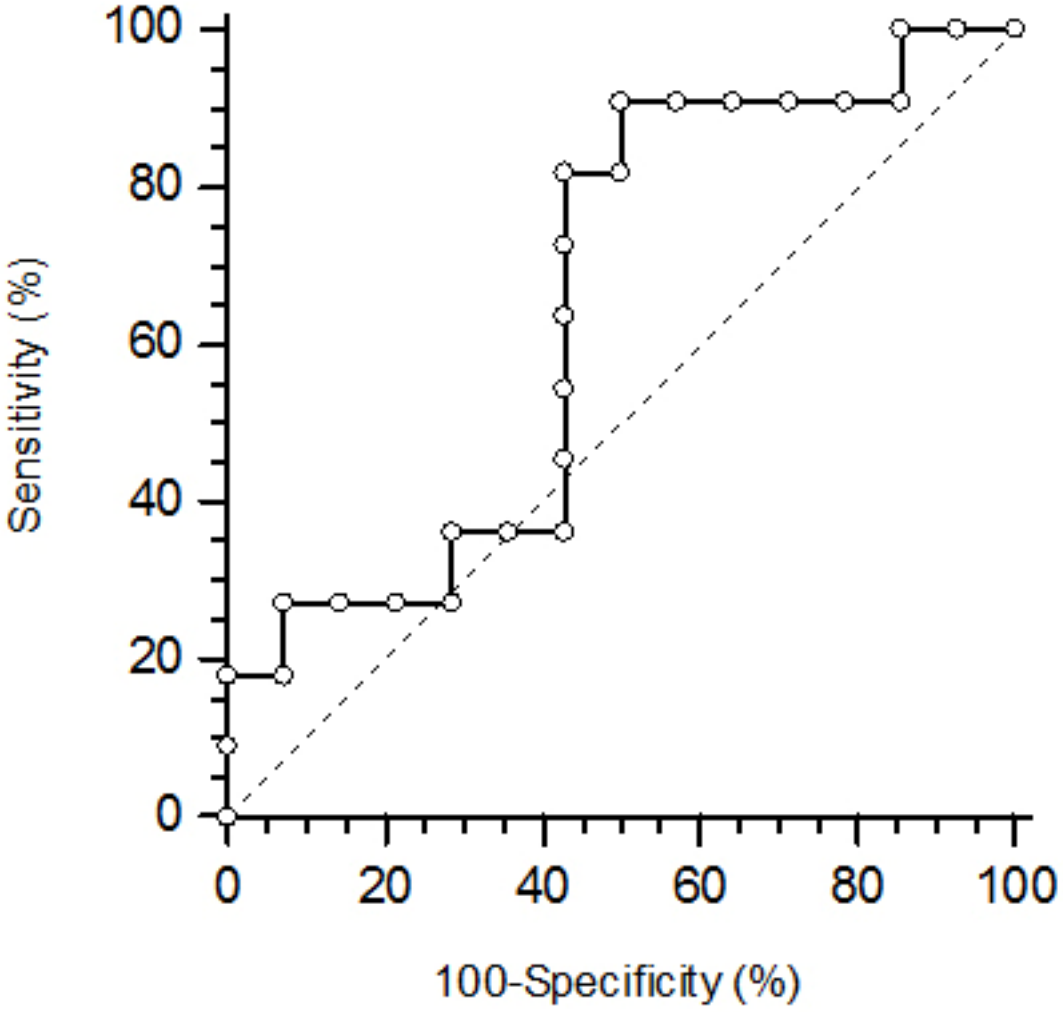
Note: SDNN (p = 0.22), RMSSD (p = 0.16) and SDSD (p = 0.16)

Graph C2 Receiver Operating Characteristic Curves for SDNN in controls and smokers



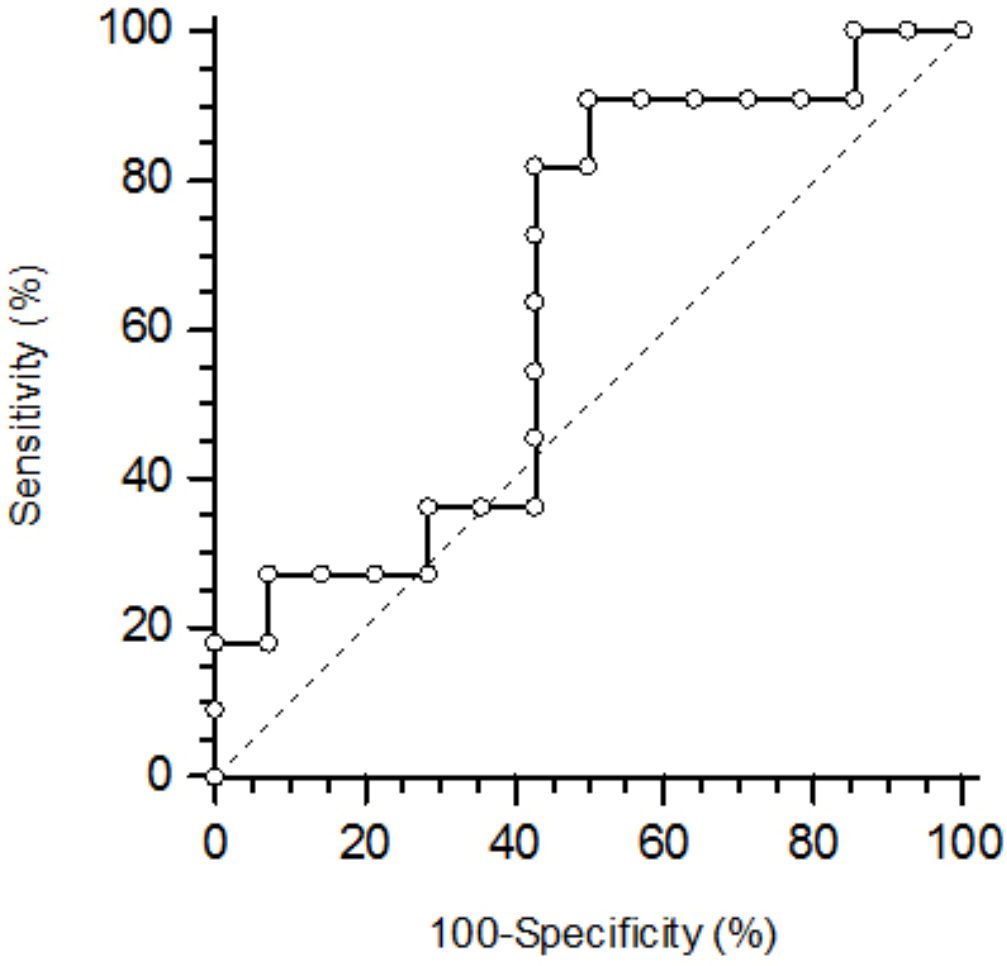
Note: AUC: 0.604

Graph C3 Receiver Operating Characteristic Curves for RMSSD in controls and smokers



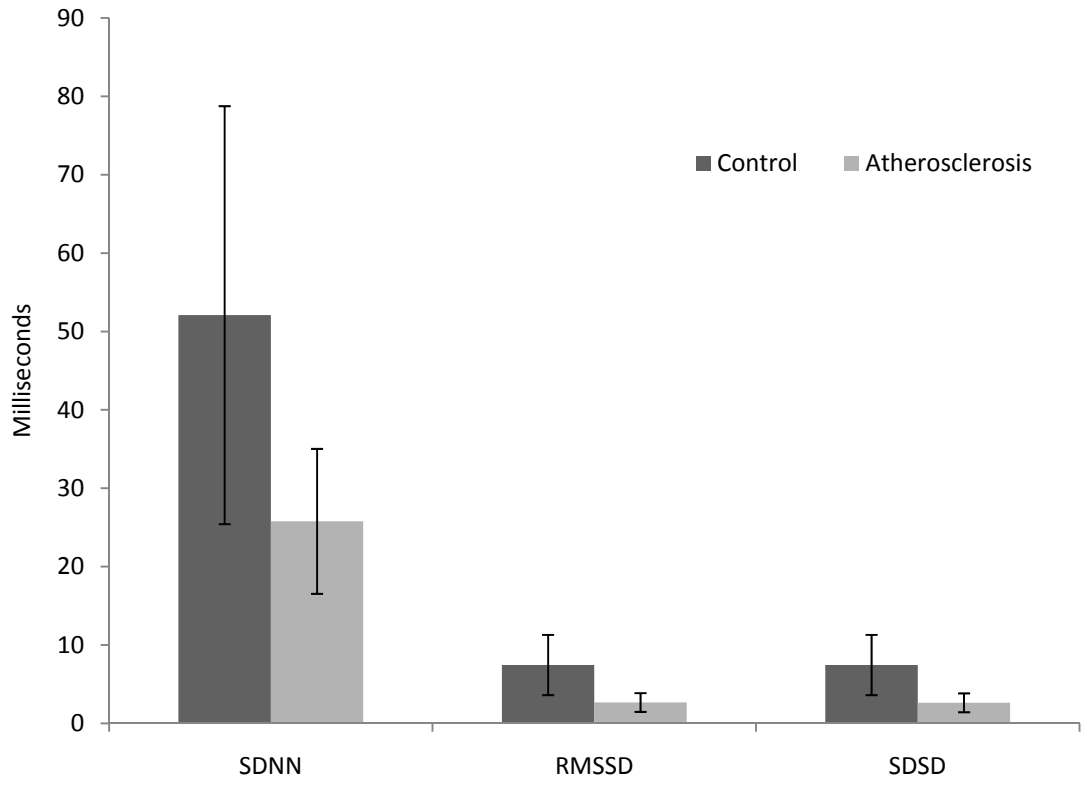
Note: AUC: 0.649

Graph C4 Receiver Operating Characteristic Curves for SDSD in controls and smokers



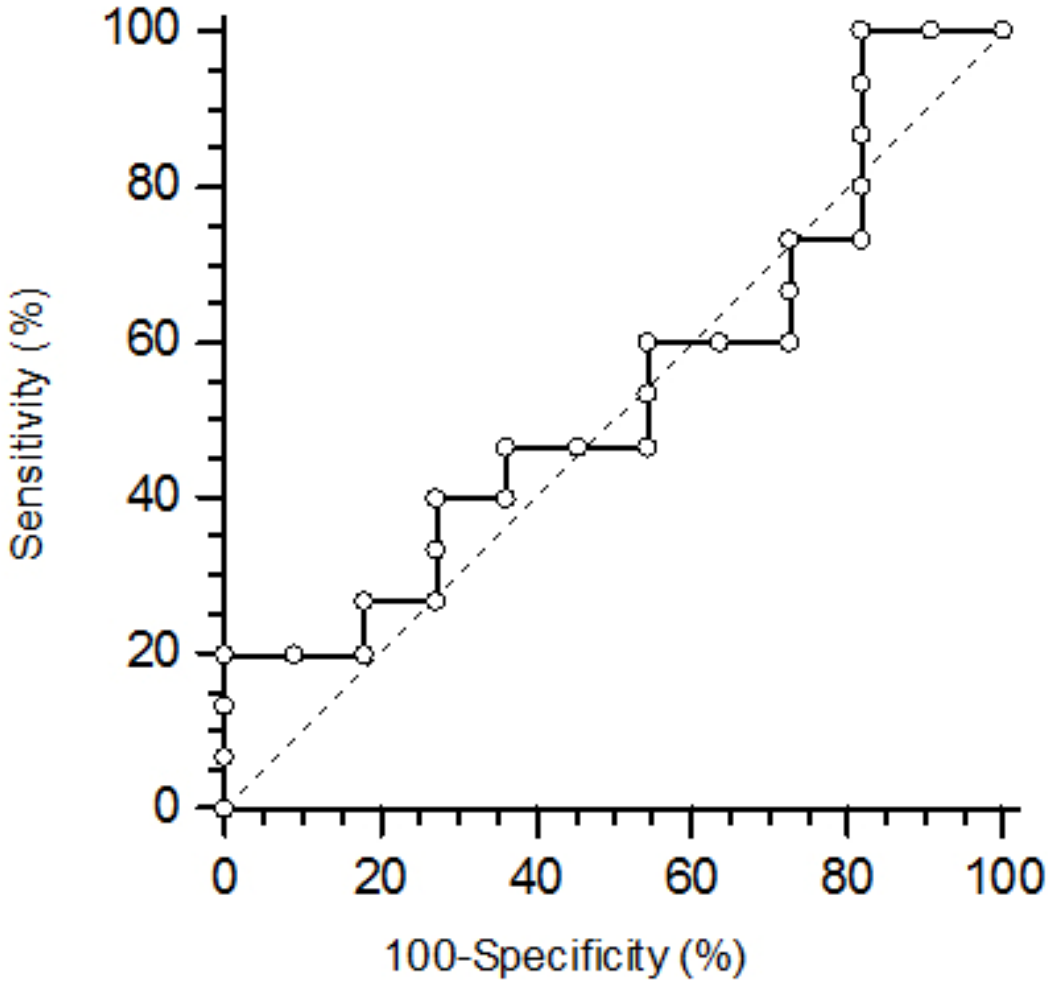
Note: AUC: 0.649

Graph C5 Heart rate variability in atherosclerotic patients and control subjects during baseline recording



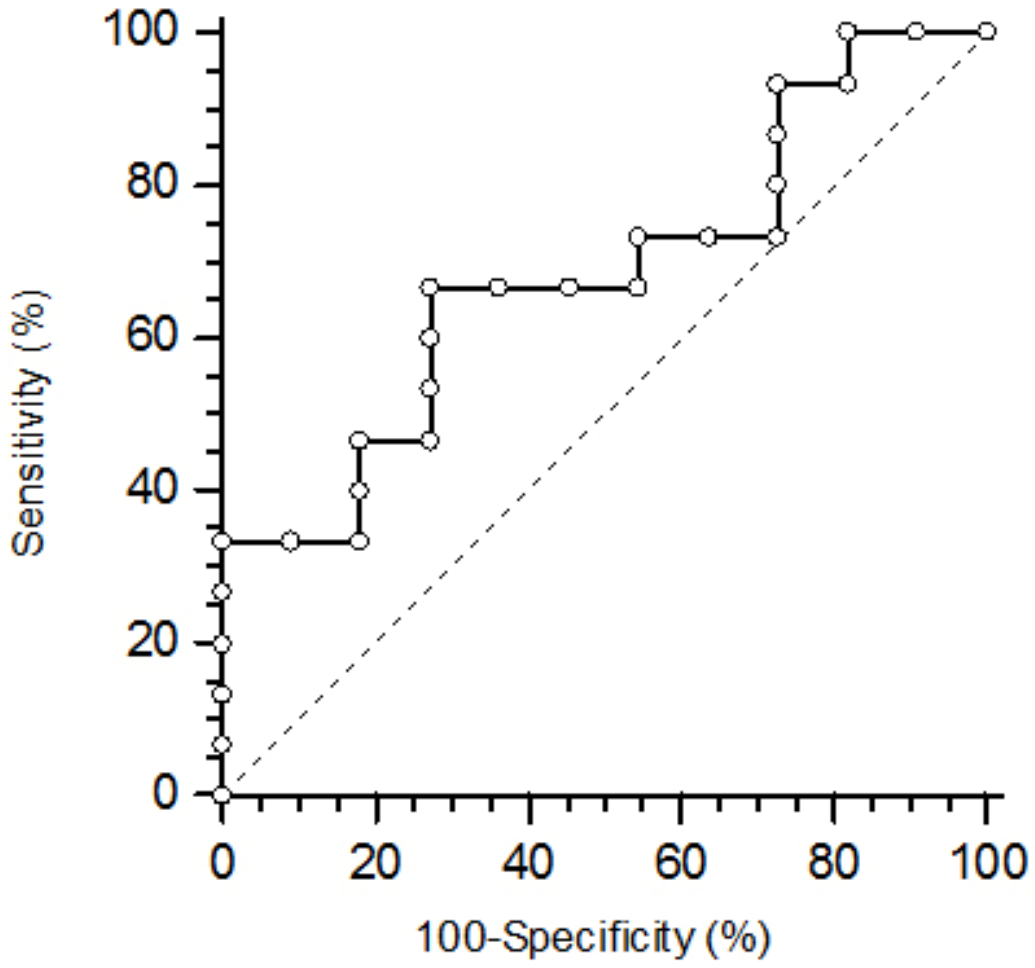
Note: SDNN (p = 0.31), RMSSD (p = 0.19) and SDSD (p = 0.19)

Graph C6 Receiver Operating Characteristic Curves for SDNN in atherosclerotic patients and control subjects during baseline recording



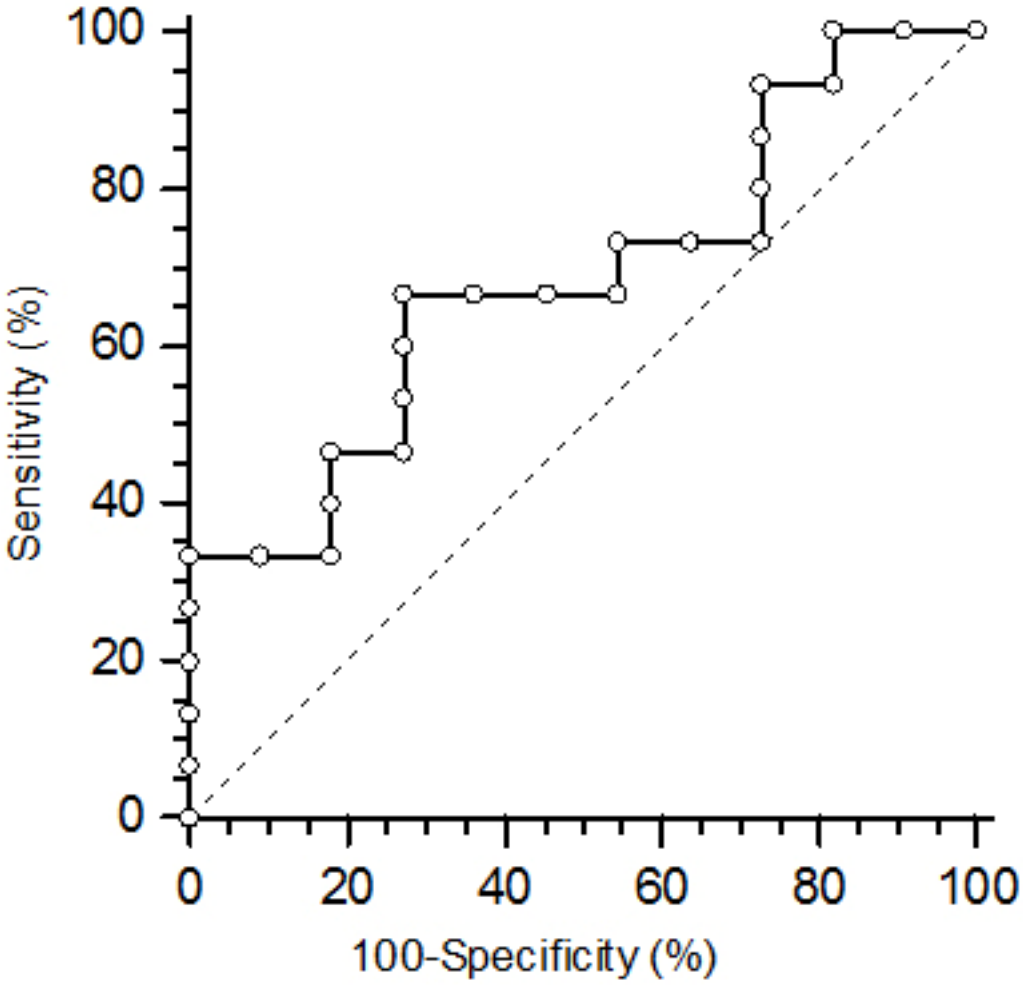
Note: AUC: 0.509

Graph C7 Receiver Operating Characteristic Curves for RMSSD in atherosclerotic patients and control subjects during baseline recording



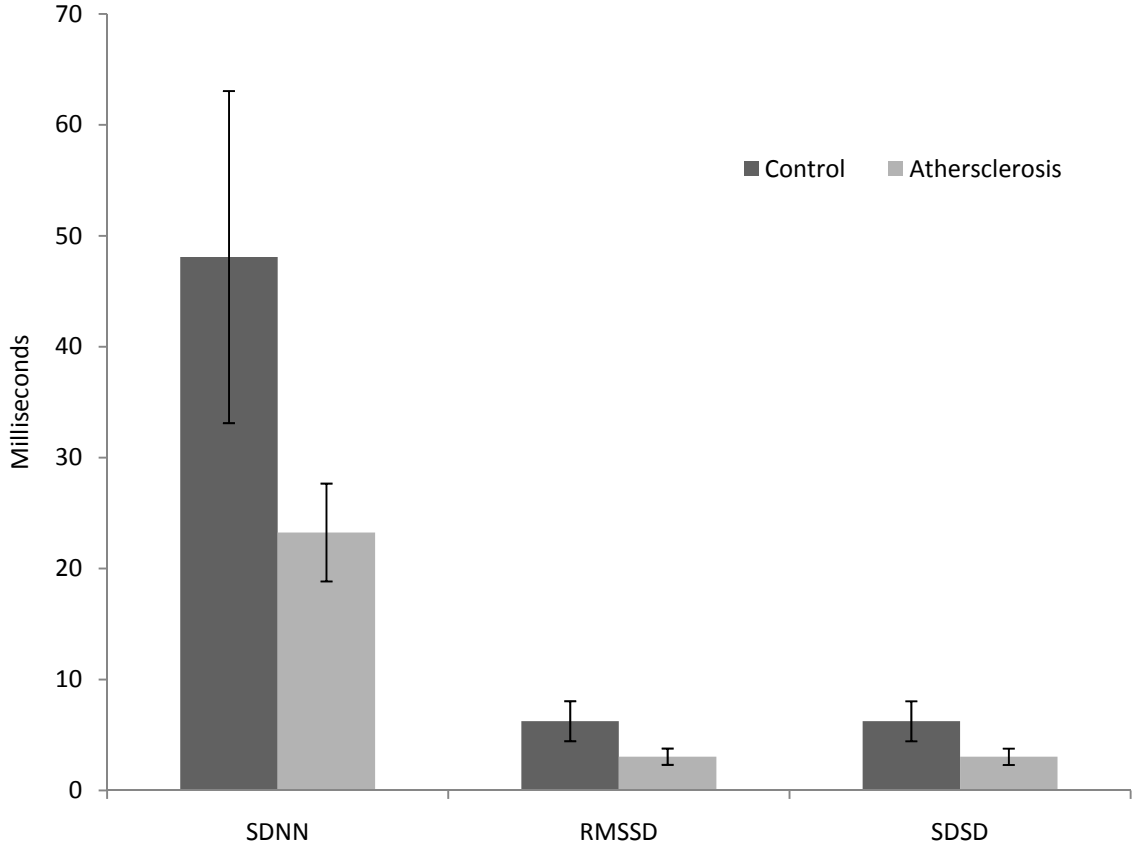
Note: AUC: 0.685

Graph C8 Receiver Operating Characteristic Curves for SDSD in atherosclerotic patients and control subjects during baseline recording



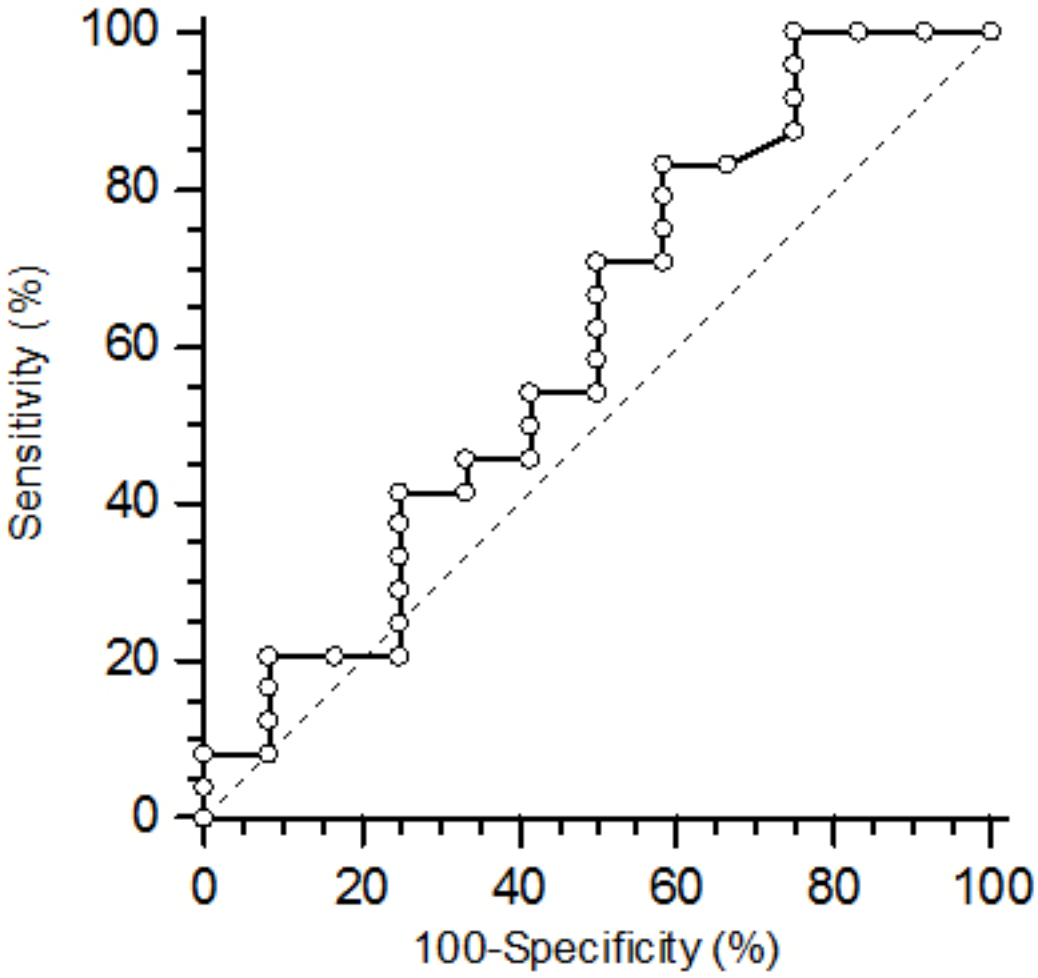
Note: AUC: 0.685

Graph C9 Heart rate variability in atherosclerotic patients and control subjects during local acetylcholine infusion



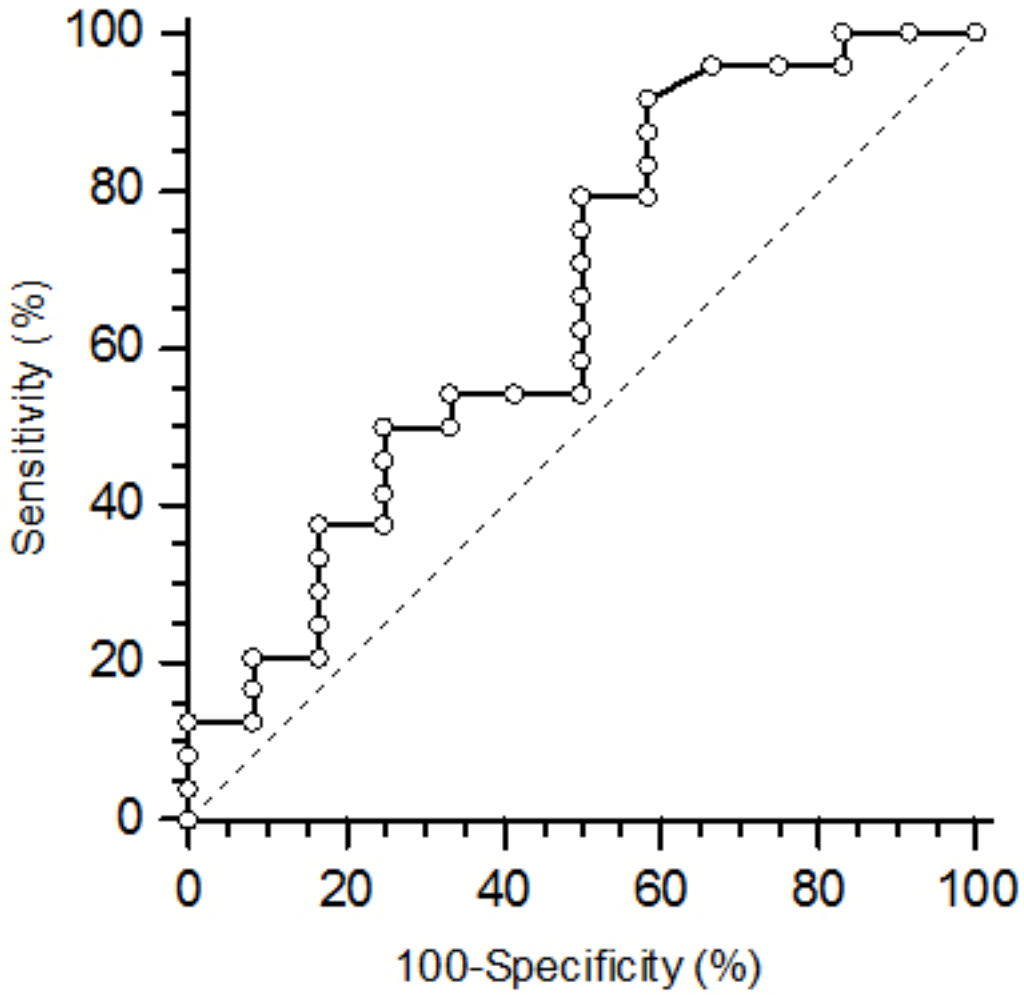
Note: SDNN (p = 0.049), RMSSD (p = 0.059) and SDSD (p = 0.059)

Graph C10 Receiver Operating Characteristic Curves for SDNN in atherosclerotic patients and control subjects during local acetylcholine infusion



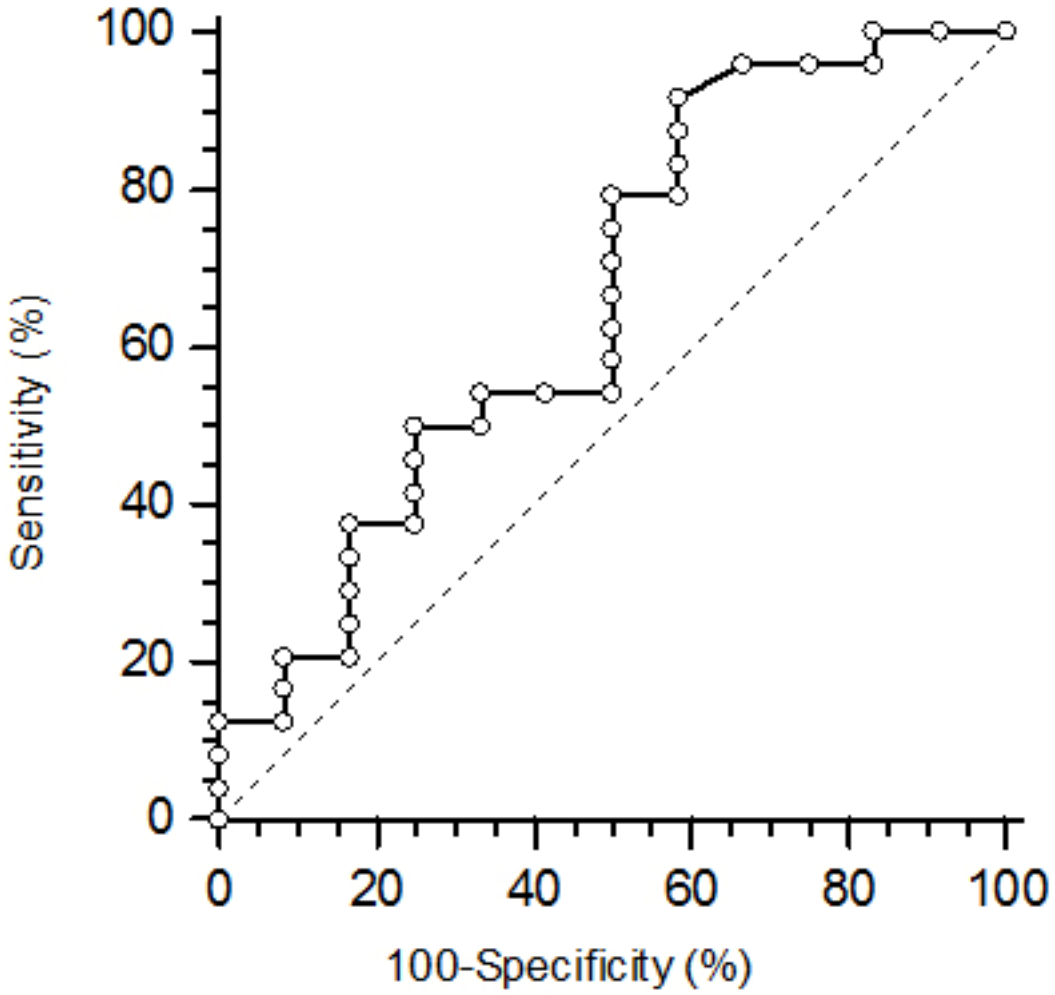
Note: AUC: 0.609

Graph C11 Receiver Operating Characteristic Curves for RMSSD in atherosclerotic patients and control subjects during local acetylcholine infusion



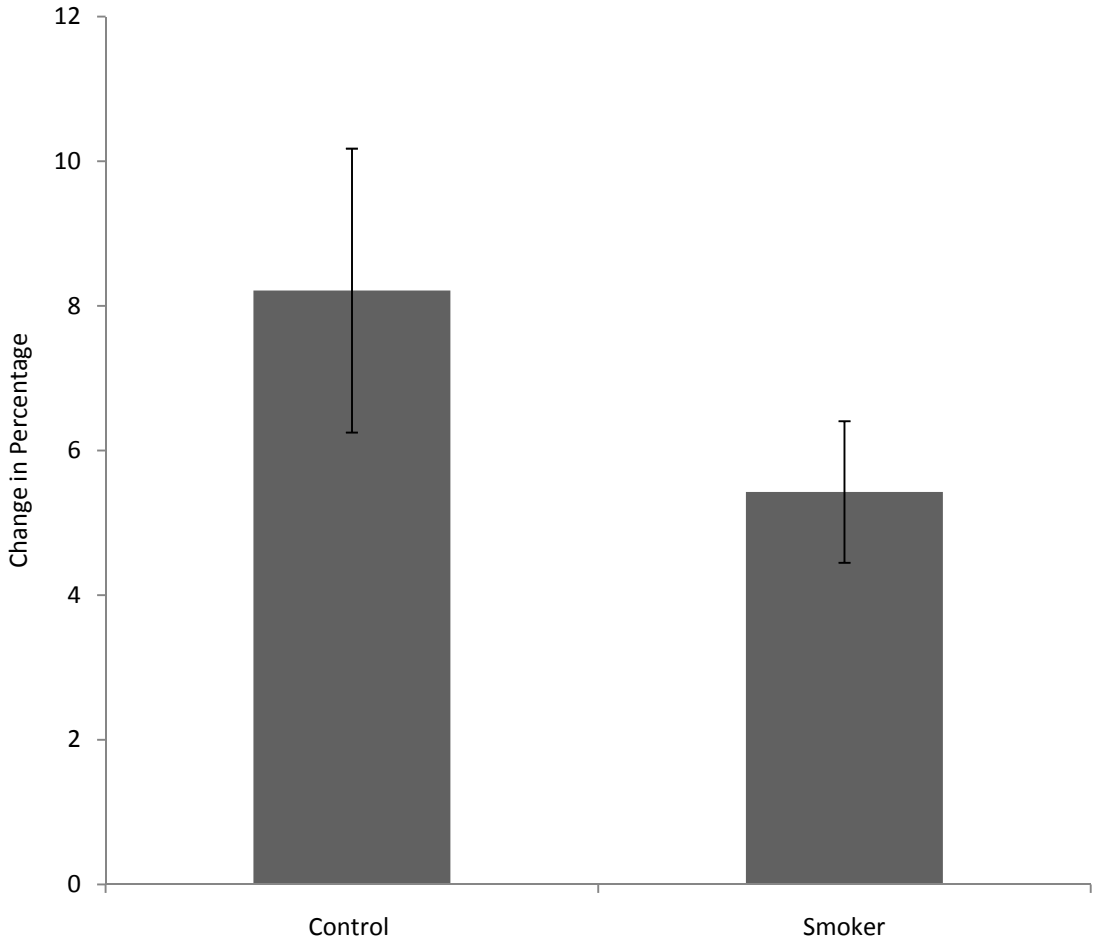
Note: AUC: 0.661

Graph C12 Receiver Operating Characteristic Curves for SDSD in atherosclerotic patients and control subjects during local acetylcholine infusion



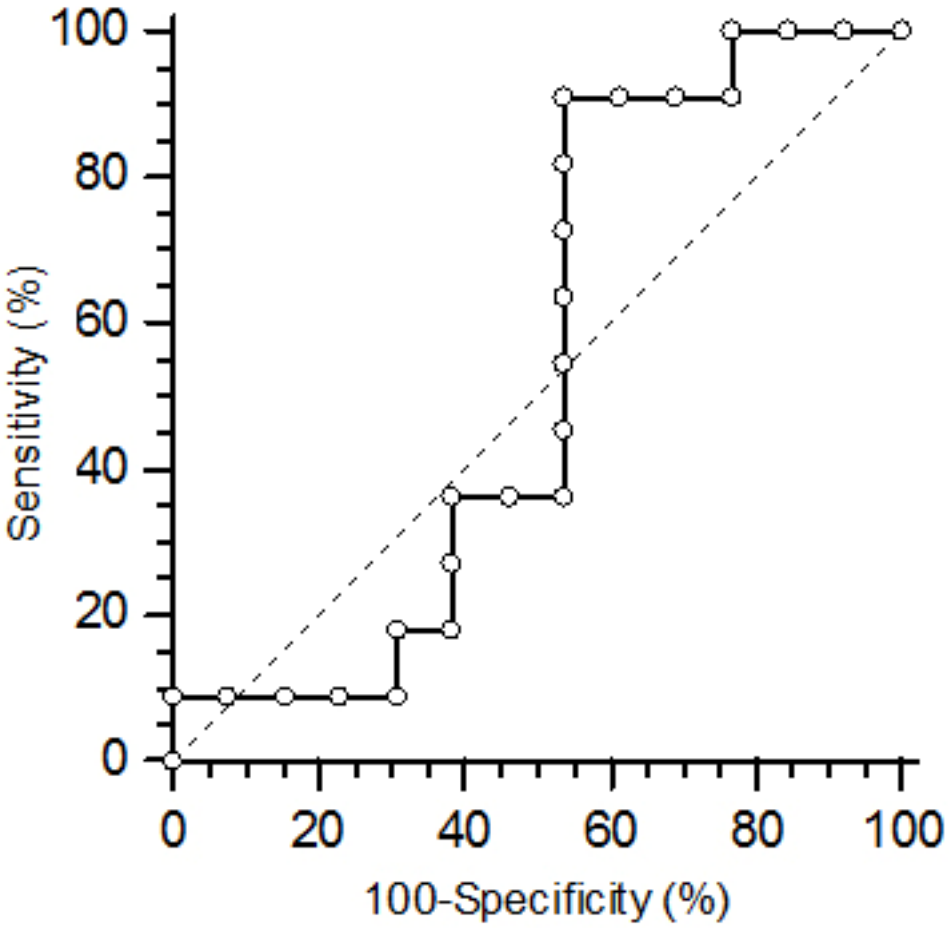
Note: AUC: 0.661

Graph C13 Flow mediated dilatation in smokers and controls



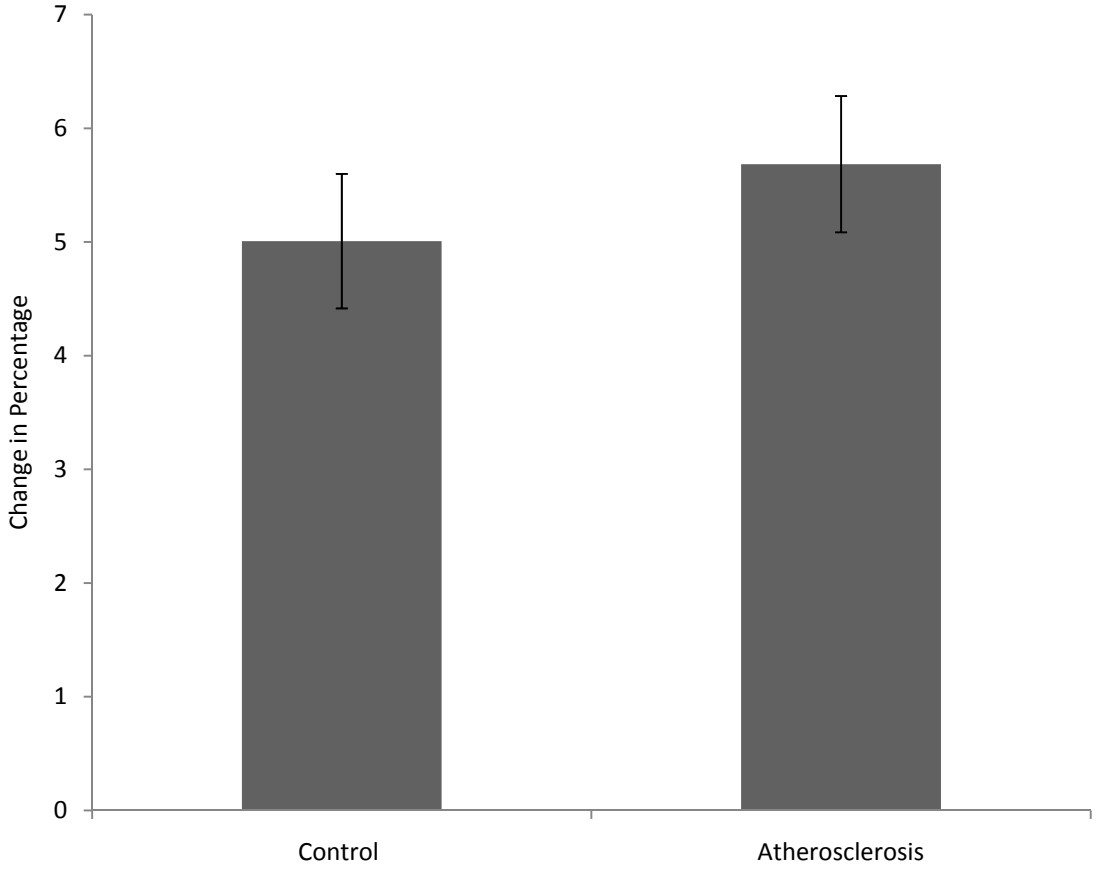
Note: (p = 0.24)

Graph C14 Receiver Operating Characteristic Curves for flow mediated dilatation in smokers and controls



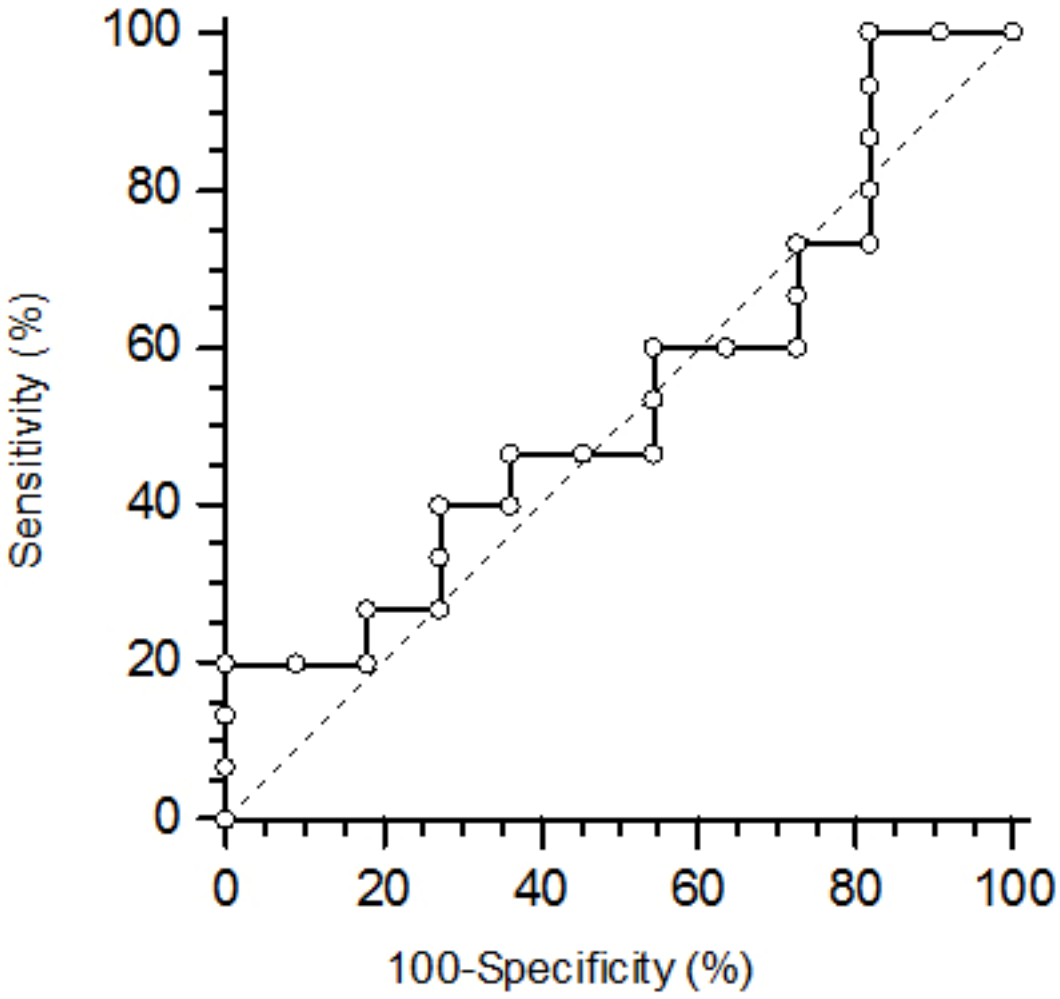
Note: AUC: 0.538

Graph C15 Flow mediated dilatation in atherosclerotic patients and control subjectsFlow mediated dilatation in atherosclerotic patients and control subjects



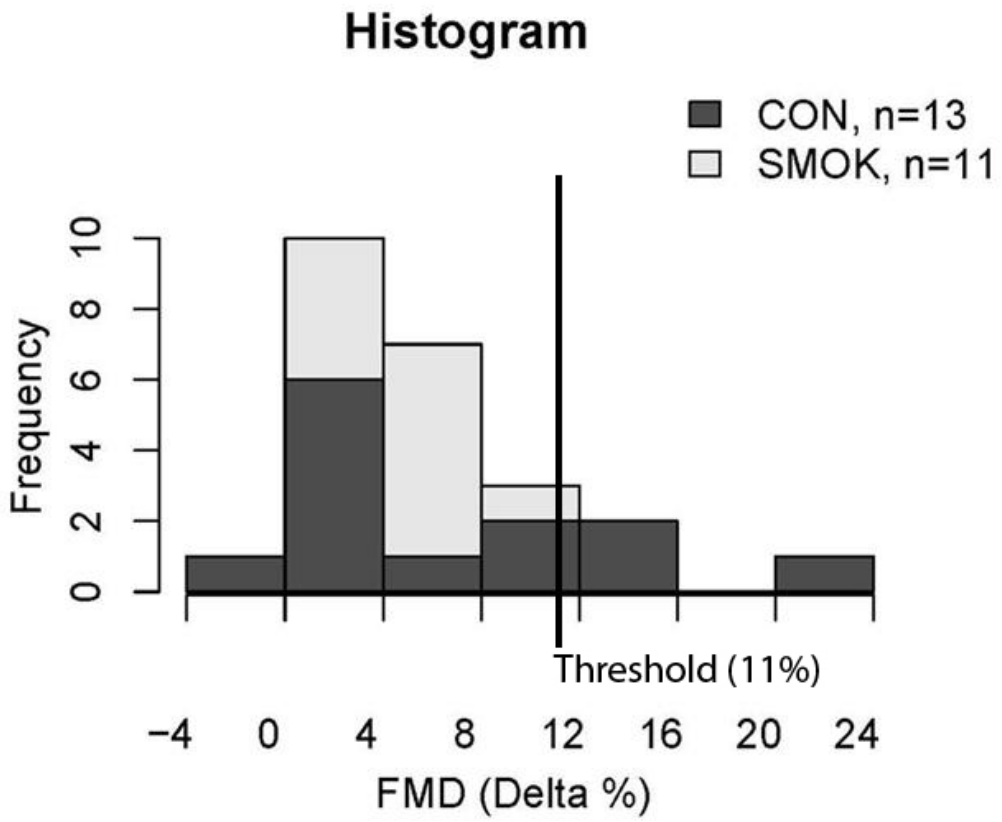
Note: (p = 0.48)

Graph C16 Receiver Operating Characteristic Curves for flow mediated dilatation in atherosclerotic patients and control subjects



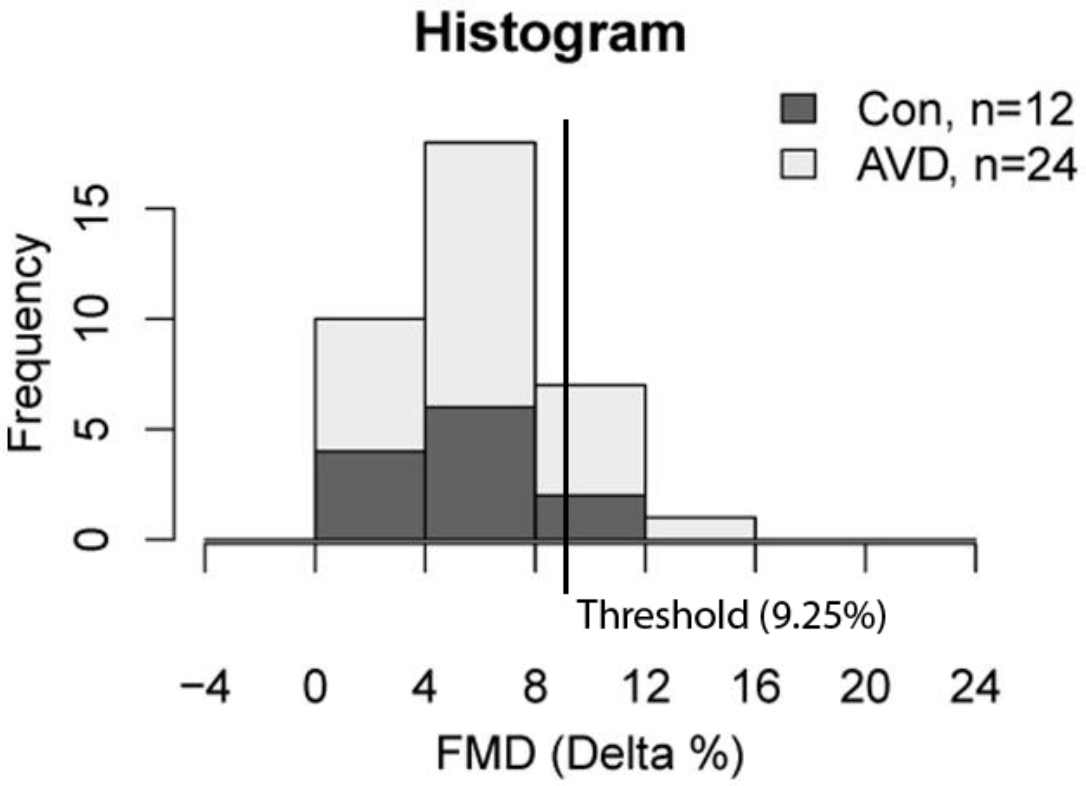
Note: AUC: 0.563

Graph C17 Flow mediated dilatation histogram for smokers



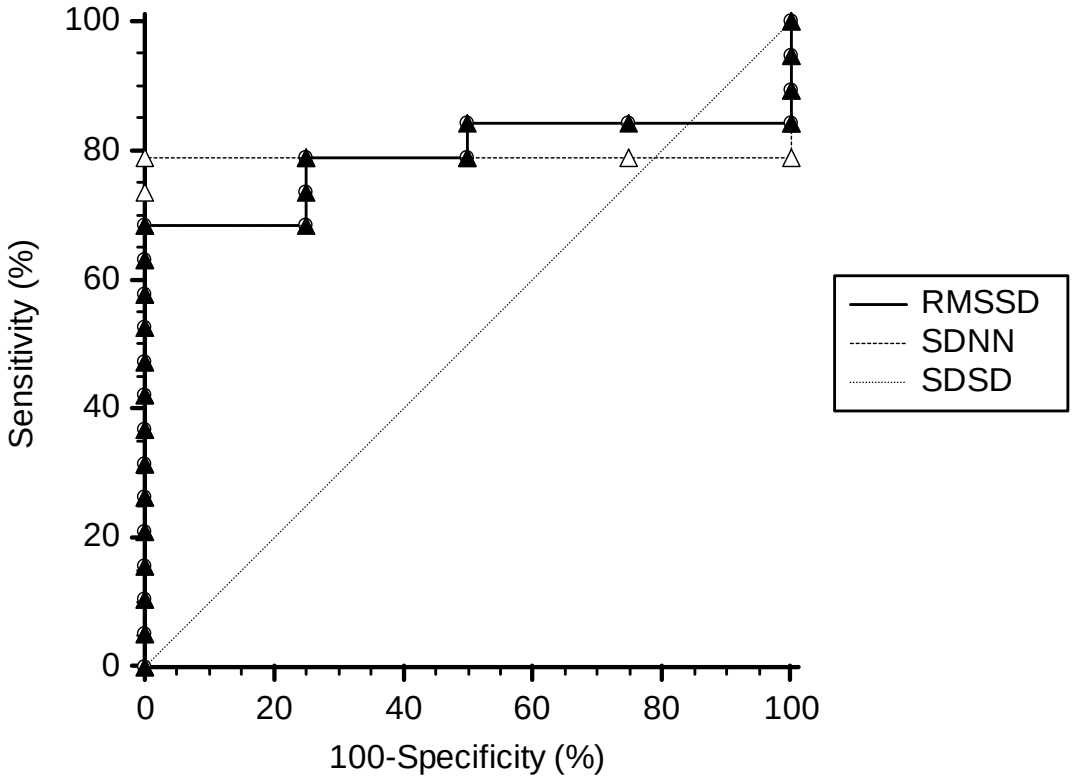
Note: Smokers (SMOK) and control subjects (CON). The distrution reveals an unimodal distrubiton.

Graph C18 Flow mediated dilatation histogram for atherosclerotic



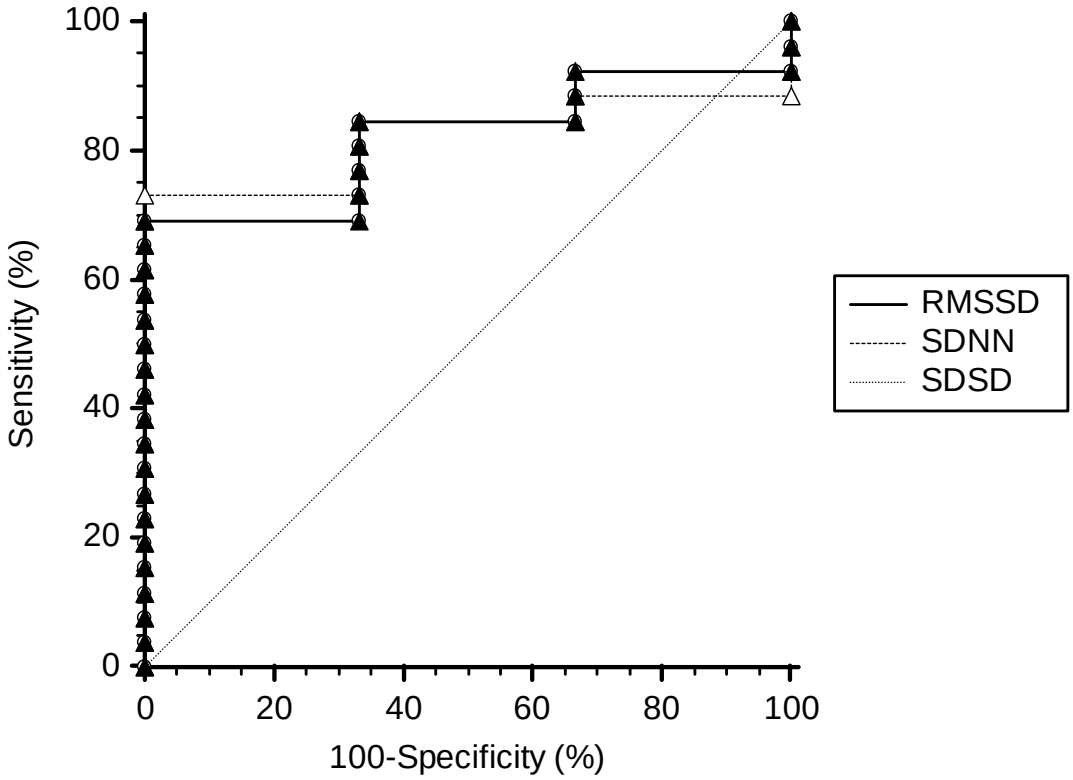
Note: patients (AVD) and control subjcets (Con). The distrution reveals an unimodal distrubiton.

Graph C19 Predictability of endothelial dysfunction using heart rate variability parameters derived from smokers and control subjects



Note: The ROC curves of all three heart rate varaibility parameters predicting endothelial dysfunction are shown. The AUC values for all three parameters are 0.789 are identical for all three heart rate variability parameters.

Graph C20 Predictability of endothelial dysfunction using the heart rate variability parameters derived from atherosclerotic patients and control subjects



Note: The ROC curves of all three heart rate varaibility parameters predicting endothelial dysfunction are shown. The AUC values for all three parameters are 0.821 and are identical for all three heart rate variability parameters.

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