ANALYSIS OF ELEMENTAL CONCENTRATION AND SPECTRAL BIOMARKERS IN ISCHEMIC MUSCLE OF PATIENTS WITH PERIPHERAL ARTERY DISEASE

A Thesis by

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The following faculty members have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirement for the degree of Master of Science with a major in Industrial Engineering

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DEDICATION

To my family and my loved ones

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ABSTRACT

Peripheral arterial disease (PAD) is a chronic progressive narrowing of the arterial lumen due to atherosclerosis (fat, plaque deposition and hardening of arterial walls), which affects approximately 8 million lives in the United States. Patients diagnosed with PAD have increased risks of limb loss and mortality. The classic symptom of PAD is intermittent claudication (IC), defined as calf pain associated with increased metabolic demand on the muscle. Functional testing, such as the ankle brachial index (ABI), measured as the ratio of systolic blood pressure in the ankle to that in the arm, is the most common test for the diagnosis of PAD. The ABI can identify reduced blood flow (due to blockages in the arteries) based on blood pressure differences. However, there is a need to measure more than just abnormal blood flow, it is also critical to measure the effects of compromised blood flow on the skeletal muscle. In this study, we evaluate the hypothesis that differences in muscle elemental composition and biochemical alterations in the diseased tissue could be correlated with clinical diagnosis and may be used to characterize muscle damage severity. These findings may aid in the development of specialized preventive and rehabilitative treatment plans by providing new biological targets based on the specific stage of disease and muscle damage. This will also be a stepping stone toward the development of improved monitoring techniques of muscle damage repair following treatment intervention.

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1. INTRODUCTION

1.1 BACKGROUND OF PERIPHERAL ARTERY DISEASE

Peripheral artery disease (PAD) is a vascular disease characterized by narrowing the arteries of the lower extremities and subsequently leading to decreased blood flow to the legs (Ostchega et al., 2007). Atherosclerosis, hardening of the arteries, which occurs when fatty deposits, cholesterol, and plaque buildup on the inner layer contributes to arteries stenosis. (Aronow, 2010). The larger range of arteries are affected in the regions of the legs, pelvis, and abdominal aorta (Hirsch et al., 2006). Thus, the decreased blood flow causes the lower extremities not to meet the needs of resting tissue metabolism and increases the pain (Aronow, 2010). The classical symptom of PAD is intermittent claudication (IC), an ambulatory disorder which is defined as lower extremity pain that causes the patient to stop walking and resolves after rest (Rolando et al., 2009).Claudication is an early indicator of muscle disease, in which myofibers begin to deteriorate due to altering metabolic processes resulting in changes of muscle morphology (Pipinos et al., 2008).

PAD is a progressive disease affects more than 8 million lives in the United States and it is perceived to be increasing in the elderly population (Steffen et al., 2008). When the disease is untreated, the severity of the disease increases and leads to foot ulcers, gangrene, and even leg amputation. Moreover, PAD is associated with a significant increase in cardiovascular morbidity and mortality (Hirsch et al., 2006). Unfortunately, there are limited treatments for PAD consisting of surgical revascularization, life style changes, risk modification, and regular exercise proposed by PAD management (Pellegrin et al., 2014). Consequently, the complexity of PAD, as well as pathophysiological mechanisms are still largely unknown. Although blood flow limitation to active muscle is of critical importance, little is known about the factors independent

of blood flow and intrinsic to skeletal muscle that may also contribute to the disease process and functional limitations in PAD patients (Pellegrin et al., 2014).

1.2 STATEMENT OF THE PROBLEM

Peripheral arterial disease (PAD) is a leading significant healthcare problem linked with progressive morbidity and mortality rate in many countries (Allison et al., 2007; Norgren et al., 2007). The atherosclerotic blockage results in reduced blood flow, consequently a mismatch between the oxygen supply and metabolic demand resulting in symptoms of intermittent claudication causing biochemical alterations which results in muscle damage (Stewart et al., 2002). Individuals with symptomatic PAD classically present with intermittent claudication, which is the pain they feel when try to perform even minor exercises (Bhasin & Scott, 2007). The classical methods for screening PAD includes ankle brachial index (ABI), ultrasonography, venous plethysmography, and angiography (Bhasin & Scott, 2007). The fundamental principle behind the methods are, to measure the blood flow pressure differences for monitoring and treatment interventions of PAD (Regensteiner & Hiatt, 2002). However, all these methods have limitations in monitoring progression or regression of PAD because there is a need to measure more than just blood flow; there is a need to measure muscle degeneration as well.

The contribution of this thesis is to measure the changes in the elemental concentration difference between control, claudicating, and critical limb ischemia and identify the critical spectral biomarkers, susceptible to muscle damage. This contribution is significant because it exemplifies a next step in PAD muscle degeneration research, which is expected to advance the field in directing novel therapeutic intervention and monitoring clinical treatment interventions of PAD. “Chronic disease will never reach its clinical horizon to compromise health if it is attacked at its origin”(Booth et al., 2000). The severity of the disease can be manifested by the

optimal diagnosis, which requires measurable quantity and type of damage present in a PAD patient. This study proposes to identify differences in elemental concentration and specific spectral biomarkers of the muscle damage, to provide objective criteria of muscle PAD muscle damage for therapeutic interventions.

1.3 RESEARCH OBJECTIVES

In this research, we will evaluate the hypothesis that differences in muscle elemental composition (as determined by scanning electron microscope with energy dispersive X-ray spectroscopy) and spectral biomarker differences (determined by Fourier Transform Infrared Spectroscopy) between control, claudicating and critical limb ischemia varies and it can correlate with clinical diagnosis and may be used to characterize the severity of muscle damages in PAD patients.

Specific objectives of this research are:

1. To identify critical differences in elemental composition, including sodium, potassium, calcium, magnesium and sulfur in myofibers of gastrocnemius biopsies from control, claudicating, and CLI patients
2. To identify acute spectral biomarkers of muscle damage in patients with peripheral artery disease using FTIR

1.4 THESIS ORGANIZATION

The thesis is structured into four chapters. Chapter 1 provides the background of PAD, statement of the problem, contribution and the research objectives. Chapter 2 presents a literature review that includes risk factors of PAD, impacts of PAD, evaluation of PAD and diagnostic methods. The study also includes review on diagnostic methods, studied by scanning electron microscope, and spectral biomarkers using FTIR. Chapter 3 addresses objective 1 of the research work which deals with the examining of gastrocnemius muscle samples from control,

claudicating, and critical limb ischemia. The muscle samples were analyzed by SEM- EDS and the data are analyzed statistically by analysis of variance. Chapter 4 demonstrates the objective 2 of the research work which deals with analyzing the biomarkers of muscle damage in patients with peripheral artery disease using FTIR and the data are analyzed statistically by analysis of variance and Box-whisker plot.

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2. LITERATURE REVIEW

Peripheral artery disease (PAD) is a chronic, life threatening disease that affects morphology and function of muscle (Regensteiner et al., 1993) and is also a growing health problem for people in the United States. Approximately 12% to 14% of the population suffer from a form of PAD and experiences pain with narrowing of arteries, arterial dysfunction, impaired leg muscle perfusion, and musculoskeletal abnormalities (Al-Qaisi et al., 2009; Norgren et al., 2007). PAD severity increases the risk of limb loss and mortality also increases. Non-coronary arterial syndromes are influenced by the structure and functional change of the arteries that supply blood to the brain, limbs and other organs (Hirsch et al., 2006; Steffen et al., 2008).

2.1 EPIDEMIOLOGY OF PAD

PAD is a serious growing health condition that increases an individual's risk for heart attack, stroke, and leg amputation. Importantly, the presence of PAD confers significant cardiovascular morbidity and mortality which is high in people with prior myocardial infarction or stroke (Leng et al., 1996). Patients with indications of PAD typically lead to intermittent claudication (pain in the leg due to cramps) that occurs in the affected limbs during exercise and causes the patient to stop and rest until the pain subsides (Kojima et al., 2014). Secondary to intermittent claudication, PAD is associated with significant functional impairment, and exercise capacity is reduced approximately by 50% compared with healthy subjects (Hiatt et al., 1994).

2.1.1 PAD Surveillance

As the population age increase, the frequency of PAD increases and is predominantly seen in ages later than 40 years old (Criqui et al., 1985; Murabito et al., 2002; Selvin & Erlinger, 2004). As a result, PAD is growing as a clinical problem among the increasingly aged population in developed countries. The prevalence of PAD, defined as an ankle-brachial index (ABI) <0.90 in either leg, was 0.9% between the ages of 40 and 49, 2.5% between the ages of 50 and 59,

4.7% between the ages of 60 and 69, and 14.5% for age 70 and above (A. T. Hirsch, M. H. Criqui, et al., 2001; Selvin & Erlinger, 2004). The age-specific annual incidence of intermittent claudication for ages 30 to 44 years was 6/10,000 men and 3/10,000 women, and this incidence increased to 61/10,000 men and 54/10,000 women within the ages of 65 to 74 years (Kannel et al., 1970). Ostchega et al. estimated that PAD affects approximately 8-12 million lives in the United States, including 12-20% of the population over age 65 (Ostchega et al., 2007). .

2.1.2 Potential regulator

Over the last decade, exercise training has emerged as a very important intervention for primary and secondary prevention for vascular diseases (Linke et al., 2006). The prevalent forms of vascular disease are cerebrovascular disease (CVD) that affects the brain, coronary heart disease (CHD) that affects the heart, and the most prevalent vascular diseases are PAD that affects the lower extremities. Exercise, a form of control measure should promote all aspects of physical conditioning, including aerobic capacity and muscular endurance, range of motion and flexibility, and muscular strength (Pate et al., 1995). Importantly, despite the evidence attained from benefits of regular physical activity in the management of individuals with cardiovascular disease, the manner in which the exercise is prescribed is often “generic” rather than tailored to an individual’s state, severity and type of cardiovascular disease (Pina et al., 2003). Thus, PAD is associated with serious health problem among growing population.

2.1.3 Economic Impact

Cardiovascular diseases remain the leading cause of death in the United States and most western countries. Mortality data shows that about 58% of all deaths were caused due to cardiovascular disease in the year 2002 (Minino et al., 2006). Furthermore, cardiovascular disease is the underlying cause of death accounted for 36.3% of all deaths in 2004, or 1 of every 2.8 deaths in the United States. In fact, an estimated 79 million American adults (1 in 3) have 1

or more types of cardiovascular disease. Of these, 37 million are estimated to be 65 or older (Weatherley et al., 2007). The economic burden of cardiovascular disease is enormous (Go et al., 2014). In fact, the costs of cardiovascular disease totaled an estimated 315.4 billion dollars in the United States in the year 2014 (Go et al., 2014). Heidenreich et al. estimated the cost of all cardiovascular disease and it projected to be increasing in years Figure 2.1

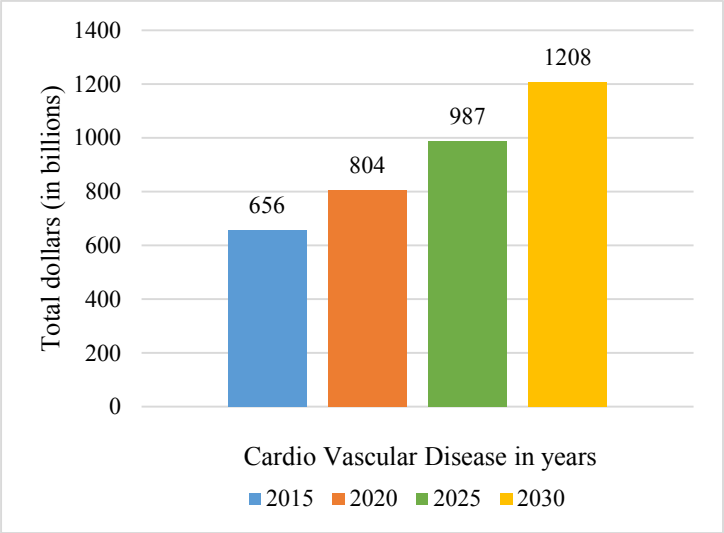

Figure 2.1 Projected total cost of cardiovascular disease.
Extracted from (Heidenreich et al., 2013)

2.2 PATHOPHYSIOLOGY AND RISK FACTORS

Conventional cardio risk factors such as smoking, age & gender, race, diabetes mellitus, hypertension, dyslipidemia, hyperhomocysteinemia, and chronic renal insufficiency are associated with PAD (Norgren et al., 2007; Steffen et al., 2008). Figure 2.2 graphically represents the odds ratio of few primary risk factors associated with PAD. Diabetes mellitus and smoking are the strongest modifiable risk factors for PAD, predominantly in white population (American Diabetes, 2003; Garcia, 2006). Age and sex are the most important non-modifiable

risk factors in all ethnic groups (J. Dormandy et al., 1999). Factors that reduce the risk of getting PAD include regular physical activity and moderate alcohol intake.

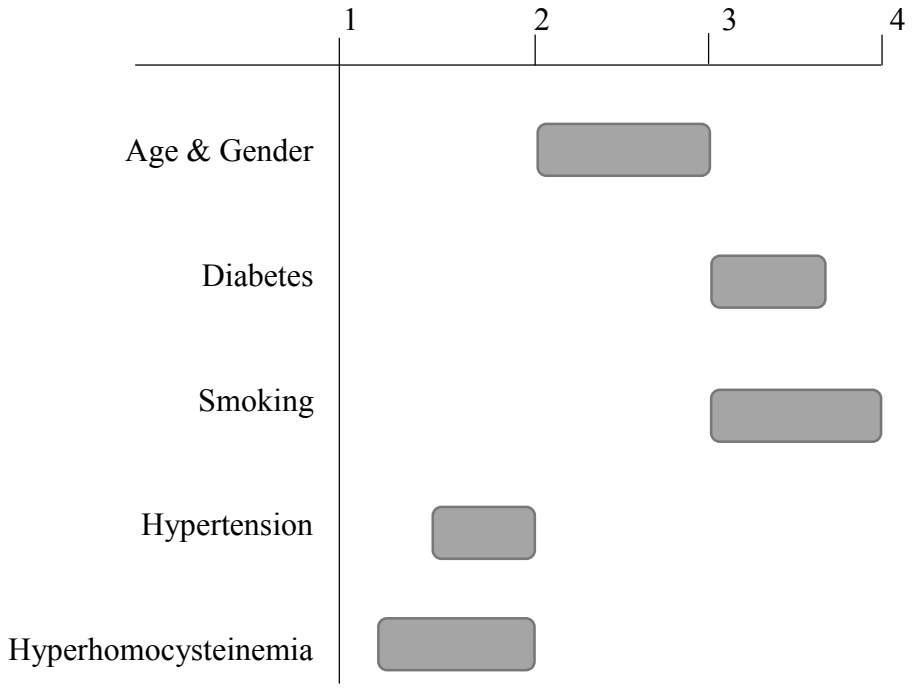

Figure 2.2 Odds ratio of peripheral arterial disease. Reformed from: (Norgren et al., 2007)

2.2.1 Smoking

Many studies have revealed that the most powerful risk factor associated with PAD is cigarette smoking (Norgren et al., 2007; Selvin & Erlinger, 2004; Willigendael et al., 2004). Interestingly, previous studies have consistently shown that the strength of the association between active cigarette smoking and PAD is stronger than the association between active smoking and coronary heart disease (CHD) (J. A. Dormandy & Rutherford, 2000). Smoking, however, is an important source of exposure to lead and cadmium (Hoffmann et al., 2001). Cadmium in cigarettes has been proposed as a causative agent for cigarette smoke–induced cardiovascular disease (Hoffmann et al., 2001). The risk of developing claudication, limb-threatening ischemia, and amputation is higher with the chain smoking patients (Donnelly & Yeung, 2002). Recent research by Go et al. demonstrated that among 440 PAD patients, there

was a larger demographic of male smoking patients, with higher exposure to risk of PAD (Go et al., 2014). Furthermore, the amputation rate related to PAD among smokers has been shown to be around twice than that among nonsmokers (J. A. Dormandy & Rutherford, 2000). Researchers found that the effect of smoking on PAD is partly reconciled by the cadmium content of cigarettes by adjusting the cadmium level in the cigarettes (Navas-Acien et al., 2004). Records from NHANES 1999–2000 (NCHS) predicts that high blood levels of lead and cadmium leads to increase the risk of PAD by 2.8 fold for cadmium and 2.9 fold for lead (Rosamond et al., 2008). The predominance of PAD is strongly associated with the lead and the cadmium level in blood. Willett et al. examined the number of cigarettes smoked per day was positively associated with the risk of fatal coronary heart disease (CHD) (Willett et al., 1987). When the number of cigarettes smoking per day increases, the risk of CHD associated also increases by 2 to 3 fold (Figure 2.3)

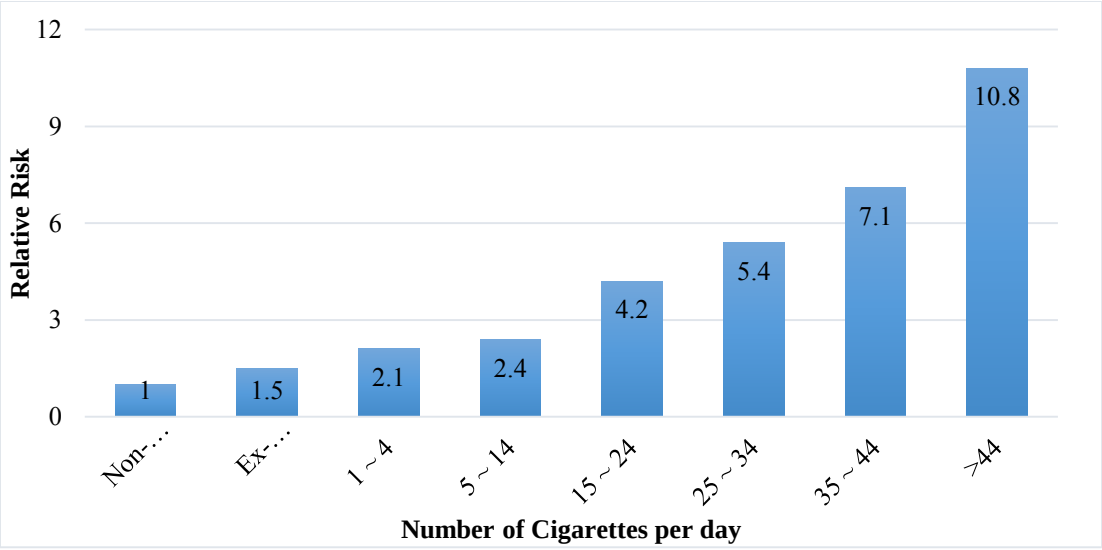

Figure 2.3 Relative risk of CHD according to the number of cigarettes consumed. Modified from: (Willett et al., 1987).

2.2.2 Age & gender

As people age advances, the prevalence of peripheral arterial disease increases and nearly 20% of people over the age of 70 years have PAD (Regensteiner & Hiatt, 2002). The frequency of disease is observed higher in men than women (Figure 2.4) and also increases as one gets older (Allison et al., 2007; Norgren et al., 2007). Ostchega et al in their study, claims that more than 5 million U.S. adults aged 60 and older have PAD and that more than two-thirds of the cases are asymptomatic and PAD increases with age from 7% to 23% among people of age 60 to 80 and older (Ostchega et al., 2007). Among the elderly people, non-Hispanic blacks, and women are observed to have high predominance of PAD (Go et al., 2014).

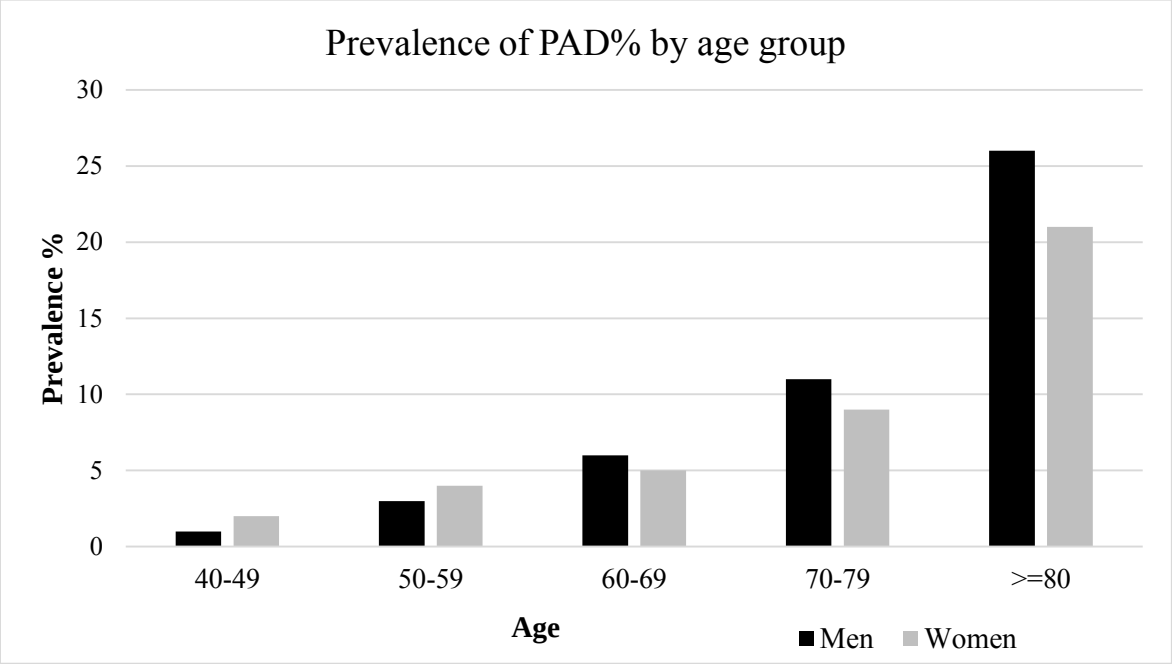

Figure 2.4 The prevalence of PAD in population. Revised from: (Allison et al., 2007)

2.2.3 Diabetes

About 20% to 30% of PAD patients in US are found to be affected with diabetes (Marso & Hiatt, 2006). The development of symptomatic PAD is more likely to occur in diabetic patients. The risk of PAD progression is higher in patients with both PAD and diabetes than with

patients without diabetes. The severity of the arterial disease is strongly related to the presence of diabetes in individuals. The rate of leg amputation in individuals with PAD and diabetes is correlated to regional variation (Steffen et al., 2008). Individuals with diabetes have a higher risk for atherosclerotic diseases. Atherosclerosis causes death and disability in most patients with diabetes. Diabetes will independently increase the risk for PAD, in addition to known cardiovascular risk factors (Marso & Hiatt, 2006).

2.2.4 Hypertension

Hypertension is associated with two to three times increased risk for PAD (Regensteiner & Hiatt, 2002). The relative risk for developing PAD is lower for patients with hypertension than patients with diabetes or smoking (Norgren et al., 2007). While critical limb ischemia and loss of tissue are an impeding consequence of PAD, it indicates 20-60% increased risk for myocardial infarction; 2 to 6 fold increased risk of death due to coronary events and 4 to 5 times higher risk of a cerebrovascular event (Hirsch et al., 2006)

2.2.5 Hyperhomocysteinemia

Homocysteine is an amino acid produced by the catabolism of methionine or cysteine, which are present in all individuals, being higher under certain conditions (Wolosker, 2009). Experimental studies confirmed that the vascular lesion is associated with the exposure to high levels of this catabolite, and these high levels of homocysteine are caused by oxidative stress, endothelial lesion, endothelial dysfunction, inflammation, thrombosis, and cell proliferation (Welch & Loscalzo, 1998). The prevalence of hyperhomocysteinemia is high in the vascular disease population (Norgren et al., 2007). In a meta-analysis, khandanpour et al. proved the level of homocysteine is significantly higher in PAD patients compared to control subjects (Khandanpour et al., 2009). Bergmark et al. found that the increase in the levels of homocysteine is associated with the increase in the extent of PAD, since homocysteine levels were lower in

patients with only one level of the disease regarding those with several levels of the disease (Bergmark et al., 1993).

In recent years, several new plausible risk factors for atherosclerosis have been proposed, including homocysteine (Khawaja et al., 2007; Smith et al., 2004), C reactive protein (CRP) (Khawaja et al., 2007; Selvin E et al., 2006), fibrinogen (Khawaja et al., 2007; Selvin E et al., 2006) lipoprotein (a) (C.H, 2004; S. W. Cheng et al., 1997; Smith et al., 2004) Tseng et al. 2004, increased platelet activity (Dieter et al., 2002) and hypercoagulability (Dieter et al., 2002). The relationship between PAD and these cardiovascular risk factors have not been fully elucidated, although some may explain the ethnic variations in susceptibility to PAD (Khawaja et al., 2007).

2.3 IMPACT OF PAD

In a study conducted by REACH, high annual cardiovascular event rates of outpatients are found to be atherosclerotic arterial disease and risk of atherothrombosis and it is also predicted to be one of the life threatening diseases in 2020 (Rosamond et al., 2008). The risk factor profiles of atherothrombotic patients are similar throughout the world; the risk factors like hypertension (81.8%), hypercholesterolemia (72.4%), and diabetes (44.3%) are the life threatening factors (Deepak L. Bhatt et al., 2006). A study by Rajagopalan et al. showed that the vascular diseases like atherosclerosis and thrombotic, platelets play a major role. The study shows that platelet reactivity is more prevalent in PAD patients than in healthy controls. Therefore the progression of severity is predicted not only by its symptoms, ABI and marks, but also by the increased reactivity level of platelets (Rajagopalan et al., 2007). PAD can be caused by degenerative disorders that lead to a decrease in arterial wall integrity and ensuing dilation. As a result, arterial wall degeneration can cause aneurysm formation that may result in destructive arterial ruptures (Hirsch et al., 2006). Individuals with no pre-existing cardiovascular diseases will frequently

experience cardiovascular and degenerative events including myocardial infarction and stroke (F. G. R. Fowkes, 2008).

2.4 CLINICAL PRESENTATION AND CALASSIFICATION

2.4.1 Claudication

Intermittent claudication (IC) is characterized by exertion pain or discomfort in the calves, thighs or buttocks and relieved by rest (Rolando et al., 2009). The discomforts are in the form of cramping, aching, and tiredness. These symptoms dissipate after a few minutes of rest and it rises again while climbing stairs, walking inclined, and moving quickly. Patients with IC are hindered by pain resulting in limited walking distance or speed. Inadequate blood supply, due to narrowing of arteries and building of plaques, fats, and cholesterol to the calf muscles and buttocks cause IC. This impairment in function results to reduced quality of life. (Coutinho et al., 2011).

2.4.2 Critical Limb Ischemia

The more advanced and final significant stage of PAD is Critical Limb Ischemia (CLI) which may lead to amputation of limb if not properly treated. The prevalence of CLI is likely to increase with an aging population, the rising incidence of diabetes, chronic kidney disease and the rate of tobacco use among adults (Falluji & Mukherjee, 2014). The symptoms of the CLI are pain at rest, non-healing ulceration and gangrene. When resting tissue metabolism does not receive sufficient blood flow, causes pain during rest and eventually leads to tissue loss (Aronow, 2010). This stage is called as critical lower extremity ischemia. Traditional methods for classifying the level of PAD beyond ABI screening include Fontaine's stages and Rutherford's categories (Table 2.1).

2.2 Classification of PAD-Fontaine's stages and Rutherford's categories.

Modified from: (Norgren et al., 2007)

Fontaine		Rutherford		
Stage	Clinical	Grade	Category	Clinical
I	Asymptomatic	0	0	Asymptomatic
IIa	Mild Claudication	I	I	Mild Claudication
IIb	Moderate to Severe Claudication	I	2	Moderate Claudication
		I	3	Severe Claudication
III	Ischemic rest pain	II	4	Ischemic rest pain
IV	Ulceration or Gangrene	III	5	Minor tissue loss
		III	6	Major issue loss

Regardless of advances in technology, a large number of patients are subjected to amputations in the United States and worldwide for CLI. If early uncovering strategies of PAD with appropriate medical and procedural interventions are tracked, then the majority of these amputations may be avoided (Falluji & Mukherjee, 2014). Finally, the annual incidence of critical limb ischemia approximates 500 per one million population (J. A. Dormandy & Rutherford, 2000). While these methods are a common approach to diagnose degenerative PAD not responsive to medical therapy, lesser is known about the impact of such symptomatic PAD on muscle function and morphology. Further, little is known about the impact of revascularization on muscle function in patients with PAD. Many raised a question, ABI and other techniques just measures the flow of blood and do not consider the end organ effect due to plaque buildup in the arteries (Kramer, 2007). There is a need to measure the secondary effect caused by abnormal blood flow, elemental concentration differences, and biomarkers of the disease as it progresses.

2.4.3 Amputation

Amputation, an end stage in the disease progression, at which the tissues are beyond rescue or there was wide tissue death. The majority of the amputations are performed only if that can improve the quality of life (Norgren et al., 2007). Amputation was considered as an optimistic impact in life, however there is still an increase in morbidity and mortality rate even after amputation (Norgren et al., 2007). It was estimated that about 150,000 amputations annually in the US, with perioperative mortality rates of 5–10% for below-knee amputation and up to 50% for above-knee amputation because of comorbid conditions (Hirsch et al., 2006). Among patients with CLI, approximately 25% progress to leg amputation (J. Dormandy et al., 1999). Barshes et al. claimed that PAD tops second highest among the three risk factors leading to foot ulcer - structural foot abnormalities, neuropathy, and PAD (Figure 2.5) (Barshes et al., 2013).

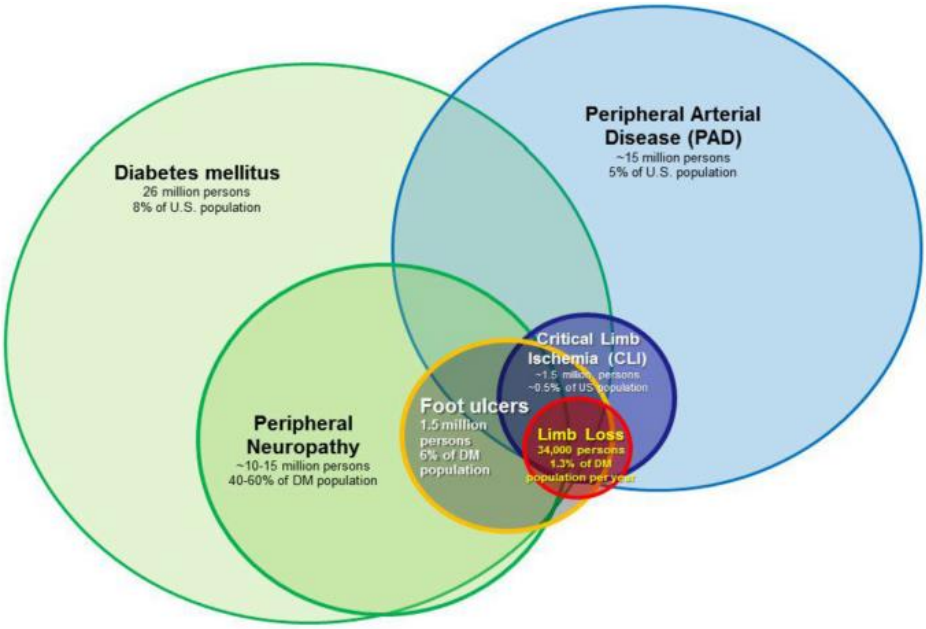

Figure 2.5 The overlapping relationship of risk factors associated with non-traumatic limb loss in the United States (Barshes et al., 2013)

2.5 DIAGNOSIS OF PAD

2.5.1 Noninvasive evaluation - ABI

Symptoms are not an accurate prediction of the presence or absence of PAD, or even the severity of the disease. For example, patients can display various degrees of PAD symptoms, but actually have more severe cases of PAD (Norgren et al., 2007). Since PAD causes critical impact on health, accurate diagnosis of PAD is very important. Careful examination and additional non-invasive testing is essential to ensure the diagnosis. The most common, non-invasive, reproducible, and reasonably accurate way to estimate asymptomatic PAD is to use the ankle-brachial systolic pressure index (ABI) in the legs (Marso & Hiatt, 2006; Norgren et al., 2007). According to Bhasin and Scott, ABI is universally accepted way of measuring PAD (Bhasin & Scott, 2007). ABI is the relative measure of systolic blood pressure of the arteries supplying the legs relative to the systolic blood pressure in arms (Al-Qaisi et al., 2009). Kim and colleagues validated and presented how to calculate the ABI in a diagrammatic representation Figure 2.6.

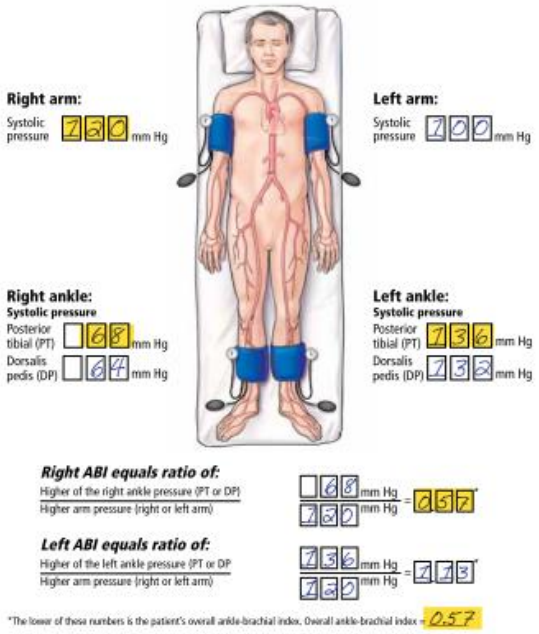

Figure 2.6 Diagrammatic representation of ABI measurement and calculation:
Extracted from (Kim et al., 2012)

From each leg, ABI is calculated and the lower value is the patient's overall ankle-brachial index. An abnormal value in either leg indicates peripheral artery disease. The diagnostic criteria for peripheral arterial disease based on ABI are as provided in Table 2.3

Table 2.3 Diagnostic criteria for PAD based on ABI. Reformed from (American Diabetes, 2003)

ABI Value	Interpretation of disease
1.0 - 1.3	Normal
0.91– 0.99	Borderline
0.70 – 0.90	Mild obstruction
0.40 – 0.69	Moderate obstruction
If <0.40	Severe obstruction
If >1.30	Poorly compressible

Patients with ABI values greater than 0.91 are considered to be healthy patient, meaning the systolic blood pressure in the brachial artery of the arm is similar to that of the systolic blood pressure of the ankle is normal, which implies there are no blockages or narrowing of arteries are present. The severity of PAD increases as the value of ABI decreases, the more severe the limit of arterial blood flow, and leads to more serious cases of ischemia (Aronow, 2010). Nearly 4 to 2 fold increase in cardiovascular mortality is associated with lower ABI. On the other hand, the presence of medial arterial calcification, common in diabetes mellitus, signifies the poor compressibility of arteries with an ABI value greater than 1.3. This is the main limitation of the ABI, which makes the diagnosis of peripheral vascular disease less reliable. The several traditional atherosclerotic risk factors are compared with the ABI value to predict the mortality and severity of PAD (Bhasin & Scott, 2007). ABI only measures the burden of plaque buildup in

the circulatory system and do not measure the end-organ effects of the plaque buildup (Kramer, 2007). This suggests the need to measure more than just blood flow. Overall, the ABI measurement is effective in the screening, diagnosis of PAD, but has limitations in providing evidence about the state of muscle degeneration.

2.5.2 Invasive techniques and Evaluation

Patients with IC have been treated conventionally for their leg symptoms with medical therapy, lifestyle modification, and exercise programs. Multiple approaches for revascularization include surgery, angioplasty, stenting and atherectomy (Goodney et al., 2014). An intense change has been observed in the patients treated with invasive endovascular techniques, the use of revascularization procedure (Slovut & Lipsitz, 2012). The use of endovascular repair increased >3-fold and the amputation rate decreased by 29% (Goodney et al., 2014). Nationally, mortality after surgical bypass decreased from 7% to 4% from the 1980s to mid-1990s (Slovut & Lipsitz, 2012). Secondarily, evidence from researches prove that endovascular therapy and surgical therapy have lowered the amputation rate over the last few years (**Error! Reference ource not found.**) (Balar et al., 2011). However, Regensteiner et al. claimed that even after revascularization procedures, patients continued to have a decline in walking parameters such as slower walking velocity, poorer standing balance, slower time to rise from a seated position, suggesting that there are continued end-organ effects (Regensteiner et al., 1993). Generally, invasive technique can be effective with proper Medicare and the costs of revascularization for patients who are at risk for amputation, as well as the costs of the amputation procedure itself, remain uncertain. These costs vary significantly according to the type of treatments patients receive (Goodney et al., 2014)

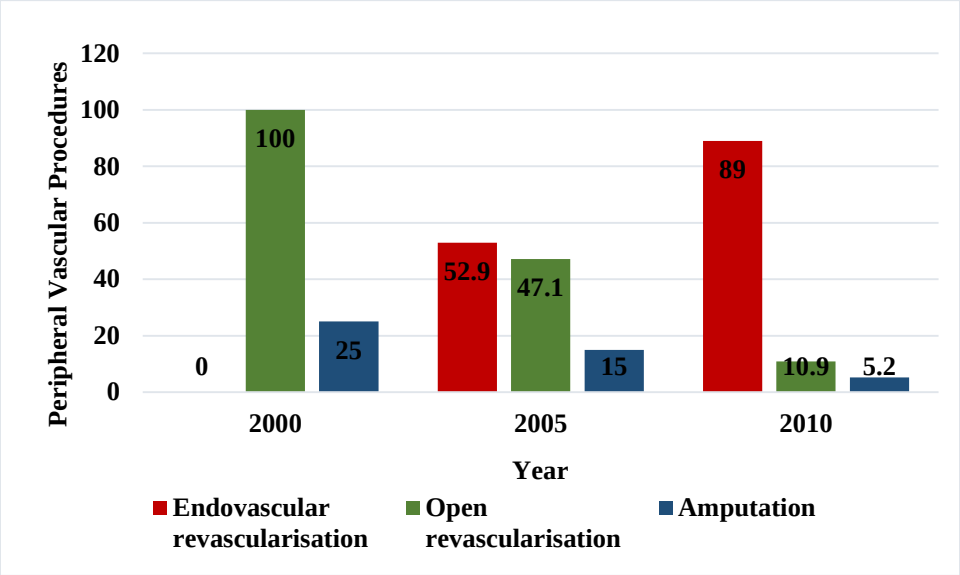

Figure 2.7 Revascularization techniques reduced the amputation over the years. Modified from (Balar et al., 2011)

ABI has been found to be not effective in detecting progression of atherosclerosis, as shown in a study conducted by McLafferty and colleagues. Using catheter angiography and duplex ultrasound imaging, the authors also found that imaging studies are superior to ABPI to monitor the progression of peripheral vascular diseases (McLafferty et al., 1997). Supported by other researchers, Hirsch and colleagues claim that clinicians who screen patients for peripheral arterial disease on the basis of finding a complaint of intermittent claudication will miss up to 90% of high-risk patients with the disease (Hirsch et al., 2006; A. T. Hirsch, S. L. Halverson, et al., 2001). Similarly, Criqui and colleagues claim that a history of intermittent claudication underestimates the presence of peripheral arterial disease by a factor of two to five (M. H. Criqui et al., 1985). Anatomic studies like, duplexsonography, magnetic resonance angiogram, and contrast angiography are the few methods for those patients in whom anatomical localization of stenosis or occlusions is important and revascularization is reflected in the data (American Diabetes, 2003).

2.6 ELEMENTAL CONCENTRATION

2.6.1 Electron microscope and its advancement

Over the last few decades, electron microscopy was engaged as an effective instrument in examining the cell structure (Kuto et al., 1982), study of cell function, and investigating metabolic diseases (Aleu & Afifi, 1964; Bacaner et al., 1973). In a study conducted by Van Breemen, electron microscopy was used to study the detailed morphological changes in the dystrophic muscle. Electron microscopy was used to investigate the ultra-structural alterations in muscle during dystrophic degeneration, with their possible correlation with dysfunction (Van Breemen, 1960). Muscular dystrophy, a myogenic metabolic disease characterized by degeneration of skeletal muscle fibers as the disease progress. From this study, detailed morphological modifications between normal and muscular dystrophic muscles including early alterations in the sarcoplasmic reticulum, mitochondria, vacuoles, and interfiberillar spaces in fibers were revealed (Van Breemen, 1960). In 1966, Gauthier and Padykula used electron microscopy to make note of heterogeneity that exists in mitochondria within the diaphragm and knowledge of muscle mitochondrial morphologies (Gauther & Padykula, 1966). The orientation of the myofibril, thick & thin filaments of squid muscles and obliquely striated organization of funnel retractor muscles are demonstrated with transmission electron microscope (Rosenbluth et al., 2010). All these studies helped in detailed study of the ultrastructure of the samples. Moreover, scanning electron microscope along with X-ray microanalysis allows the determination of chemical elements (Bacaner et al., 1973) and the sample spectrum with peaks (Figure 2.8).

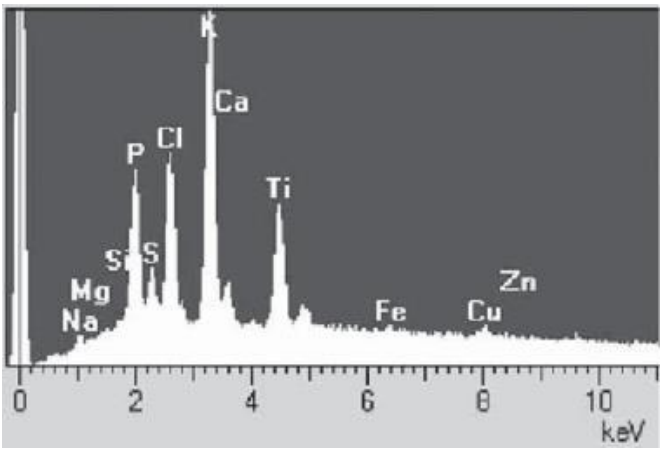

Figure 2.8 Example of X-ray spectrum with characteristic peaks.
Courtesy of (Godfried et al., 2007).

Maunder et al. compared the elemental concentration of specific subcellular organelles in normal and diseased (Duchenne muscular dystrophy) human muscle (Maunder et al., 1977). They proposed X-ray microanalysis as a sensitive method in correlating the element concentration with ultrastructure changes and to obtain chemical information without destroying the cell structure. The study focused on the elemental concentration of myonuclei and interstitial cell nuclei of control and diseased patients. It exposed the elemental distribution for phosphorous, chlorine, sulfur and calcium. The authors compared experimental data of intracellular calcium to phosphorous ratio of the diseased muscle (Figure 2.9) to a known ratio from previous studies. The study revealed age and biopsy technique did not appear to influence the concentration ratio between control and diseased muscle (Maunder et al., 1977).

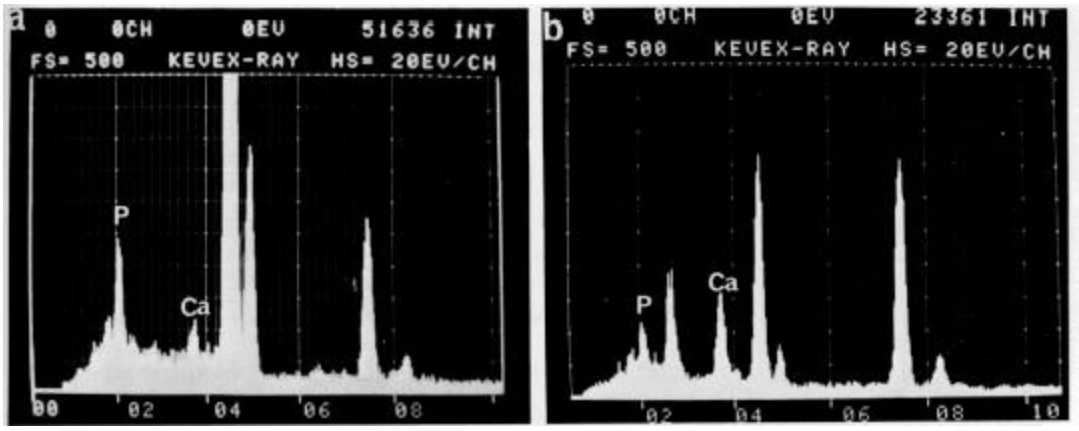

Figure 2.9 Spectra of myonuclei of (a) normal muscle and (b) diseased (Duchenne muscular dystrophy) muscle. Source: (Maunder et al., 1977)

Further, in a study conducted by Wroblewski et al. the elemental composition of different human muscles such as slow twitch and fast twitch muscles were analyzed using X-ray microanalysis (Figure 2.10). Elements such as phosphorous, sulfur, sodium, and chlorine were analyzed and was worthwhile in correlating the morphological and chemical parameters among the two muscle types (Wroblewski et al., 1978).

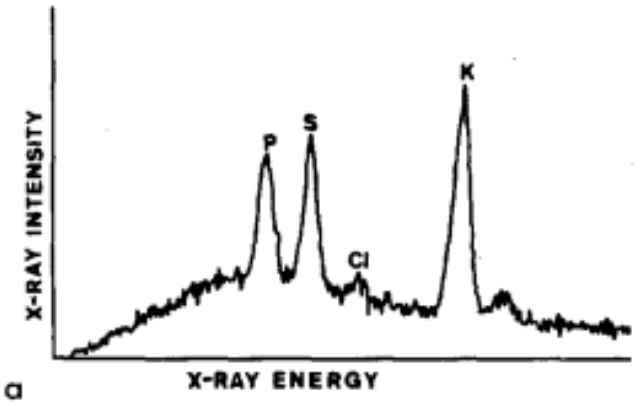

Figure 2.10 X-ray spectrum from muscle fiber with its characteristic peaks.
Source: (Wroblewski et al., 1978)

The study revealed the concentration of sulfur and phosphorous was differing between slow and fast twitch muscles and the concentration of chlorine and potassium does not vary

among the muscle types. The phosphorous concentration was slightly higher and is of type-I muscle due to the higher content of phospholipids in sarcotubular system. Thus, with the help of X-ray microanalysis technique, they were able to characterize the histochemical features of the muscle tissue sample (Wroblewski et al., 1978).

Later in 1987, Wroblewski et al. studied the elemental concentration of human muscle fibers before and after a knee surgery followed by immobilization. The study quantified the changes in intracellular chemical concentration which includes chlorine, sodium, potassium, phosphorous, and sulfur (Wroblewski et al., 1987). It was observed that the chlorine concentration was gradually increasing after the surgery and the insight was suspected to be surgery, muscle inactivity, or hemorrhagic shock. Hence, the study helped in analyzing the elemental concentration of human skeletal muscle before and after the knee surgery and stated the fact behind those alterations.

2.6.2 SEM & EDS, a high resolution technique

Energy dispersive X-ray spectroscopy (EDS) analysis, a successful analytical detection technique for elements and its composition in tissues (Humble et al., 2003; Jonas et al., 2001; Kametani & Nagata, 2006). SEM along with EDS utilizes an electron beam to relocate electrons from their higher energy levels and occupies the position vacated by an ejected inner shell electron. The elements are identified based on the atoms of each element release X-rays with unique amounts of energy (Patri et al., 2009). Furthermore, the X-ray spectral uses pseudo-colors to depict the two-dimensional spatial distribution which in turn can be used to identify the chemical composition. This type of elemental analysis offers more conclusive evidence of particle identity than other analytical methods (Patri et al., 2009).

2.6.2.1 Elemental Mapping of cardiac tissue

EDS associated with scanning electron microscopes has a potential applicability to the spatial mapping and evaluation of the relative distribution of chemical elements in biological tissues, including cardiac tissue (Patri et al., 2009). In a study conducted by Novaes et al. the tissue electrolytes such as calcium, sodium, magnesium, and potassium are determined as these are the critical elements in determining the contractile property performance in rat muscles (Novaes et al.). The study revealed the elemental map of cardiac tissue and found the differences between normal (Figure 2.11) and infected myocardium (Figure 2.12). These data suggest for further perspectives concerning the role of these minerals in functioning and heart structure in rats (Novaes et al., 2013).

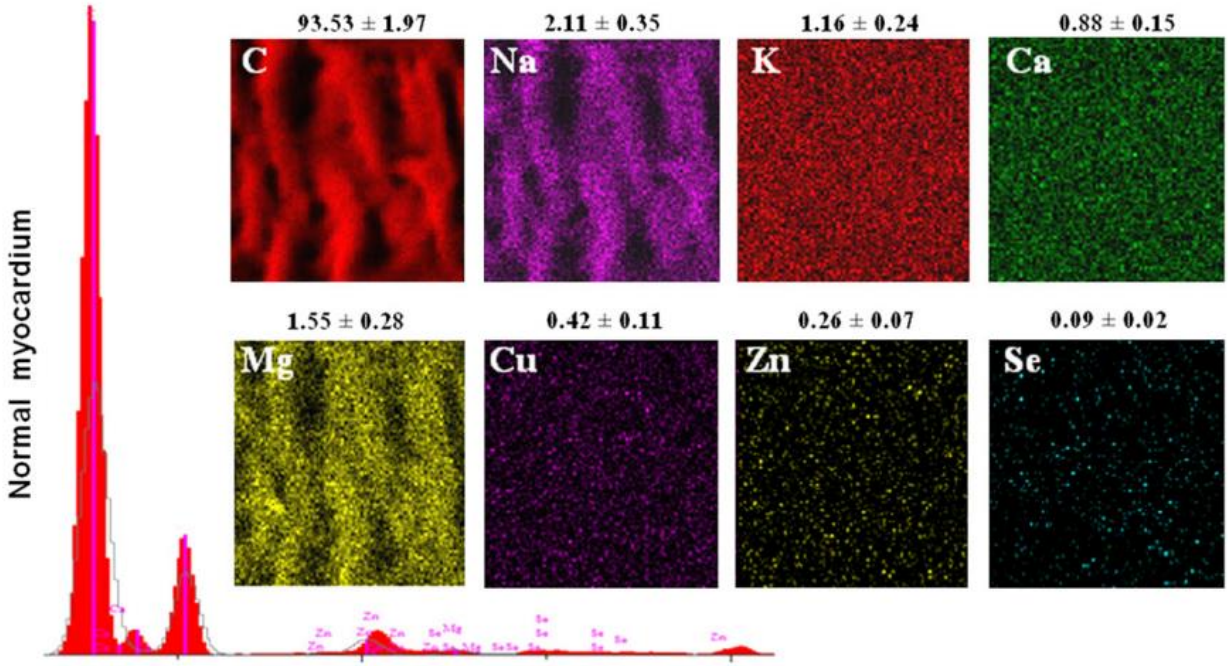

Figure 2.11 Elemental map of normal cardiac tissue. Image Courtesy: (Novaes et al., 2013)

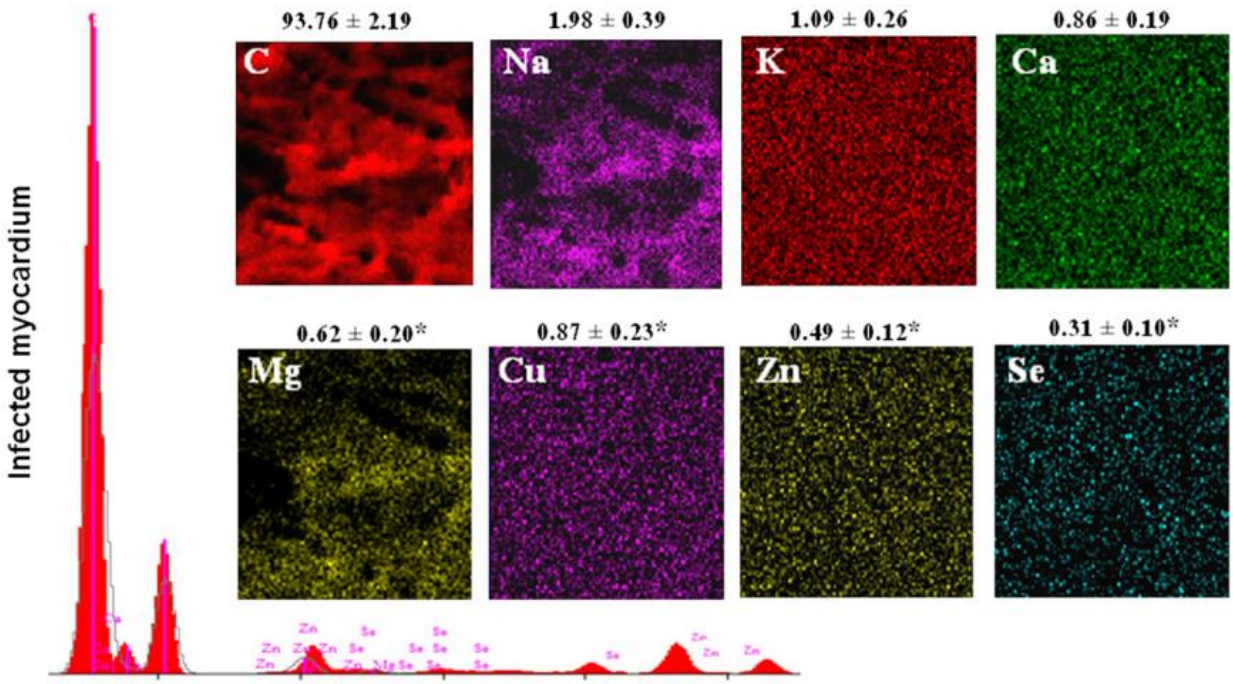

Figure 2.12 Elemental map of infected cardiac tissue. Image Courtesy: (Novaes et al., 2013)

In another study, SEM-EDS helped in detailed topographical microstructure, elemental distribution, and chemical composition which provides the detailed information on human cardiac calculi and spatial distribution of the elements (Chang et al., 2014). The study revealed the significant composition of cardiac calculi are cholesterol and calcium hydroxyapatite. By SEM-EDS analysis, the elemental composition of cholesterol was discovered to be major concentration of C, minor concentration of N, O, trace amount of P, Ca and calcium hydroxyapatite are discovered to rich area exhibited greater amounts of C, O, P, and Ca as well as trace amounts of N, Na, Mg, and Al (Chang et al., 2014) (Figure 2.13). Apparently, the elemental mapping of the calcified area also determined and reveals the elemental distribution (Figure 2.14).

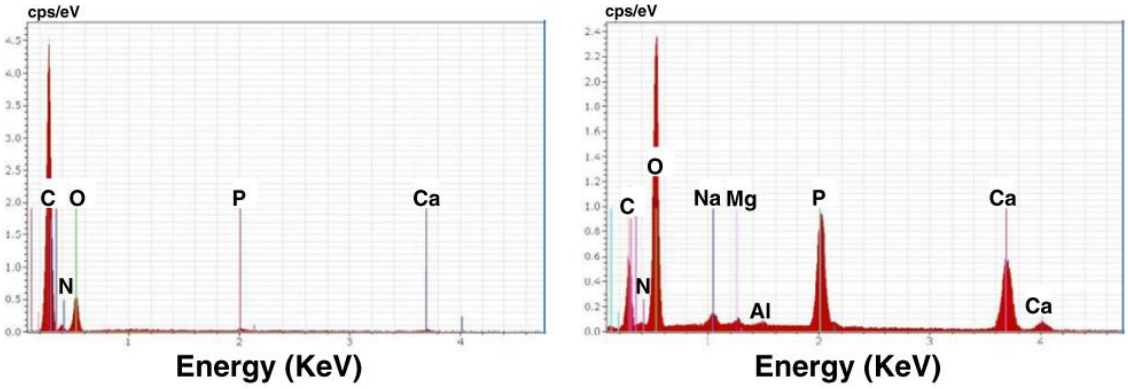

Figure 2.13.SEM-EDS spectrum of a) cholesterol and b) calcium hydroxyapatite from the cardiac calculi. Source: (Chang et al., 2014)

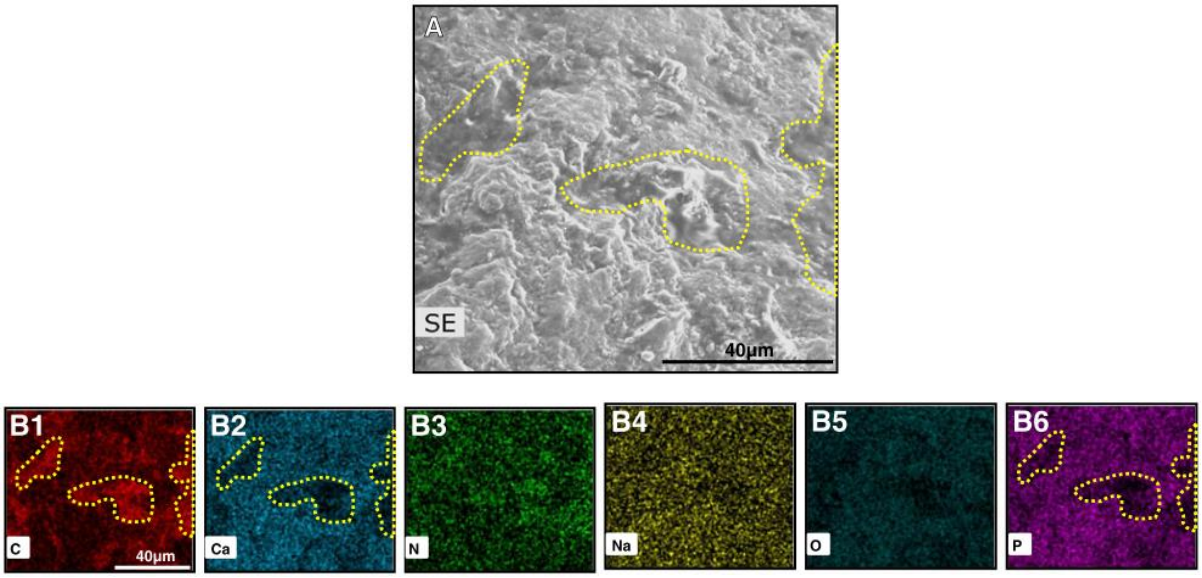

Figure 2.14 SEM-EDS elemental mapping of cardiac calculi. Source: (Chang et al., 2014)

Although the pathology and lithogenesis of cardiac muscles are not considered in this paper, it paves a way to analyze the elements present in the cardiac calculi and based on which the treatment can be improved by the physicians to nullify the effect of calculi.

2.6.2.2 Evaluation of concentration by EDS method:

Using energy dispersive X-ray spectroscopy, the skeletal disorder associated with reduced bone mineral density variations are studied. In this study, the concentration of calcium and phosphorus at different sites of normal and diseased (Osteoporosis) bones are evaluated by

using two spectroscopic techniques: Auger electron spectroscopy (AES) and energy-dispersive X-ray spectroscopy (Kourkoumelis et al., 2012). The study revealed the EDS spectral profile of Ca and P concentration at different sites of normal and diseased bones Figure 2.15.

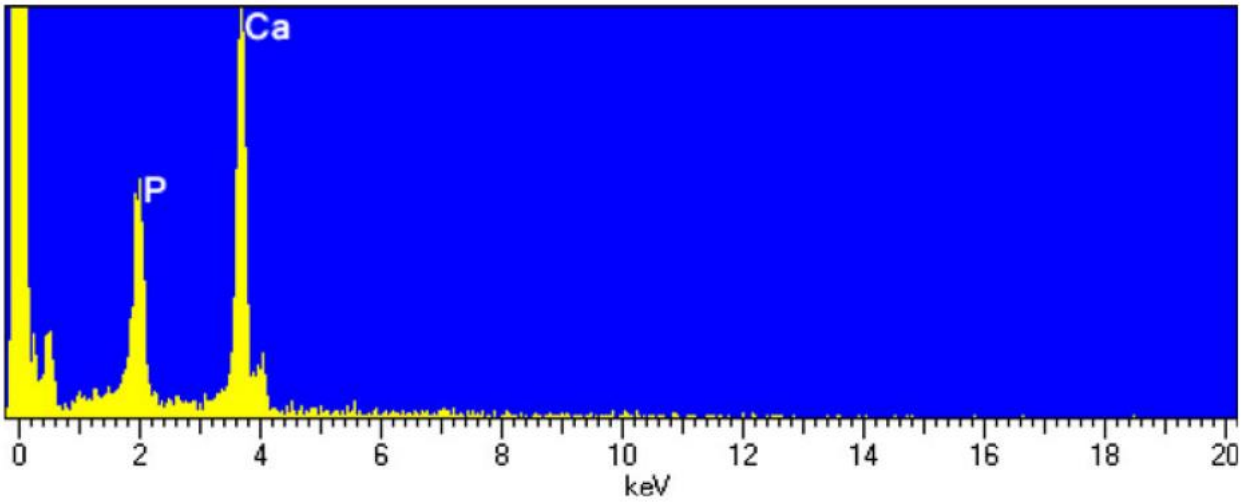

Figure 2.15 EDS spectral profile of normal and diseased rabbit bones.
Image courtesy: (Kourkoumelis et al., 2012)

The study presented the Ca/P ratio is lowered in diseased bone and leads to reduced bone loss. The outcomes recommend that the bone quality and bone density, assumes an essential part in bone strength and is unequivocally related to the Ca/P proportion. A curiosity of this study was the presentation of two analytical spectroscopic systems that are suitable explanatory routines for evaluating the fundamental components containing bone mineral in the structure of Ca/P proportion.

2.7 SPECTRAL BIOMARKERS

2.7.1 Fourier Transform Infrared (FTIR) Spectroscopy

For more than decades, infrared spectroscopy (IR) has been widely used in the study of material analysis. FTIR spectroscopy is a powerful method of IR spectroscopy widely used in biological sciences to study the biochemical composition of microscopic tissues (Kong et al.,

2010). In this method, IR radiation is passed through a sample and the spectrum is produced by absorbing and transmitting the infrared radiation through the sample. The resulting spectrum represents the molecular absorption and transmission, creating a molecular fingerprint spectrum of the sample (Figure 2.16). The absorption spectrum of the fingerprint region corresponds to frequencies of vibrations between the bonds of the atoms making up the material and produce a unique combination of atoms and no two materials produce the same spectrum. For this exceptional reason, FTIR was widely used to identify different kind of materials.

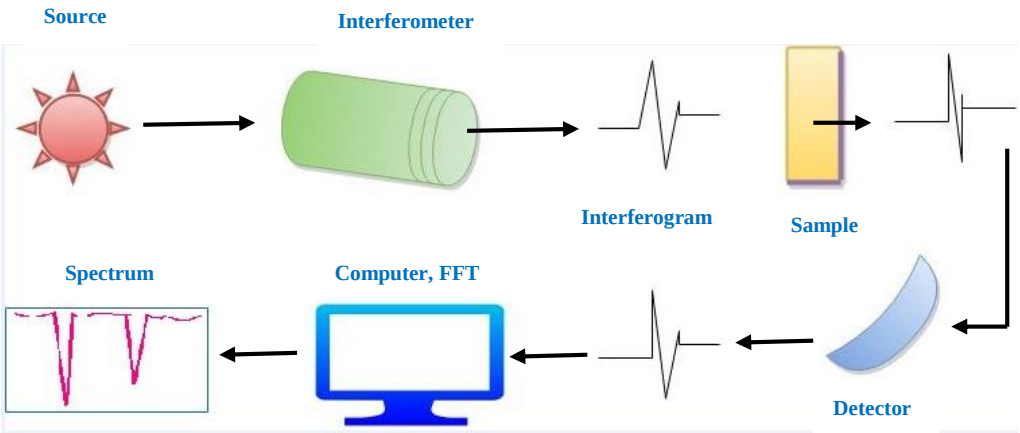

Figure 2.16 Schematic representation of FTIR system. Modified from: (http://epic.ms.northwestern.edu/KeckII/ftir1.asp)

2.7.2 FTIR application on biological tissues

FTIR is a non-invasive and emerging technique for the biochemical analysis of tissues and cellular materials. It provides objective information on the extensive biochemistry of a cell or tissue sample in many research areas (Zohdi et al., 2015). A reliable diagnostic tool for investigating changes in the biochemical constituents, protein structure (Palaniappan & Pramod, 2010), damaged tissues (C. G. Cheng et al., 2004), and toxicological studies (Sivakumar et al.,

2014). FTIR spectroscopy has emerged as a valuable tool for the characterization of protein structure with great accuracy (Surewicz et al., 1993; Susi & Byler, 1986).

In several studies, researchers employed FTIR as a promising technique for clinical diagnosis and investigated the physical and chemical features of the tissue samples. In a study conducted by Morato et al, the changes in the absorption spectrum were used to identify the biological phenomena, including cell death and proliferation in normal or diseased cells (Morato et al., 2013). The absorption spectrum of the healthy tissue of mice is shown (Figure 2.17).

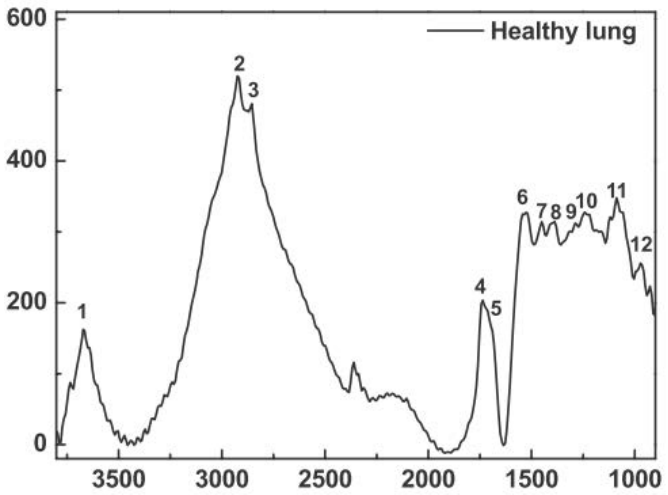

Figure 2.17 FTIR absorption spectrum of healthy lung tissue from mice. Image Courtesy: (Morato et al., 2013)

The changes in the absorption peak (Figure 2.18) in the infected mice are identified in the following groups: 1683cm^{-1} - amide I, 1455cm^{-1} - CH3, 1234cm^{-1} - asymmetric phosphate and 972cm^{-1} C–C; C–O groups (Morato et al., 2013).

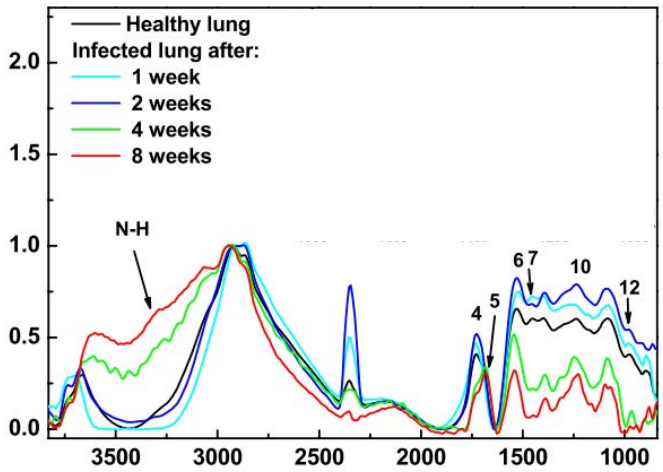

Figure 2.18 FTIR spectrum of infected mice lung tissue after 1, 2, 4, and 8 weeks. Image Courtesy: (Morato et al., 2013)

Thus, the study revealed the structural changes that varied according to the time period and were analyzed. The changes in the absorption bands of different chemical groups (amide I, amide II, phosphate groups, amine groups) are the result of morphological changes (Morato et al., 2013).

Krimm & Bandekar studied in detail the vibrational spectroscopy and provided a baseline for the conformation of peptides, polypeptides, and proteins. The ranges of amide bands for the conformation of the different types of proteins are shown. The study showed that amide II, a sensitive band is a mixture of N-H bond and from the C-N stretching bond. Amide III and IV bands are very complex in nature resulting from a mixture of so many coordinate displacements (Krimm & Bandekar, 1986).

In another study conducted by Ebenstein, et al., FTIR was used to study the nanomechanical properties of calcification, fibrous, and hematoma from atherosclerotic plaques. FTIR spectroscopy was used to quantify the amount of mineral and lipid in each tissue region (Ebenstein et al., 2009). Human carotid bifurcation plaque samples were collected and used for

FTIR-ATR (Attenuated Total Reflectance) analysis. Spectrum of interferograms was obtained with the spectrum range of 4000 cm^{-1} to 600 cm^{-1} (lipid, matrix, and mineral) (Ebenstein et al., 2009).

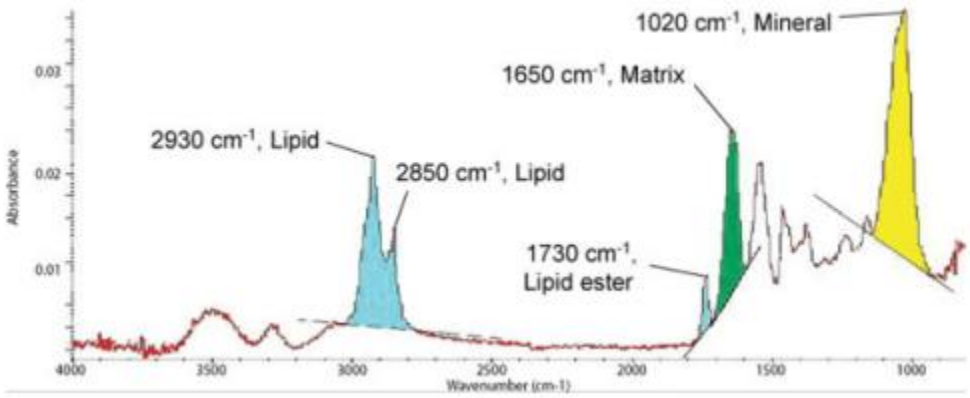

Figure 2.19 FTIR spectrum depicting the composition of plaque (mineral, lipids, and matrix). Image Coutesy: (Ebenstein et al., 2009).

The range 1720–1585 cm^{-1} was defined as the absorbance peak of amide I, a region where collagen shows strong absorbance. The mineral area was defined as the absorbance peak of phosphate at 1180–900 cm^{-1}. Finally, 2972-2845 cm^{-1} area was defined as the absorbance peak of CH2 symmetric and asymmetric stretch peaks, a region where lipids show absorbance, combined with the area under the lipid ester peak at 1730 cm^{-1}. Consequently, studies (Morato et al., 2013; Palaniappan & Pramod, 2010; Sivakumar et al., 2014; Zohdi et al., 2015) proved FTIR as the fundamental principle of molecular spectroscopic method to reveal the spectral information of protein structure, amide groups, peptide carbonyl groups, and alterations in DNA structure.

2.8 SUMMARY

PAD, a type of cardiovascular disease that presents major public health concern worldwide, which also increases the rate of morbidity and mortality (Heneghan & Sultan, 2008). PAD becomes more likely as one gets older and generally expresses symptoms such as cramping, pain, or tiredness in the lower extremities (Norgren et al., 2007).

The Ankle brachial index (ABI) is a simple and noninvasive test to diagnose PAD, practiced by physicians by measuring the ratio of systolic blood pressure in the ankle to that in the arm. Current methods have limitations in monitoring progression and regression of PAD because it is necessary to measure both abnormal blood flow and the atherosclerotic effects on the skeletal muscle in order to fully observe the degree of muscle damage or repair. There is a fundamental gap in the quantification of muscle recuperation/degeneration before and after vascular reperfusion surgeries. Hence, there is a need to investigate the effects of PAD on muscles at a cellular level. The changes leads to complex problem and reuslts in reduced blood flow, altered metabolic processes, skeletal muscle degeneration, and oxidative damages (Stewart et al., 2002).

In this thesis, PAD muscle cell degeneration is characterized by measuring the changes in elemental concentrations, which can be used to correlate with clinical diagnosis of PAD. These findings may aid in providing a foundation for the development of specialized preventive and rehabilitative treatment plans by providing new targets for treatment based on the underlying altered elemental concentrations. In addition to the elemental changes, this thesis with the help of FTIR, a vibrational spectroscopic method also used to analyze the altered spectral biomarkers in the muscles, which reflects the critical biochemical changes in the diseased muscles.

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3. ANALYSIS OF ELEMENTAL CONCENTRATION IN ISCHEMIC MUSCLE OF PATIENTS WITH PERIPHERAL ARTERY DISEASE, USING ENERGY DISPERSIVE X-RAY SPECTROSCOPY

3.1 ABSTRACT

Peripheral arterial disease (PAD) is a vascular occlusive disease of the lower extremities caused by atherosclerosis. The disease causes lower extremity pain because of a decreased blood supply to the limbs and, if allowed to progress, eventually results in limb loss. The ankle brachial index (ABI) is a standard PAD diagnostic test; however, it can only identify reduced blood flow (due to blockages in the arteries) based on blood pressure differences. The early signs of PAD manifest themselves not only in physical form but at a biochemical level as well, so there is a need to measure both the blood flow and the effects of compromised blood flow on the skeletal muscle cells. The aim of this research is to compare the changes in elemental compositions such as calcium (Ca), sodium (Na), potassium (K), magnesium (Mg), and sulfur (S) between control, claudicating, and ischemic muscle tissue. The gastrocnemius biopsies from three subjects including one control (person without PAD), one claudicating patient ($0.4<ABI<0.9$) and one critical limb ischemia patient ($ABI<0.4$) were evaluated. Using a scanning electron microscope (SEM) and energy dispersive X-ray spectroscopy (EDS), differences in elemental concentrations between control, claudicating, and PAD muscle samples were quantified. In total, 15 myofibers were analyzed, 5 from each tissue specimen. An analysis of variance (ANOVA) was performed to identify significant differences in muscle elemental concentrations. SEM and EDS were able to characterize the changes in elemental concentrations of PAD muscle. An analysis of variance (ANOVA) was performed and revealed significant differences ($p <0.05$) in elemental concentrations for sodium, potassium, calcium, magnesium, and sulfur between control, claudicating and critical limb ischemic muscle. These findings may aid in the development of specialized preventive and rehabilitative treatment plans by providing new therapeutic targets

and by quantifying the different stages in the disease process and muscle damage. This will also be a stepping stone toward the development of the improved monitoring practice of muscle repair in response to treatment.

3.2 INTRODUCTION

Peripheral arterial disease is a chronic disease caused by atherosclerosis, affecting more than 8 million lives in United States (Steffen et al., 2008). PAD can result in the narrowing or blockage of major arteries that flow into the lower extremities (Figure 3.1). If untreated, the severity of the disease can even lead to limb loss (Norgren et al., 2007). The most identifiable symptom of PAD is intermittent claudication (IC), which is characterized by lower extremity pain that is exhibited during mild exercise such as walking, but resolves after rest (Rolando et al., 2009). Patients with PAD may not walk fast due to reduced muscle force and may even sometime fail to mention their symptoms to their physician as they may have other collateral arterial channels to tolerate their arterial obstruction (Aronow, 2010).

The Ankle brachial index (ABI) is a simple and noninvasive test to diagnose PAD, performed by measuring the ratio of systolic blood pressure in the ankle to that in the arm. Current methods have limitations in monitoring progression and regression of PAD because it is necessary to measure both abnormal blood flow and the atherosclerotic effects on the skeletal muscle in order to fully observe the degree of muscle damage or repair. There is a fundamental gap in the quantification of muscle recuperation/degeneration before and after vascular reperfusion surgeries. Hence, there is a need to investigate the effects of PAD on muscles at a cellular level. Cluff et al. studied morphological parameters at the level of myofibers including area, roundness, perimeter, equivalent diameter, major and minor axes, solidity and fiber density of muscles classified as control, claudicating, and critical limb ischemia (CLI) samples (Cluff et al., 2013). Studies revealed the role of ionic concentration changes during cell injury, tenotomy

in rat muscles, and after surgeries. The studies gave strong evidence that the elemental fluctuations are due to the disturbed homeostasis of intracellular cells (Trump et al., 1979; Wroblewski et al., 1987; Wroblewski et al., 1978). Thus, the effects of PAD on muscle functions, such as the change of elemental composition in myofibers, can be studied by using SEM-EDS. By using previously prepared tissue samples, high resolution topographic images were obtained and processed to reveal the elemental compositions for control, claudicating and CLI muscle tissue. The elements (Ca, Na, K, Mg, and S) were selected after careful examination of literature on how the composition of certain elements will differ between control, claudicating, and CLI muscle (Novaes et al., 2013). A potential benefit of this research would be the creation of a quantitative method for analyzing damage to ischemic muscles at a cellular level. A more accurate model to be used clinically based on elemental composition to diagnose PAD. By monitoring the progression of PAD in this manner, it may helpful in developing specialized treatment plans based on identified elemental fluctuations. As a result, different stages of the disease can be approached with personalized treatment methods specific for the person and progression of the disease.

3.3 METHODS AND MATERIAL

3.3.1 Tissue collection

Muscle tissue biopsies including the demographics for three patients, each with three stages of PAD (control, claudicating, and CLI), were obtained. The previously prepared tissue samples were acquired from an ongoing study, “Muscle evaluation in vascular disease.” The study was approved by the University of Nebraska Medical Center "IRB 509-01" on May 21, 2009 and was made available for this protocol as anonymous specimens. The muscle tissue specimens were biopsies of the gastrocnemius, extracted with a Bergstrom needle. Tissue samples were fixed in methacarn, and embedded in paraffin. Two 4 µm cross sections, taken approximately 40 µm

apart, were mounted on glass slides for subsequent scanning electron microscopy image acquisition. Prior to collecting scanning electron microscopy images, the specimens were deparaffinized with xylene and a series of ethanol washes and allowed to air dry.

3.3.2 SEM-EDS Data collection

Using a scanning electron microscope (Figure 3.2) with energy dispersive X-ray spectrometry, an elemental microanalysis technique extensively applied in the field of engineering and biological sciences, for analyzing and quantifying all the elements, with the exception of H, He and Li (Newbury & Ritchie, 2013). The existence and percentage of the ions in muscle tissues were examined by high resolution SEM (Carl Zeiss Sigma VP Field Emission Scanning Electron Microscope), in lens detector, variable pressure system, and energy dispersive x-ray functions to characterize the surface of the specimen. The number of X-ray counts produced from the sample were compared to the number of known X-ray counts for specific elements of interest, and from this the mass fraction of the element in the sample was derived. X-rays are generated from the electron beam focusing on the desired area. Imaging and elemental analysis is carried out in a specimen chamber filled with nitrogen gas, at a variable pressure of 60 Pascal with 17.5KV as working voltage. For ion assessment, the Point & ID feature was used to specify the rectangular region for x-ray acquisition to measure the concentration of intracellular regions of the muscle sample, with the percentage of detected elements representing an atomic percentage. The atomic percentages for, Ca, Na, K, Mg, and S were compared between the three levels of muscle biopsies. Figure 3.3 presents an example spectral profile for the selected myofiber.

3.4 RESULTS

SEM-EDS revealed the spectral profile for the selected myofiber (Figure 3.3).Using Design of expert 7.1.6 analysis software, a statistical ANOVA revealed significant differences in

elemental concentrations for calcium (p=0.003), magnesium (p=0.0001) sulfur (p=0.004), potassium (p=0.0094), and sodium (p=0.0001) among control, claudicating and critical limb ischemic muscle samples (Figure 3.4). Scanning electron microscopy and EDS were able to characterize changes in the elemental concentration of PAD muscle, which correlated with a clinical diagnosis of PAD.

3.5 DISCUSSION

The results indicate decrease in intracellular magnesium, potassium, sodium, and calcium, which are the important mineral integrals to maintain cellular homeostasis. Researchers' proved presence and distribution of these elements influence the electrical properties of the cell by creating chemical concentration gradients (Gedrange et al., 2005). As an outcome, the specific changes noticed in the elements such as calcium, magnesium decrease and sulfur increase in CLI muscles are interesting and may offer insight into underlying muscle pathology which is a critical contributions from this study. Consequently, the significance of this findings may aid in the development of specialized preventive and rehabilitative treatment plans and also be a stepping stone toward the development of improved monitoring techniques for muscle repair.

In ischemic muscles, cell injury and necrosis are indicated by significant chemical fluctuations in the cells as the disease progresses (Maunder et al., 1977). Trump et al. demonstrated that in ischemic muscle cells there is a significant decrease in oxidative phosphorylation, which leads to a decline in available adenosine triphosphate (ATP) and altered diffusion of ions across the membrane (Trump et al., 1979). The physical changes in the muscle tissues are controlled by key elements such as potassium, sodium, magnesium, sulfur, and calcium. Potassium is a critical mineral that plays a key role in cell growth, muscle contraction, electrolyte homeostasis, and muscle anabolism (Pohl et al., 2013). Its importance is highlighted in the sodium potassium pump, or Na^+/K^+-ATPase. This functions to maintain the cells resting

membrane potential, thus affecting the membrane permeability to various ions. This potential difference of potassium across the cell membrane causes faster depolarization, which speeds the rate that the muscle fiber contracts (Gedrange et al., 2005). It also plays a significant role in regulating cell signaling pathways as well as controlling calcium concentrations inside the cell. Disruption of major homeostatic elements such as potassium can have devastating effects on the body (Pohl et al., 2013) . According to the data, there was a significant decrease in potassium concentration in patients with critical limb ischemia, known as hypokalemia. This causes muscle weakness due to the increased stimulus required to trigger action potentials in cells, a mechanism that is completely dependent on cell membrane potential (Pohl et al., 2013). In addition to the potassium deficiency, it was also observed the lower concentrations in magnesium, calcium and sodium. Coronary vasoconstriction caused by deficiency in magnesium causes oxygen levels to decline in skeletal muscles (Altura et al., 1993). A decrease in magnesium concentration impairs the Na^+/K^+-ATPase as well as calcium ion channels and Na^+/Ca^+ pumps in the cell membrane (Fischer & Giroux, 1987). Na^+/K^+-ATPase is driven by ATP in the form of a magnesium-ATP complex. In ischemic conditions, ATP is quickly used up in the cell in an attempt to establish chemical equilibrium and reverse the cellular damage. Because of the low concentrations of ATP, magnesium can no longer bind to its complex and leaves the cell, further disturbing the membrane potential and impairing the sodium potassium pump in the process. The result is a marked decrease in potassium concentration in the cell.

Magnesium is also known as a chemical antagonist to calcium, typically exhibiting an inversely proportional relationship to calcium (Greville & Lehmann, 1943). This intracellular-extracellular activity is one of the key factors for the generation of nerve and muscle action potentials (Gedrange et al., 2005). Interestingly, the data showed a decrease in calcium and sodium concentrations in ischemic patients instead of a steady increase (Murphy, 2008; Wallace

& McNally, 2009). One possible explanation for the calcium reduction, or handling, could be the actions of hydrogen sulfide (H_2S). Recently discovered and coined as the third known gasotransmitter, hydrogen sulfide has been the topic of many areas of research, including ischemic injury (Calvert, 2013). Such research indicates that hydrogen sulfide can have strong myoprotective effects when administered during reperfusion preconditioning (Nicholson & Calvert, 2010; Sodha et al., 2008). H_2S works both endogenously and exogenously; as an exogenous neuromodulator the gasotransmitter relaxes the smooth muscle cells of the arterial wall by increasing the lumen diameter, which increases blood flow to ischemic tissue. Endogenously hydrogen sulfide exhibits anti-apoptotic and anti-inflammatory properties, as well as the ability to normalize the high calcium concentrations through calcium handling. It does this by inducing the expression of protein kinase C (PKC) in the cell. The protein accelerates reuptake of calcium into the sarcoplasmic reticulum of the cell as well as its extrusion out of the cell through sodium calcium channels (Ting-Ting Pan et al., 2008). This transmitter is produced by 3 enzymes in the body: cystathionine β-synthase (CBS), cystathionine γ-lyase (CGL), and 3-mercaptopyruvate sulfur transferase (3MST). These control how much and when hydrogen sulfide is expressed in cells (Nicholson & Calvert, 2010).

The decrease in sodium concentration is unclear; however a decrease in abundance of sodium potassium pumps could be the primary cause (Kwon et al., 2000). Aldosterone is a key regulating hormone in sodium concentrations in the cell, but little research has been done on how it is affected by ischemic conditions (Graudal et al., 2011).Many studies observed that during simulated ischemia, ATP declines and anaerobic glycolysis increases the intracellular and extracellular pH to a state of acidosis, causing an increase in mitochondrial calcium. Sequentially, an increase in calcium will cause an increase in intracellular Na^+ through NHE exchanger (Murphy, 2008). However little is understood about the mechanisms through which

calcium is handled through hydrogen sulfides bio signaling pathway, such as how the expression of the key enzymes generating this gasotransmitter is affected by ischemic conditions or precise mechanisms of the chemical inside the cell. Also, less is known on how the capacity for calcium to be sequestered in the sarcoplasmic reticulum through calsequestrin is affected by ischemic conditions. This leads us to believe that further study needs to be performed on these key protein mechanisms in order to better understand the true nature of the effects of ischemia on homeostasis in cells. If one can elucidate these mechanisms, this could lead to new therapeutic targets for treatment of a large spectrum of ischemic vascular disorders.

3.6 CONCLUSION & FUTURE WORK

The findings from this research may reflect damage to the muscles ability to sequester calcium in the sarcoplasmic reticulum and the mechanisms through which calcium is handled through the hydrogen sulfide bio signaling pathway. At this phase, any conclusions drawn from the results are hypothetical. More data from different samples are required to confirm our initial findings and to establish the normal variation of elemental concentrations. However, the results provide unique information on the distribution of the elements and highlight interesting differences (calcium and magnesium) between healthy and ischemic muscles. To our knowledge, this is a new approach to clarify some of the processes, related to peripheral artery disease.

3.7 ACKNOWLEDGEMENT

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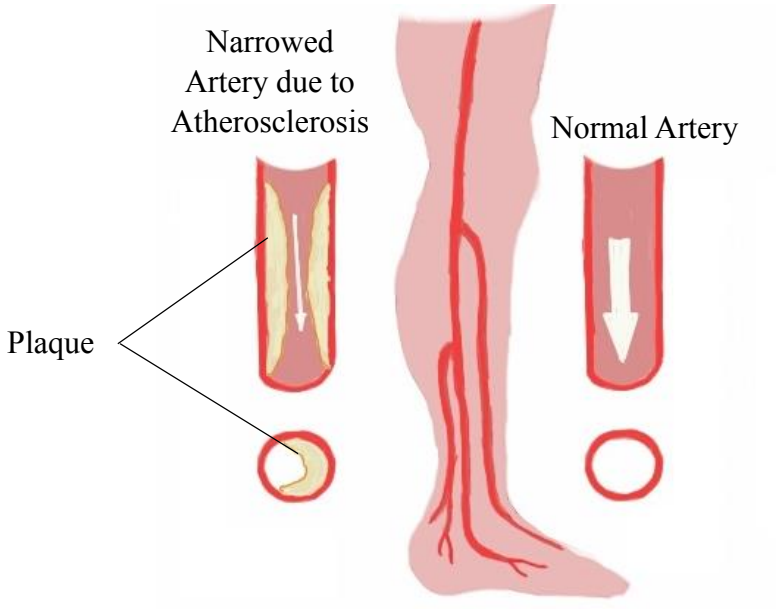

Figure 3.1 Atherosclerosis in arteries impedes blood flow

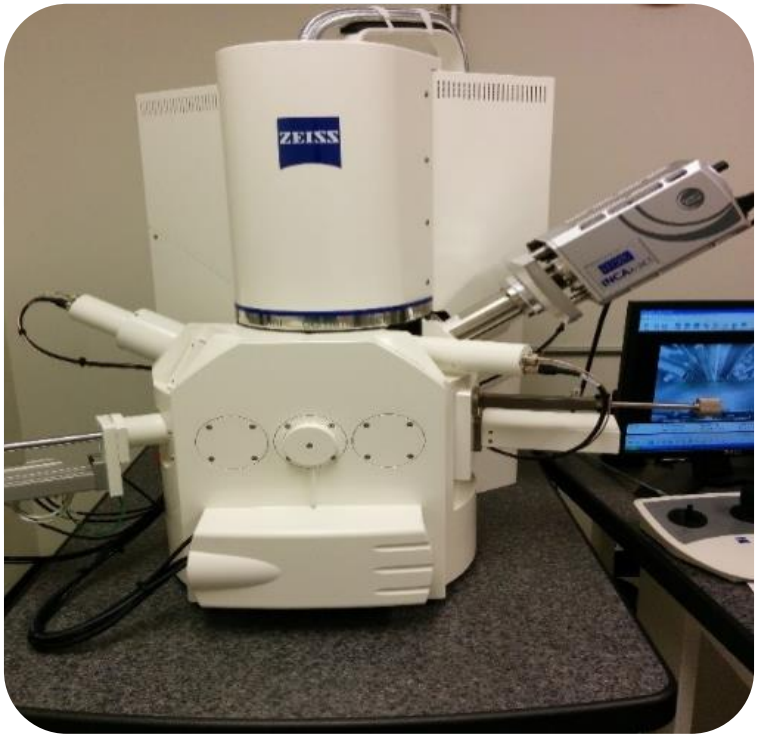

Figure 3.2. Scanning electron microscope

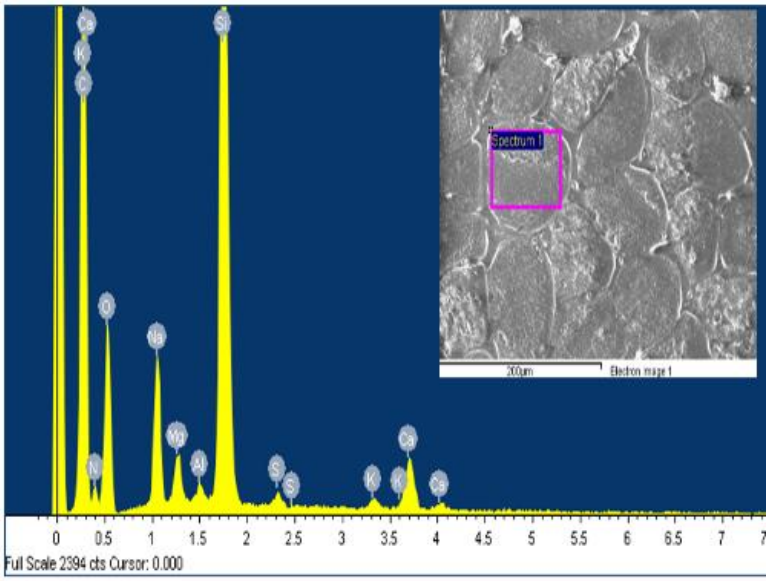

Figure 3.3. Energy dispersive X-ray spectral profile from a selected myofiber.

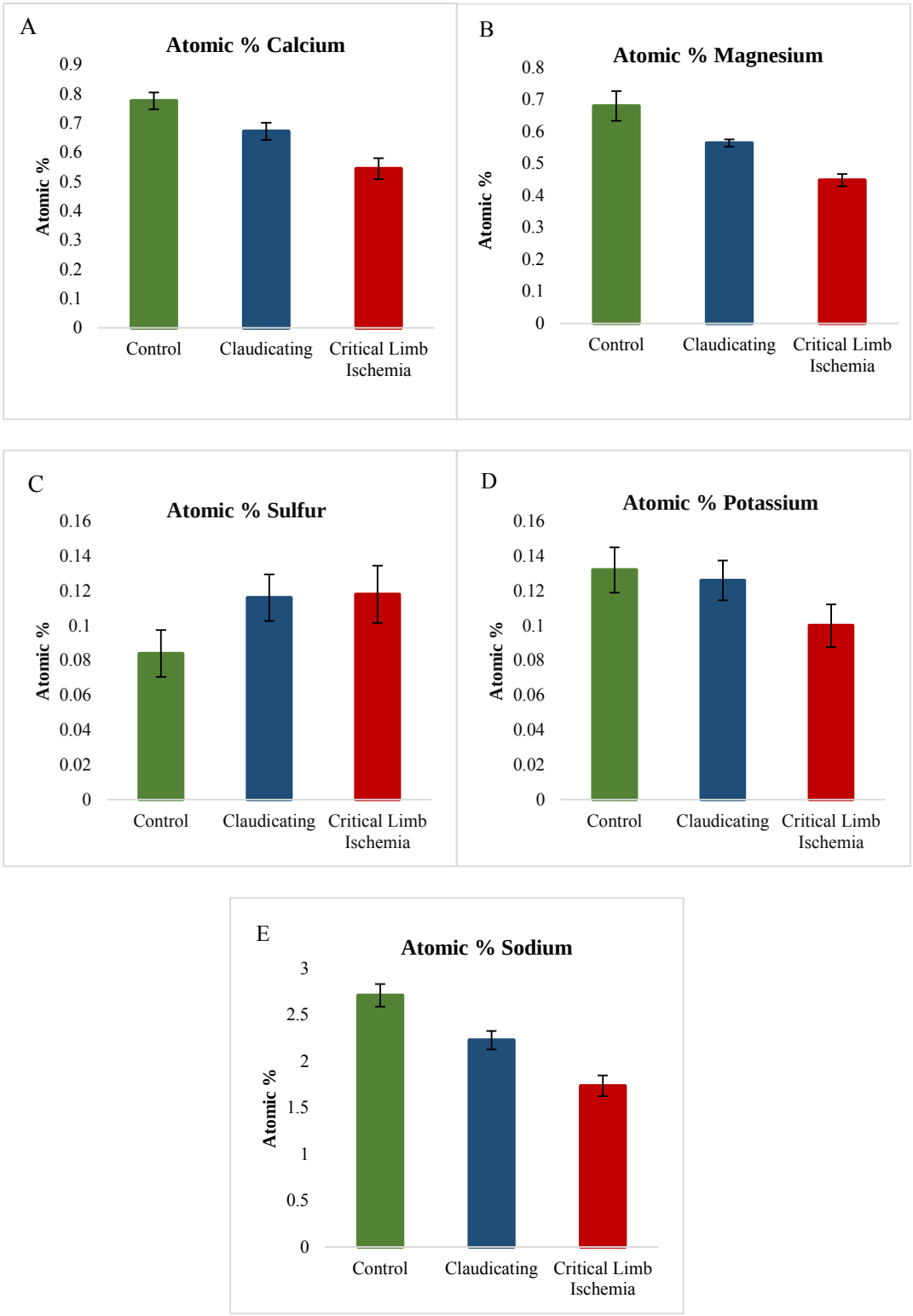

Figure 3.4 (A-E). Significant differences found in PAD muscle elemental concentration. A) Calcium, p=0.003 B) Magnesium, p=0.0001 C) Sulfur, p= 0.004 D) Potassium, p =0.0094 E) Sodium, p= 0.0001.

4. BIOMARKERS OF MUSCLE DAMAGE IN PATIENTS WITH PERIPHERAL ARTERY DISEASE

4.1 ABSTRACT

Peripheral artery disease (PAD), defined as atherosclerotic blockage (deposition of fat, plaque, cholesterol and hardening) of the arteries supplying blood to the legs affects approximately 8 million lives in the United States. This progressive disease leads to approximately 160,000 leg amputation per year and patients diagnosed with PAD have increased risk of morbidity and mortality. Intermittent claudication (IC), a typical symptom of PAD is defined as lower extremity pain induced calf ache and walking dysfunction, which is relieved after rest. Ankle brachial index (ABI) and other hemodynamic methods are the current diagnostic tools used detect PAD based on measuring the blood flow. However, skeletal muscles undergo unexpected changes in response to ischemic muscle damage. The injured muscles experience altered metabolic processes including variations in the levels of proteins, enzymes, lipids and nucleotides. Subsequently, the current biochemical tests being used cannot quantitatively evaluate the degree of muscle damage in affected areas, so the desire for new biomarkers makes this a very large field of research. In this study, we evaluated the hypothesis that Fourier Transform Infrared (FTIR) spectroscopy could be used to identify biochemical alterations in PAD muscle and characterize the severity of muscle damage. The muscle (gastrocnemius) biopsies were assessed from thirteen subjects including four control patients (person without PAD), five claudicating patients ($0.4<ABI<0.9$) and four critical limb ischemia patients ($ABI<0.4$). Using FTIR, the biochemical alterations were quantified between control, claudicating and PAD muscle samples. Statistical analysis of the data included an analysis of variance, and a partial least squares regression to identify significant differences in spectral peaks and correlate them with a clinical diagnosis. The spectral biomarkers revealed the significant

difference ($p<0.05$) between control, claudicating, and critical limb ischemia (CLI) in the finger print region of wavenumber (1200-1250 cm^{-1}). The identified spectral peaks are attributed to alterations in protein content, lipids, and phospholipid groups. The difference in identified signature peaks can discriminate the control from that of PAD muscle and correlate with the clinical presentation of the PAD patient. FTIR spectroscopy proves to be a technique that can yield novel spectral biomarkers that may complement the existing diagnosis and treatment monitoring methods for PAD.

4.2 INTRODUCTION

Peripheral artery disease (PAD) results in the impairment of blood flow due to atherosclerotic plaque buildup in the arterial walls (Schirmang et al., 2009) causing stenosis or obstruction of the lower limb arteries, the most widely recognized etiology (Flu et al., 2010). The blockages are due to the development of fat, plaque, and cholesterol on the walls, in turn interrupting the blood flow. Atherosclerosis is a complex process that involves endothelial dysfunction, lipid disturbances, platelet activation, thrombosis, oxidative stress, vascular smooth muscle activation, altered matrix metabolism, re-modelling and genetic factors (Faxon et al., 2004). The most classical symptom of PAD is intermittent claudication (IC), which is usually identified by muscle cramps, fatigue or pain in the lower legs; often the symptom location indicates the level of arterial involvement (Schirmang et al., 2009). The pain increases when patients try to walk, climb, or do work and subsides after few minutes of rest. Metabolic demands of the ischemic tissue, location of the affected artery, and the degree of fat deposition and size influences the presence of symptoms in PAD (Hills et al., 2009). The ankle-brachial index (ABI) is a simple and non-invasive tool to diagnose PAD (McLafferty et al., 1997). It is calculated by dividing the higher systolic blood pressure of each ankle artery by the higher systolic blood pressure of the upper limbs (McLafferty et al., 1997). Other diagnostic tests

include, pulse volume recording, segmental pressures, toe plethysmography, and transcutaneous oxygen measurement, which may provide more information beyond the ABI (McLafferty et al., 1997). Current systems have limitations in observing the atherosclerotic consequences of both the reduced blood flow and end organ damage of the skeletal muscle. At the molecular level of skeletal muscle damage due to disease progression, structural abnormalities show elemental differences (Busch et al., 1972; Cullen & Fulthorpe, 1975) and ionic differences, both of which could indicate possible biochemical defects (Maunder et al., 1977).

Fourier transform infrared (FTIR) spectroscopy allows acquisition of the biochemical information of molecular compositions, based on molecular bond vibrations (Baker et al., 2008). FTIR spectroscopy is a vibrational spectroscopic technique that uses infrared radiation to vibrate molecular bonds within the sample that absorbs it and exposes chemical information of the sample (Baker et al., 2008). FTIR spectroscopy is a simple analytical method for early and accurate differentiation of premalignant stages (Bogomolny et al., 2008). It is a reliable diagnostic tool for investigating changes in the biochemical constituents, protein structure (Palaniappan & Pramod, 2010) damaged tissues (Cheng et al., 2004), and toxicological studies (Sivakumar et al., 2014). FTIR exhibited spectral differences (in protein structure) between the normal and cancerous tissues of gastrointestinal tract (stomach, colon, and esophagus) (Peng et al., 1998), and has emerged as a useful tool for the characterization of protein structure with great accuracy (Surewicz et al., 1993; Susi & Byler, 1986).

The objective of this study was to identify the biochemical alterations in PAD muscle and characterize the severity of muscle damage. Using FTIR spectroscopy, the prepared tissue samples were used to reveal the spectral biomarker in the muscle samples. When comparing the spectral peaks between control, claudicating and CLI, significant differences ($p<0.05$) were identified in the fingerprint region of spectral peaks which are attributed to proteins, lipids, and

phospholipid groups. The recognized biochemical signatures can discriminate healthy muscle from PAD muscle and correlate with the clinical report of the PAD patient. FTIR spectroscopy provides spectral biomarkers of muscle damage, which offer a valuable insight into areas of research that need to be explored.

4.3 METHODS AND MATERIALS

4.3.1 Tissue preparation

Muscle tissues (gastrocnemius) samples (Figure 4.1) were collected from 13 patients consisting of 4 controls, 5 claudicating patients, and 4 critical limb ischemia (CLI) patients. The samples were fixed in methacarn, and embedded in paraffin. Two 4 µm cross sections, taken approximately 40 µm apart, were mounted on glass slides for FTIR analysis.

4.3.2 FTIR Data Collection

Fourier Transfer Infrared Spectroscopy (FTIR), a vibrational spectroscopic technique (Baker et al., 2008) used to identify the chemical functional groups. Pathologists used FTIR coupled with attenuated total reflectance (ATR) to reveal the histopathological evaluation of the cancer tissues (Dukor et al., 1998). FTIR spectral signatures were collected from the finger print region (1200-1250 cm^{-1}) and functional group region. The identified spectrums were used for data pre-processing.

4.3.3 Data Pre-processing

Data preprocessing of FTIR raw data included baseline correction and normalization. Matlab code was generated to perform baseline correction and normalization before proceeding to the data preprocessing, all raw ATR-FTIR spectra were graphed with Matlab (Figure 4.2). Statistic toolbox was used to perform baseline correction and normalization (Figure 4.3) in Matlab. Due to environmental disturbances, the baseline tends to shift from its original base.

Noise removal from signal is an essential component of the bio-signal processing and the need for accurate method is essential to obtain good results in order to interpret it (Sweeney et al., 2012). Removing noises will result in substantial improvement in the precision of prediction (Chen et al., 2004). Normalization was done in order to compare the results and understand the relation of each spectra to the total number of spectra. Most common approach to normalize is using the ratio of the peaks to the distance measured from the baseline (Azzalini et al., 2012). All the spectra were normalized using peak ratio value of (2000 cm^{-1}).

4.4 RESULTS & DISCUSSION

Significant differences ($p<0.05$) were found in the fingerprint and functional group regions at a number of spectral peaks, between 500-3000 cm^{-1} (Figure 4.4). Many of these signature peaks have been identified as certain molecular components of a cell. For example, peaks 1200-900 cm^{-1} range (Rehman & Bonfield, 1997) are associated with phosphate groups, whereas the region from 1500-1200 cm^{-1} is considered a mixed region, comprised of certain proteins, lipids, and other phosphate containing groups such as DNA (Baranska, 2013; Payne & Veis, 1988) . Any peaks at 1800-1500 cm^{-1} are strongly associated with proteins, with the amide group of a peptide bond being observed at 1650 cm^{-1} (Packer, 1994). Carbonyl groups are detected at 1750 cm^{-1} (Wang et al., 1993), and are strongly associated with ester bonds found in lipids, such as those comprising the cellular membrane. The significant differences ($p<0.05$) between the control, claudicating, and CLI were determined and represented in Box-whisker plot (Figure 4.5) Box-whisker plots revealed the most significant difference. These FTIR spectral biomarkers for muscle also correlated ($r=0.91$) with a clinical diagnosis of PAD.

The results indicated significant changes for a number of bands in the FTIR spectra; namely an increase in the peak representing phosphate groups, and a decrease in the peaks representing proteins, lipids, lipid esters, amides and DNA. These differences signify important

changes in the biochemical composition of the muscle cells as they sustain damage and eventually undergo apoptosis or necrosis, causing a concomitant change in the muscle pathology. Many of these changes are used to detect the presence of muscle damage or cardiovascular disease in affected individuals.

The FTIR analysis indicated a significant increase in intracellular phosphate levels with increasing muscle damage. This is an interesting and critical observation, considering the importance of phosphate in cellular health and metabolism. While the exact mechanism of this increase is currently unknown, there are some proposed causes for this. Recently, there has been research on the mechanisms of AMP kinase (AMP-K), and its role in phosphate regulation in the cell (Baird et al., 2012; Xiao et al., 2013). AMP-K uses complex mechanisms to activate or deactivate a large number of proteins in the cell, in order to regulate the usage of ATP. In the event of muscle damage, its expression and activity could reveal important mechanisms of muscle damage and cardiovascular diseases. It could regulate certain phosphate pathways such as creatine kinase or sphingosine-1 phosphate. It is known that creatine kinase (CK) levels are increased in the blood, a result of leakage from damaged muscle cells (Baird et al., 2012; Totsuka et al., 2002). This is so well established that CK tests were a standard in diagnosing cardiovascular diseases through muscle damage, until the focus on testing shifted to evaluating Troponin levels (Baird et al., 2012; Baoge et al., 2012; Friedman et al., 2003). The function of CK is to regulate phosphorylation of creatine, providing an acute supply of phosphate for use in ATP synthesis to provide the cell with energy. However, this accumulation of phosphate could ultimately be detrimental to a cell under ischemic conditions, where it needs a higher supply of ATP to attempt to maintain metabolic homeostasis. AMP-K could be deactivating creatine kinase in an attempt to retain more phosphate, therefore creating more ATP.

Sphingosine-1 phosphate is a biosignaling lipid, which serves an important role in muscle regeneration. Research has shown that this lipid increases regeneration by activating muscle satellite cells, which allows the damaged muscle to regenerate new cells (Donati et al., 2013). Part of the regulations that AMP-K govern may be responsible for providing phosphate to sustain sphingosine-1 phosphate synthesis to heal the surrounding damaged muscle, or to other phospholipids involved in muscle cell repair (Donati et al., 2013; Saiardi, 2012). The increased phosphate levels could also be due to bone resorption, which releases both phosphate and calcium into surrounding tissues. Bone resorption has been shown to be linked to cardiovascular diseases, though there has not been a lot of research done on how it affects muscle damage (Farhat & Cauley, 2008; Farhat et al., 2007).

Overall, there was a decrease in the total lipid content in the samples. This was to be expected, since part of the process of necrosis includes fragmentation of the lipid bilayer of the cell. However, there could be other reasons that a decrease in lipid content was observed. The oxidation of phospholipids could play an integral role in the total lipid decrease in muscle tissue, due to a multitude of reasons. Many phospholipids, when cleaved, produce fatty acids such as arachidonic acid. Arachidonic acid causes inflammation, which is increased in damaged muscle tissue. Sphingosine is also a component of many phospholipids; cleavage of these phospholipids would aid in the synthesis of sphingosine-1 phosphate, which would ultimately help regenerate lost muscle tissue. Intramyocellular lipid content decrease could also be a result of muscle damage. Little research has been done on INML in damaged muscle, but the extreme degree of oxidation occurring within the cell could potentially cause oxidation of these fats, or even jettison them from the cell (Powers & Jackson, 2008; Schrauwen-Hinderling et al., 2006; Schrauwen-Hinderling et al., 2003) , nor has much research has been done on the concentrations or activity of peroxidation enzymes such as lysosomes during chronic muscle injury. These could

be the key components in the regulation of lipid bilayer homeostasis in ischemic conditions, and warrant further research into the role they play in PAD.

The decrease in protein content is also expected, as it has been shown that there are increases in quite a few different muscle proteins in the blood serum, which is one of the primary methods for detecting the presence of muscle damage. Creatine kinase is one of those proteins, and was the standard for testing in hospitals until the mid-90s, where hospitals shifted to Troponin as a biomarker for detecting muscle damage. Along with Troponin, other muscle proteins constituting some of the most essential structures of the muscle cell, including actin, myosin, and desmin are detected in the serum content.

Research is currently focused on developing new biomarkers for more accurate and easier detection for muscle injuries, especially those induced by cardiovascular diseases such as PAD. However, not much research has been accomplished regarding the protein Calsequestrin (CSQ), a protein critical for proper calcium regulation in the sarcolemma of muscle cells. It has been shown that there is an intracellular decrease of this protein in damaged dystrophic muscles, but to our knowledge there is no literature regarding CSQ in skeletal muscle that have sustained ischemic injury (Doran et al., 2004). If this protein decreases in these muscle cells as well, the results could be used to correlate precise degrees of muscle damage by measuring calsequestrin activity, though further research is needed to be able to test this hypothesis. The spectra also indicates lower intensities for amide bonds, which is expected, as the amide bonds comprising the proteins that are being damaged would also decrease along with these compounds.

The decrease in phosphodiester bonds indicate a decrease in DNA content in the cell. Recent research has been focusing on the damage mitochondria sustain in damaged muscle, and the role they play in the overall health of these muscle cells (Bhat et al., 1999; Pipinos et al.,

2006). Significant damage to the mitochondria would elicit corresponding damage to mitochondrial DNA, which would be present in the FTIR spectra. Damage to the mitochondrial membranes and inner proteins would also be present in spectra; if the mitochondria lose their function the muscle cells would experience significant oxidative damage, and also further promote ATP conserving methods, such as those proposed earlier by AMP-K. It is also possible that the damage to muscle cell's DNA was incurred, as DNA fragmentation would be observed in the beginning stages of apoptosis.

This research is interesting that advocates further study into the biochemical mechanisms as it involve damages in ischemic muscles. While many of these have been discussed, more research needs to be performed to further elucidate the fundamental causes of ischemia induced muscle damage, as seen in PAD patients. FTIR is a powerful technique for imaging biological tissue, was able to provide critical insight into biomarkers of muscle damage and how it affects the tissues at a molecular level. Muscle cells are comprised of a vast number of complex mechanisms, many still unsure, that could be used to develop new therapies or tests for early diagnosis and more effective treatment for the disease.

4.5 CONCLUSION & FUTURE WORK

FTIR spectroscopy was able to characterize the secondary effects of PAD on the gastrocnemius muscle by identifying unique biochemical signatures of diseased PAD skeletal muscle. While the spectra do not immediately reveal the vast array of mechanisms or their degree of complexity in damaged muscle cells, they do offer valuable insight into areas of research that need to be explored, as well as play an integral part in methods for discovering new therapeutic targets for treating and diagnosing muscle damage in affected patients. The identified signatures can discriminate control spectra from PAD muscle tissue and correlate with the

clinical presentation of a PAD patient, providing the potential for novel spectral biomarkers that can complement existing diagnosis and treatment monitoring methods for PAD.

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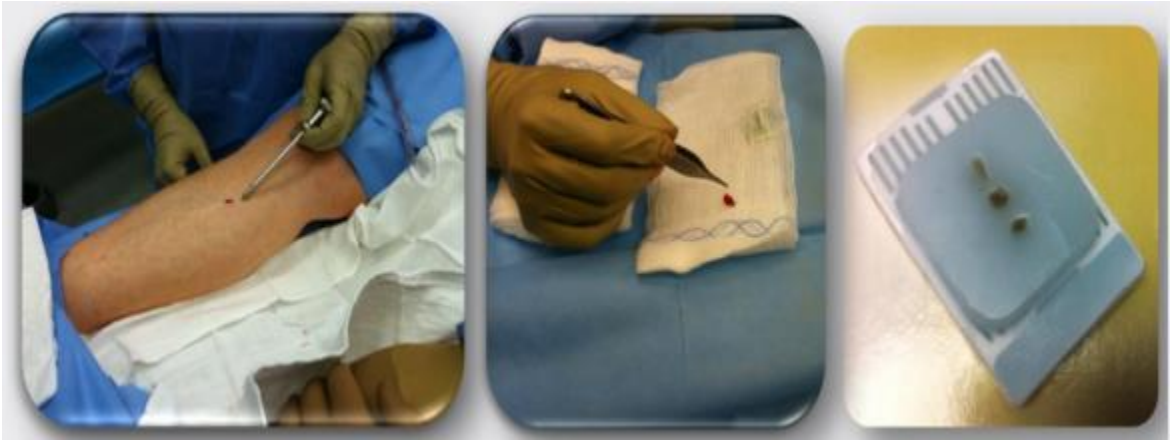

Figure 4.1. Tissue Preparation

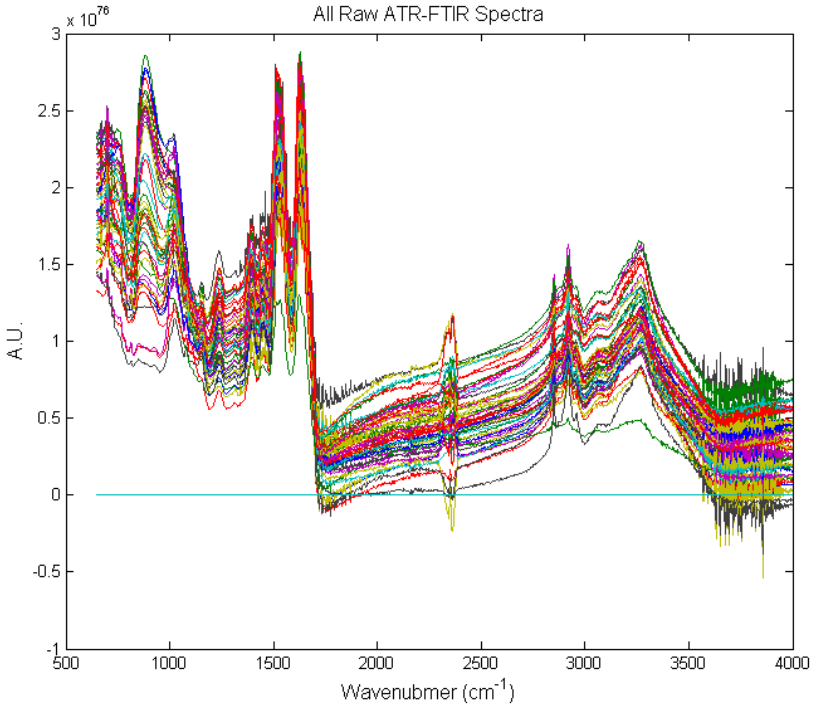

Figure 4.2. All Raw ATR-FTIR Spectra

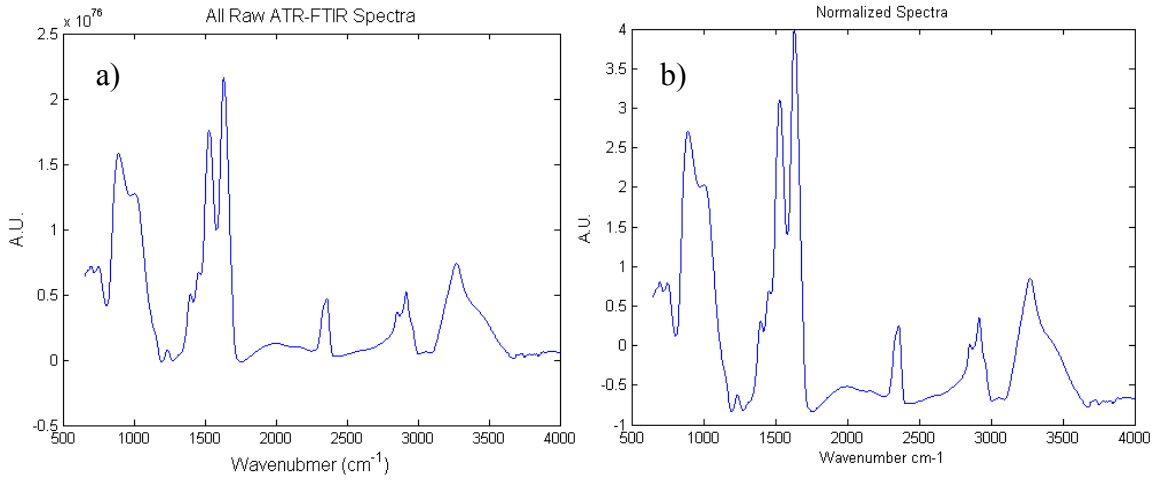

Figure 4.3. a) Baseline Correction, b) Normalization using median value (2000 cm^{-1})

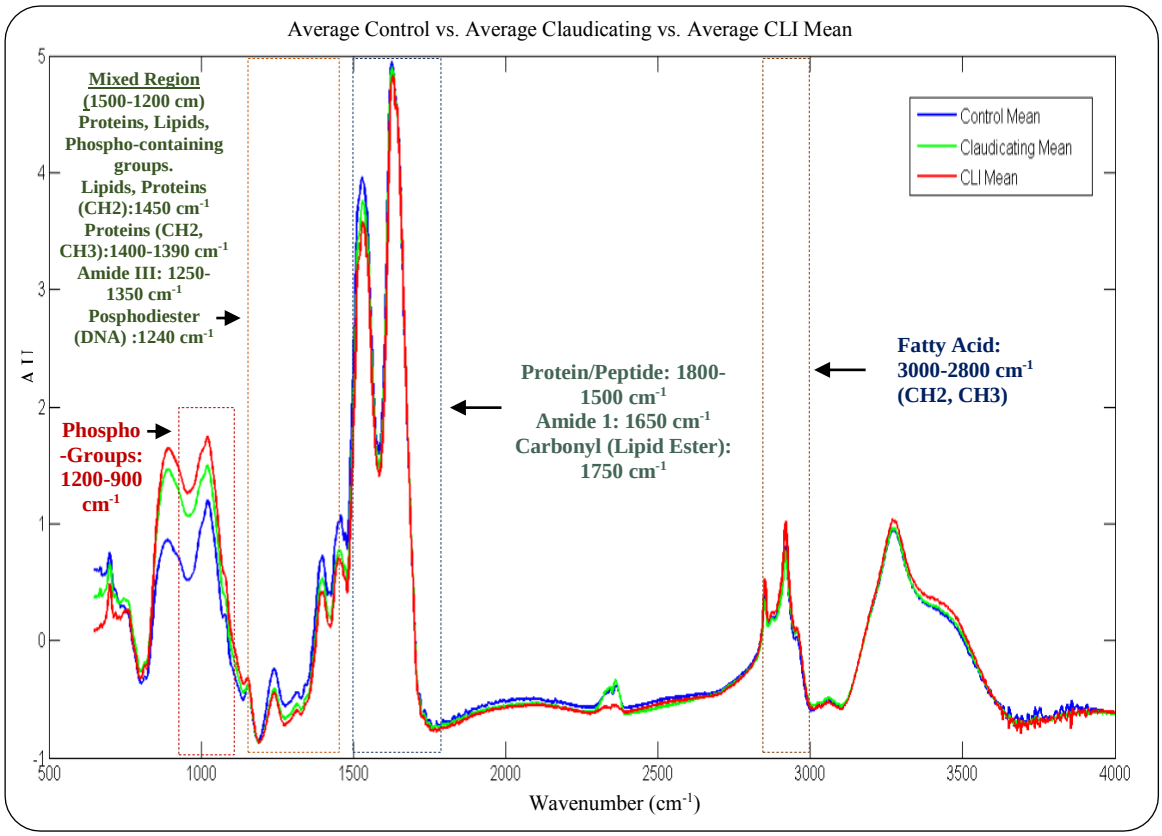

Figure 4.4. FTIR spectra of Gastrocnemius muscle tissue

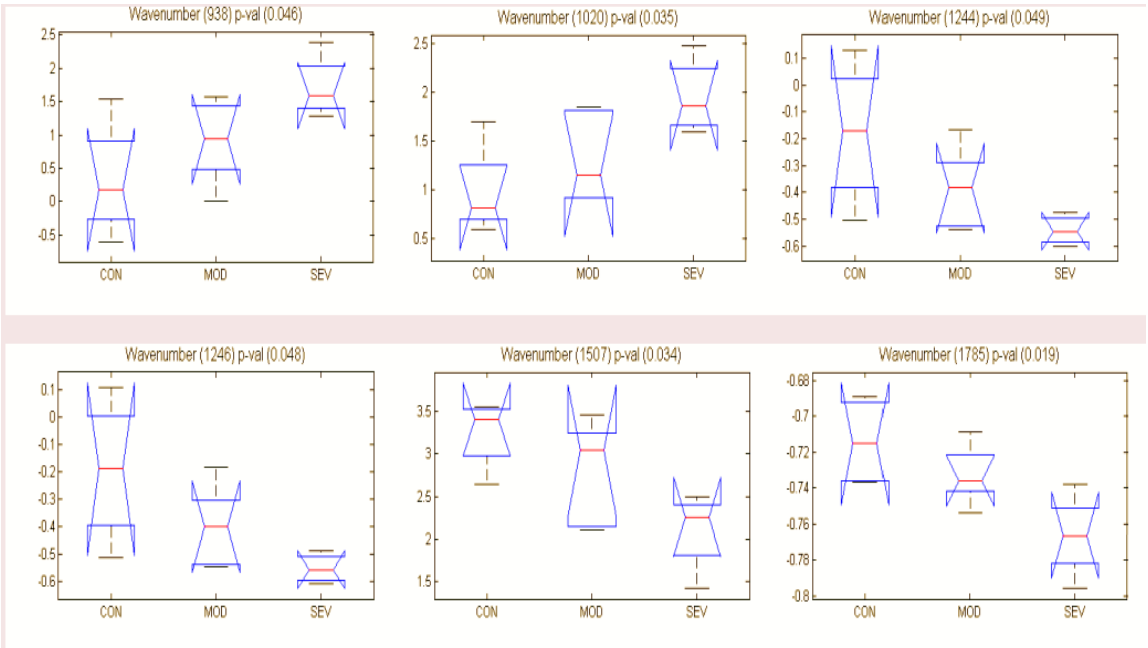

Figure 4.5. Box Whisker plot