Dietary Intake of Peripheral Artery Disease Patients

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

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Abstract

Peripheral artery disease (PAD) is one of the most common cardiovascular diseases. Despite high prevalence of PAD, data regarding the dietary patterns of Canadian individuals with PAD is required. Biomarkers are used as an alternative to dietary assessment methods and are generally used to measure true dietary intake. A total of 30 participants with established PAD were recruited for this study. Dietary intakes were estimated using a 3 day food record (3DFR) and food frequency questionnaire (FFQ). Docosahexaenoic acid (DHA) status was analyzed in plasma, plasma PL and RBC membrane PL. The determined mean dietary macronutrient distribution consisted of 18% protein, 33% fat and 47% carbohydrate with 3DFR and 19% protein, 36% fat and 43% carbohydrate with FFQ. The mean intakes using 3DFR and FFQ, respectively, were: saturated fat = 24.2, 22.8 g; sodium = 4156.6, 2852.3 mg; eicosapentaenoic acid (EPA) = 90.0, 50.0 mg and DHA = 128.5, 110.0 mg. FFQ showed significant ($p<0.05$) correlation ($r=0.48$) with plasma and plasma PL DHA. As compared to national guidelines, the participants consumed a diet that was 61% higher than National Cholesterol Education Program (NCEP) recommendations for saturated fat. Also, the participants consumed a diet which was 177% and 246% higher than NCEP and the Institute of Medicine (IOM) recommendations for sodium, respectively. Hence, PAD patients should be encouraged to eat a diet that is lower in saturated fat and sodium. None of the participants met the American Heart Association (AHA) recommendations for established cardiovascular disease of 1g of combined EPA and DHA.

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List of Abbreviations

PAD	Peripheral artery disease
CVD	Cardiovascular diseases
CHD	Coronary heart disease
ABI	Ankle-brachial index
IC	Intermittent claudication
NO	Nitric oxide
IL-6	Interleukin-6
MCP-1	Monocyte chemotactic protein
VCAM-1	Vascular cell adhesion molecule
TNF	Tumor necrosis factor
VCAM-1	Vascular cell adhesion molecule
CRP	C-reactive protein
FFQ	Food frequency questionnaire
ω-3	Omega-3
ω-6	Omega-6
3 DFR	3 day food record
Vitamin D_2	Ergocalciferol
Vitamin D_3	Cholecalciferol
25(OH)D	25-hydroxyvitamin D
Hcy	Homocysteine
NHANES	National Health and Nutrition Examination Survey

PUFA	Polyunsaturated fatty acids
ALA	Alpha-linolenic acid
LA	Linoleic acid
AA	Arachidonic acid
EPA	Eicosapentaenoic acid
PL	Phospholipid
RBC	Red blood cell
DHA	Docosahexaenoic acid
LDL	Low density lipoprotein
TC	Total cholesterol
HDL	High density lipoprotein
BHT	Butylated hydroxytoluene
PAL	Physical Activity Level
NCEP	National Cholesterol Education Program
IOM	Institute of Medicine
RBC	Red blood cell
mg	Milligram
g	Gram
Kcal	Kilocalorie
ml	Millilitre
µl	Microlitre

1.0. Introduction

Peripheral artery disease (PAD) is a highly prevalent, silent, and at times asymptomatic manifestation of atherosclerosis (1). It is one of the most common cardiovascular diseases (CVD) frequently accompanied by inflammation and oxidative stress in humans (2). PAD is related to the increased incidence of cardiovascular and cerebrovascular mortality and morbidity (3, 4). PAD affects about 800,000 Canadians (5), and is particularly prevalent in older adults (6) and increases with age (7).

There is a wide variation in the prevalence of PAD with age 40 and above. Various studies have reported different prevalence rates for PAD. The prevalence of PAD is about 16% in North America and Europe. Approximately 27 million people in North America and Europe are affected by this disease. A total of 16.5 million PAD patients out of 27 million patients are asymptomatic (1). In Canada, it is estimated that 4% of those aged 40 plus (1), 10% (8) to 20% (9) of individuals over 55 years, and 20% over 75 years (7) are affected by PAD. According to the recent statistics from the U.S., PAD affects 5.9% of the United States population over the age of 40 and roughly 16.5 million Americans (10).

One study (11) has reported a higher prevalence of PAD in men as compared to other studies (12, 13), which reported the prevalence of PAD in men and women was equal.

PAD is characterized by formation of fatty deposits that build up along the wall of arteries that supply blood to the upper and lower extremities (arms and legs). These deposits lead to a narrowing of the arteries (atherosclerosis or hardening of arteries), thereby affecting the blood circulation in the legs and feet at rest or during exercise (14).

Clinically, PAD is defined by the presence of an ankle-brachial index (ABI) of <.90 (15, 16).

ABI is a simple, non-invasively measured and reproducible method of diagnosing PAD (16). ABI has a sensitivity of about 100% in the normal population (1). ABI also has a sensitivity of 80-95% and a specificity of 95-100% in detecting hemodynamic lesions (17, 18). An abnormal ABI is a strong risk predictor for early cardiovascular events and mortality (19).

Symptoms of PAD also include painful cramping of leg and hip muscles, numbness, weakness and cooling of skin in specific areas of legs (14). In addition to walking intolerance, PAD patients suffer from toe and/or foot sores.

PAD may be asymptomatic (1), or present with intermittent claudication (IC) (walking induced pain in one or both legs that is only relieved by rest) (16). IC may be present in 15-40% of patients with PAD (20). Chronic IC may also lead to gangrene (localized tissue death), and in advanced stages amputation and limb loss can occur. Complications of PAD also include mobility loss, surgical revascularization and nursing home placement (21). PAD is associated with reduced walking capacity, resulting in poor quality of life with complications (2).

Moreover, many risk factors have been associated with the later development of PAD including insulin resistance/diabetes mellitus, dyslipidemia, hypertension, old age and smoking (16, 22). In addition to the symptoms described above, PAD is also linked to impairment of endothelial-dependent vasofunction (23). Other emerging risk factors such as high homocysteine levels, fibrinogen and creatinine are also associated with PAD (22). Independent of the traditional risk factors, β2 microglobulin, cystatin-C, glucose

and C-reactive protein (CRP) levels also showed association with PAD, however, only β2 microgloublin and cystatin-C showed a high correlation with ABI (24).

Treatment of PAD includes modification of traditional risk factors such as smoking, hypercholesterolemia, hypertension and diabetes mellitus through smoking cessation, low fat / low salt diet, exercise, weight reduction, dietary changes and medical treatment with cholesterol lowering drugs, antihypertensive drugs and antiplatelet agents (10, 14).

2.0. Review of Literature

Understanding the process of development of PAD is an important step towards the management of this highly prevalent and undertreated disease. The following review will focus on the mechanism of development of PAD and its progression as well as the role of lifestyle and dietary nutrients in PAD.

The formation of atherosclerotic plaque in the arteries precedes the development of PAD. Atherosclerosis, a contributor of PAD, is characterized by chronic inflammation and is caused by such factors as smoking, hypercholesterolemia, high fat diets, obesity, sedentarism, diabetes mellitus and hypertension (25, 26).

Atherosclerosis affects vascular beds and can lead to the obstruction of coronary, cerebral and peripheral arteries, the latter resulting in PAD (25). In the initial stages of development of atherosclerosis, no warning signs are prominent as these are detected at later stages when 50% of the artery gets occluded with atherosclerotic plaque. Depending upon the site of development of atherosclerotic plaque within specific arteries, the plaque can lead to the development of coronary artery disease (CAD), cerebrovascular disease and PAD (15).

Development of atherosclerotic lesions: In PAD following vascular injury, oxidized LDL begins to cause endothelium damage and results in diminished production of nitric oxide (NO) by the enzyme nitric oxide synthase (27). NO, a vasodilator, has many important functions such as protection against vascular injury, inflammation, leukocyte adhesion to the endothelium, limiting platelet aggregation and preventive smooth muscle proliferation and thrombosis (28, 29, 30). During PAD, leukocytes adhere to the endothelium which is resistant to leukocyte adhesion in a normal state (31). Angiotensin

II has opposes the actions of NO and also enhances reactive oxygen species production which results in expression of inflammatory cytokines such as interleukin-6 (IL-6), monocyte chemotactic protein (MCP-1) and vascular cell adhesion molecule (VCAM-1) in the endothelium resulting in greater monocyte adhesion (32, 33, 34). Adhesion molecules are expressed by pro-inflammatory cytokines such as tumor necrosis factor (TNF), IL-1, and C-reactive protein (CRP) (35, 36, 37, 38). As the monocytes move through the intima they become lipid laden foam cells which constitute early atherosclerotic lesions. Pro-inflammatory cytokines secreted by macrophages further aggravate this process (1). CRP levels also diminish the activity of NO which has endothelial protection properties (39). A study has reported an association between arterial elasticity and the oxidative stress marker (F2 isoprostanes) in PAD providing an indication of the role of oxidative stress in altering the elasticity of arteries (40).

Another study has reported that PAD patients have higher levels of cytokines, adhesion molecules, selectins, Von Willebrand factor, CRP, fibrinogen and increased blood viscosity (41). As compared to age matched controls, PAD patients have a 3-5 fold greater risk of cardiovascular mortality (41). In fact, the survival rate for PAD patients was approximately 50% for asymptomatic patients and 25% for symptomatic patients in a 10 year follow up study (1).

Diabetes is common among men and women with PAD (21). One study (21) has shown that participants with PAD and diabetes had poor lower extremity function (higher prevalence of leg pain on exertion and rest, diabetes associated neuropathy, lesser fast pace walking speed during the 4 m walk and walked significantly fewer feet during 6 minute walk) and a higher number of cardiovascular diseases as compared to those with

PAD alone. Another study (42) has also reported that diabetic people have a 1.5 to 2.5 fold increased risk of PAD.

Understanding the relationship of diet and life style factors in PAD towards the management of PAD is crucial. Diet and life style factors such as smoking, alcohol consumption, sedentary habits are modifiable risk factors for PAD. These factors can have a great impact on disease progression.

2.1. Lifestyle factors and PAD

In one trial conducted to study the effects of lifestyle and dietary interventions, it was found that education towards lifestyle changes and improved nutritional habits (lesser saturated fat, higher monounsaturated and polyunsaturated fat), increased moderate exercise and decreased smoking by patients with PAD led to lowered levels of triglycerides and increased levels of HDL cholesterol, all contributors to the risk profile for PAD (43). With respect to dietary ω-3 fatty acid consumption, habitual exercise and social status such as education have been associated with high intake of ω-3 PUFA derived from fish among men and healthy diet among both genders (44).

2.2. Physical activity and PAD

Public awareness about PAD and its relation to risk factors like smoking and obesity is poor, which results in adverse health effects and increased health care cost (5). Exercise has also been reported to have a beneficial effect on pain free walking distance of PAD patients (45). Increased physical activity may improve blood lipid profiles (46), HDL levels, glucose intolerance (46), blood pressure (46), lower blood viscosity and plasma fibrinogen levels in men and women of age 55-75 yrs (46). In male smokers with PAD, a significant association between increased physical activity at age of 35-45 yrs and

increased mean ankle brachial index at age of 55-75 yrs have been reported (46). A significant association between femoral atherosclerosis and higher serum HDL concentrations and exercise has been reported in older adults (47).

2.3. Smoking and PAD

Smokers have lower plasma concentrations of ω-3 PUFA, EPA and DHA than non-smokers (48). Cessation of smoking resulted in increased warmth of the limbs according to thermogram analysis along with increased pulsation and blood flow in digits in patients. This explains the dilating effects after smoking cessation, and reverse effects of smoking (i.e. constriction was observed with resumed smoking) (49).

2.4. Alcohol intake and PAD

In French participants, median daily consumption of alcohol in men (24 g) and women (4 g) showed a positive significant association ($p<0.05$ ck) with HDL levels (50). With respect to alcohol (wine) consumption, low to moderate alcohol intake 13-24 g/day (1-2 drinks) in men and 7-12 g/day (0.5-1 drink) in women was associated with lower IC risk (Hazard ratio (HR) = 0.67 for men and 0.44 for women) (51). Similarly, one study has also reported an inverse association of moderate alcohol consumption and PAD risk. (Relative risk (RR) of PAD with >7 drinks/week was 0.68 during 11 years follow up study as compare to >1 drink/week) (52), while another study showed no significant PAD risk reduction with alcohol consumption (26). Some studies (12, 53) have reported positive association of alcohol consumption and some (54, 55) reported an inverse association with the risk of IC. Alcohol consumption (wine) was associated with higher ABI in males ($p = 0.03$) and no association of alcohol consumption and ABI was found in

women (55). Daily consumption of 1-2 drinks of wine was associated with decreased cardiovascular or cerebrovascular disease (RR 0.47) (56).

Beneficial effects of alcohol can be related to its role in raising HDL levels (57). Wine and beer have antioxidant rich properties due to the presence of polyphenols that may prevent LDL oxidation, a key component of atherosclerosis (58). Flavonoids have anti-antioxidant, anti-inflammatory, anti-thrombotic and endothelial protection properties (59). A study designed to investigate the relationship between wine, alcohol and atherosclerosis provided evidence that flavonoids in red wine and grape juice promote endothelium-dependent dilation by reducing endothelin-1 production (a vasoconstrictor inducing smooth muscle cell proliferation). They also increase nitric oxide synthase expression and HDL, and exhibit antiplatelet actions and antioxidant properties (resistance to LDL oxidation) thus potentially providing a beneficial effect to patients at risk of atherosclerotic events (25).

Flavones, found in leafy vegetables and herbs, and flavonols, found in many plant foods, are the two classes of flavonoids that have been linked to a decreased risk of PAD. An increase equivalent to one standard deviation of any of these two classes of flavonoids was shown to decrease the risk of PAD by 50% (59).

2.5. Diet and PAD

2.5.1. Dietary Assessment Methods

To understand the relationship between nutritional status and disease in population groups, it is important to obtain information on food intake from dietary assessment methods, and then correspond the collected information to biochemical and anthropometric measures through clinical trials. To measure the dietary intake of

individuals at any life stage is challenging. Choosing a method depends on the question to be answered and should match the answers in the desired population group. The effect of season, day of the week, and use of supplements should be taken into consideration during nutrient intake assessment of participants (60).

Limitations that are associated with assessing food intake of older people are: inability to accurately describe the food consumed, memory lapses and no involvement in food preparation methods could produce errors in recording the data (61). Various dietary assessment methods can be used to estimate the intake of specific foods, and these are discussed below.

Diet assessment methods can generally be divided into two groups: quantitative daily consumption methods and methods that obtain retrospective data on food consumption patterns. The quantitative daily consumption method includes dietary recalls, 24 hr recalls and food records (3 days, 7 days, weighed and estimated). This method also measures the quantity of individual foods consumed during a specific time period, in order to measure actual intake for recent and longer periods (habitual intake). On the other hand, retrospective methods such as diet histories and food frequency questionnaires (FFQ's) are generally used to assess usual dietary intakes (60). Use of food records and FFQ`s provides additional information on food patterns (62).

2.5.2 Twenty Four Hour Recall (24 hr)

The 24 hr recall method involves the recall of a subject's exact food and beverage intake in the previous 24 hr period. Detailed descriptions of all foods and beverages consumed are recorded by the interviewer including portion sizes, brand names, and cooking methods (63, 60). To assist the subject in estimating portion sizes, food models,

photographs or household measures are used. A single 24 hr is a more appropriate method for the estimation of average food intake for large groups, and is a quick method that can be used with illiterate participants with low subject burden and more compliance (60). One advantage of the recall method is that it is less likely to change habitual eating behaviour as the data is collected after the subject has already consumed the meals (64). On the other hand, the 24 hr recall method is generally more time consuming than FFQs, (65). This method is not suitable for usual food intake of individuals since it is based on actual intake and may not fully and accurately represent habitual intake (64). Multiple 24 hrs recalls can be used to assess habitual intake and should include several days with one weekend day to account for the food variability on the weekend (63, 60). The success of this method depends on the subject's memory, motivation of the respondent, ability of the subject to describe the accurate portion sizes and the interviewer's skills (63). Total time for the interview takes about 20-30 minutes or more depending on the complexity of food, particularly those eaten as mixed dishes, so intense training is required to acquire accurate data by the interviewer in a non-judgmental way. This method is associated with less subject burden. Quantification of portion size error is based on memory which is a source of error in the collection of food intake data (64).

2.5.3 Food Records

Food record methods require participants to record the detailed descriptions of all foods and beverages consumed for a specified period of time (usually 3, 5 or 7 days). This method also includes estimation of portion sizes, methods of preparation, the amount of each raw ingredient present for composite dishes such as spaghetti, specific brand names and the final weight of the composite dish (63, 60). The use of household

measures such as spoons, cups, rulers for the meat measurements, classical measures such as counts for eggs and bread slices are used for the estimation of portion sizes. Portion sizes are usually converted into grams and manually entered into a nutrient analysis software program in order to calculate nutrient intakes. In estimated food records, imprecision in estimation of portion size is the error that results in imprecise nutritional intake data.

Weighed food record methods are the most precise methods currently available and have the least correlated errors (63, 64). They require participants to weigh and record every food and beverage consumed during a specified time period (63). The weighed method prevents bias and errors in estimating habitual intakes by using a weighing scale instead of measuring portion sizes with cups and spoons that can alter the intake data. However, subjects can misread the scale and respondents can change the accurate value to simplify the measurement in order to save time. This is the most accurate method in estimating absolute usual intake. In order to succeed in the weighing method, participants should be literate, motivated and numerate. Due to the high burden associated with the weighed method, individuals may be less interested to record their intake creating a respondent bias (63). One major advantage of this method is that participants are not required to rely on memory, eliminating errors in recalling food items previously consumed.

2.5.4. 3-Day Food Record

Estimated 3DFR is a dietary assessment method that is used to record a subject's intake for three days. In this method the subject has to record all the food items, including the beverages they consume, for 3 complete non-consecutive days. Days includes 2

weekdays and one weekend day to account for the weekend effect on the food and nutrient intake, followed by entry of data collected and analysis by nutrient intake data software by the researcher. This method allows the subject to record their intake on the 3-day food diary instead of recalling what they had eaten on a particular day. 3DFR has a high degree of reporting and low proportion of phantom foods and missing foods as compared to the 24 hr recall thus creating less bias. This method is easier to administer than the weighed method in which a subject has to weigh every food item they consume for a complete 24 hr period. Despite that the 3DFR is costly and labour intensive as compared to the 24 hr recall (65). This method provides fewer burdens by using household measures such as cups, teaspoon, tablespoon and classical measures such as slices of bread or counts of eggs to record portion sizes of the food items consumed instead of weighing the food items that they consume. This method requires moderate subject motivation in order to bring accuracy to the portion size and food items recorded, and the need for participants to be literate and numerate in order to prevent errors. The 3-day food record (3DFR) is an open-ended method and can accommodate any food description details and diversity in the food intake of the participants. In addition to this, 3DFR data can be analyzed by nutrients, individual foods or by food groupings, thus providing flexibility to explore the data collected in many ways (64).

In comparing the nutrient intake with specific recommendations, estimates of absolute energy and macronutrients are required. The best choice of dietary assessment would be recall or record. For correlation purposes, rankings of nutrient intakes are used (64).

2.5.5. Food Frequency Questionnaires (FFQ)

The Food Frequency Questionnaire (FFQ) is a valid and highly reproducible dietary assessment method for long chain ω-3 fatty acids (LC ω-3 PUFA) (66, 67). Many FFQs have been developed to assess the intake of LC ω-3 PUFA. FFQ is now widely used to estimate usual dietary intake over a less precise period of time (60) and often used to study an association between dietary habits and disease (64). FFQ generally consists of two parts: a comprehensive list of foods that includes a questionnaire on specific foods consumed in relation to a particular season and a set of frequency of use response categories (63, 60). Initially the FFQ was designed to obtain qualitative data on food consumption patterns but also used to obtain semi-quantitative data with the inclusion of portion size estimation using photographs to report small, medium or large serving sizes of food items (60). This portion size estimation helps in developing nutrient scores of participants by multiplying relative frequency of food consumed with nutrient content of average portion sizes. This method is simple, less expensive and easy to administer (68) and can be used to collect usual intake data over an extended period of time and places fewer burdens on participants (60). It is also less costly than other dietary assessment methods and can give a quick estimate of usual intake (64). Data collected from FFQ can be used to rank the individuals on the basis of dietary intake (63). Proper understanding of participants for FFQ impacts the subject's response to the FFQ questions and quality of data obtained (69). Over/underestimation of food portions, errors in food recalling (memory), and misinterpretations of questions are other limitations of FFQ (64).

In the past, FFQs have been used to estimate intake of LC ω-3 PUFAs in comparison with 3DFR in a normal healthy population of Australian subjects (67, 70).

An existing FFQ (71) has been used in this study as described in materials and methods section. To date, FFQ has been used to estimate ω-3 intake in some patient populations and has been validated for use against biomarkers of ω-3 intake (66, 67, 70). However, estimation of ω-3 intake using 3DFR and FFQ has not been studied specifically for PAD patients. Therefore, the opportunity exists to study the 3DFR and FFQ as a dietary assessment tool in determining ω-3 intake of PAD patients and compare this with plasma DHA, plasma phospholipid (PL) DHA and Red blood cell (RBC) DHA as a biomarker of LC ω-3 PUFA intake.

Diet can play an important role in decreasing the risk factors of chronic disease. Therefore, it is essential to understand the dietary intake of PAD patients. Currently, no ideal method for the assessment of nutrient intake of PAD individuals is available because all dietary assessment methods have their own limitations and are often associated with random and systematic error (60). Therefore, none of the methods can be considered to be a 100% reliable measure of true dietary intakes.

The occurrence of random and systematic errors could be within person or between persons. Random within person error is due to the day to day variation in dietary intake and measurement error on any day. Systematic within person error could be due to consciously or unconsciously underreported or exaggerated dietary intake of foods, or when an questionnaire lacks an important food item for a subject, or the question could be misinterpreted by a subject (64). This error will not be reduced by replicating measurements. Systematic error is difficult to measure and can be understood by validation study. Systematic between person error arises from systematic within person

error such as removal of commonly eaten foods from the questionnaire and the use of the wrong nutrient value for a food (64).

Random error affects the precision of a method and is often controlled by increasing the number of observations, that is the sample size of the study to approach the true value, whereas systematic errors produce a significant bias in the results and the difference might be associated with certain respondents, specific interviewers and specific food items (60).

Randomized and systematic error can be minimized by incorporating various procedures into each step of a dietary assessment process (60) such as adequate training of interviewers and coders, the use of a standardized interview protocol, interviewing techniques and administration of pilot studies (63, 60).

Different biases are associated with dietary interviews. Respondent bias may occur in dietary assessment studies as the respondent may not understand a question or may receive expression cues from the interviewer to alter the response or give socially desirable answers, thus resulting in an under-reporting or over-reporting of food intake (63, 60). Interviewer biases can also occur due to incorrect questions, incorrect recording of responses, intentional omissions and distractions (63, 60). Respondent memory lapses may occur in diet assessment, leading to the incorrect reporting of foods consumed (60).

Incorrect estimation of portion size is a common error in dietary studies (60). Estimating portion sizes is highly variable among individuals, depends on the size of the food item and is independent of age, gender, body weight and social status (72). Measurement aids are commonly used in estimating portion sizes with the use of household measures such as drawings, photographs, food replicas, food models and even

real food samples (60). Failure to report supplement intake leads to an overestimation of the nutritional inadequacy prevalence (60). Under-reporting of energy intakes, the tendency to overestimate low intakes and the tendency to underestimate high intakes can also lead to errors in estimating the adequacy of nutrient intake. It has been suggested that energy intake should be considered when looking at the correlations between caloric intake and specific nutrients (64). This can be explained by the fact that nutrient intake tends to be associated with caloric intake, and that specific nutrients may only be correlated with disease because of their association with total energy intake (73). Thus, it becomes important to decide whether the nutritional factors can be measured as absolute amounts or adjusted for total energy intake.

Validity in dietary assessment methods refers to the extent to which a dietary method measures what it is designed to do. Methods designed to measure usual dietary intake of individuals are difficult to validate because true intakes are not known with absolute certainty. Validity is affected by systematic error. Even if two diet assessment methods yield similar results, there is no assurance that the results represent the actual dietary intakes. As absolute validity is not provided by any of the reference methods, the term relative validity is commonly used. Due to the limitations of comparing the relative validity of two diet assessment methods, nutrient biomarkers have been identified as an additional method of diet assessment (60). With the use of biochemical indices, dietary intake data can be validated as independent of nutrient intake and thus errors will be independent.

Low energy reporting is a common problem and a big challenge seen in dietary assessment studies and nutrition surveys. This is due to the underreporting of food

consumed. A review article has indicated the under reporting of energy intake in many nutritional studies and it exists across dietary assessment methods (74). Low energy reporters refer to those study participants who report their dietary intake below the minimum energy required to sustain the physiological processes and metabolism of the body (75). A study (76) conducted in Montréal adults (n=246) aged 18-82 years has reported that 43% of individuals were under reporters and a higher percentage (54%) of males were low energy reporters in comparison to women (35%). Low energy reporting by the subjects could be either due to under recording or under eating or due to both reasons during the dietary assessment period (77). Few methods exist to determine the energy misreporting in the dietary assessment studies. To determine the validity of energy intake (EI), a doubly labelled water technique is used as an independent marker. This technique is expensive, time consuming and imposes higher subject burden. Another quick and less expensive method to determine the misreporters is to calculate the ratio of EI and total energy expenditure (TEE). TEE can be calculated using the following equations that are described below developed by the Institute of Medicine, which include information on a subject's age, weight, height and physical activity level (PAL). Different PA levels are shown below:-

Table 2.1. Values of physical activity level

Activity	**PAL**
Sedentary	1.0-1.39
Low active	1.4-1.59
Active	1.6-1.89
Very active	1.9-2.5

Different cut-off points have been used to determine the number of subjects who have misreported their energy intake in different studies (78). If the ratio between energy intake (EI) and TEE was less than 0.79, the subject was considered to be a low energy reporter. If the ratio between EI and TEE was more than 1.21 (79), the subject was considered to be a high energy reporter. EI was calculated from the dietary software (80) when all the foods consumed were entered into the software.

The Canadian community health survey (CCHS 2.2) conducted in a population aged 12 and over in 2004 has shown that average under reporting of energy intake was 10%. This survey has shown that overweight, obese, physically active people, adults in comparison to teenagers and women in comparison to men were under reporters of energy intake. The different foods which are associated with under reporting are cakes, pies, savory snacks, cheese, potatoes, high fat meat products, soft drinks, fat-type spreads, snack foods high in sugar, salt (junk foods) and condiments. These foods are also associated with the tag of bad foods and socially undesirable and unhealthy foods which could be a reason for misreporting these foods and resulting in the underreporting of energy intake.

2.6. Impact of nutrition on PAD

Diet plays an important role in the vascular health. A healthy diet can improve vascular function and a high fat diet can be detrimental to vascular activities. For many decades, researchers have been interested in the relationship between diet, health and disease. The World Health Organization has set up guidelines for the prevention of cardiovascular disease as a diet low in saturated fat (<10% of calories), low in salt (<5g/day), devoid of

trans fats and low in total fat (<30% of calories). Consumption of a wide variety of fruits, vegetables and grains (>400g/day) is also advised (81). Diet can be an important modifiable risk factor in PAD as shown in research studies mentioned below. Diet can have a profound effect on the progression of atherosclerotic plaques not only due to its influence on circulating blood lipids, but also through protective anti inflammatory processes and modulation of immune responses in the intima of arteries (82).

With respect to general dietary habits, PAD patients have lower average calorie intake (1904 Kcal/day) and nutrient intake as compared to normal participants (2036 Kcal/day) (26). In addition to poor caloric and nutrient intakes, PAD patients have also been shown to have an increased number of comorbid conditions (high blood pressure, CAD and diabetes) (10). High vegetable lipid intake > 34.4 g per day, a vitamin E intake > 7.7 mg per day and elevated serum high density lipoprotein (HDL) cholesterol concentration have been associated with a reduction in the prevalence of PAD (OR=0.39 with vegetable lipid intake, 0.37 with Vit E intake and 0.76 with HDL levels respectively) (26).

Results from the National Health and Nutritional Examination Survey (10) found that nutrition has a significant impact on the prevalence of PAD. Irrespective of the traditional risk factors for CVD, higher consumption of foods rich in antioxidants (vitamin A, C, and E), folate, vitamins B_6, fibre and ω-3 fatty acids have significant protective effects with decreased prevalence of PAD (10). Furthermore, one of the studies found that none of the participants with PAD met the Institute of Medicine (IOM) and National Cholesterol Education Program's (NCEP) recommendation for sodium

consumption. More than three-quarters exceeded recommendations for saturated fat intake and very few participants met the daily recommendations for fibre (83).

2.7. Micronutrients and PAD

2.7.1. Vitamin C (Ascorbic acid)

Vitamin C is a water soluble vitamin that has antioxidant properties and maintains redox state, and immune function. It is most commonly found in citrus fruits. Vitamin C is the most effective and first antioxidant to be used against oxidative stress, a pro-atherogenic condition in blood (84), which may explain low vitamin C status observed in PAD patients (2). Reduced levels of vitamin C are associated with greater susceptibility of LDL to oxidation and increased risk of CVD (85). Also, higher levels of reactive oxygen species in inflammatory atherosclerotic lesions can lower the level of vitamin C (82). Vitamin C deficiency could also reflect an unhealthy diet and may not itself be a risk factor (26). Levels of vitamin C are reduced in PAD patients which is associated with reduced walking capacity, smoking and systemic inflammation associated with higher CRP levels (2). One of the studies has shown that vitamin C protects endothelial functioning in PAD patients and also decreases the levels of soluble intracellular adhesion molecule 1 and thiobarbituric acid reactive substances (86). Higher vitamin C levels are associated with decreased risk of PAD (OR= 0.34) (87).

Smokers have low plasma level of vitamin C as compare to nonsmokers (88) affecting absolute claudication distance that was lower in PAD patients (2). This study also suggested the anti-atherogenic role of vitamin C based on associations between PAD and vitamin C deficiency (2). The antioxidant and anti-inflammatory properties of vitaminC may explain the anti-atherogenic role of vitamin C (26). Vitamin C and ABI

showed a significant association (p=0.006) in smokers (54). An inverse association between vitamin C and PAD has been found in women; 100 mg of vitamin C intake resulted in a 0.013 increase in ABI for women (89). Beneficial effect of vitamin C on lowering odds of PAD (OR=0.51-0.65) have been reported at doses 60.1 mg and >125 mg (10). Conversely, clinical trial results showed that the beneficial effect of vitamin C on PAD is not positive (90). Also, vitamin C exhibits pro-oxidant properties that could affect the results of the clinical trials (90).

2.7.2. Vitamin E

Vitamin E, a membrane bound antioxidant, protects against cellular damage caused by PUFA oxidation by free radicals oxidation. Vitamin E is found in nuts, fish and vegetable oils (89). A 10 mg increase in vitamin E intake resulted in a 0.015 increase in ABI in men (89). A positive association of vitamin E was shown with ABI (p=0.04) (54) and reduced PAD (RR = 0.67) (89). Vitamin E, can improve IC in PAD patients by exhibiting a beneficial effect on platelet aggregation and red blood cells, thus improving blood flow to the arms and legs (91). Antioxidant, anti-inflammatory and antiaggregatory effects, and smooth muscle cell proliferation inhibition properties (92) can explain the anti-atherosclerotic effects of vitamin E. These findings suggest that there is an association between dietary antioxidants and PAD. Despite the beneficial effect of vitamin E on PAD, randomized Heart Outcomes Prevention Evaluation (HOPE) trial did not find any beneficial effect of vitamin E supplementation on cardiovascular events (93). One study has reported the possibility of antioxidants in reducing cardiovascular events in PAD patients but no improvement in lower extremity function (94). Some studies have (95, 96) reported that vitamin E does not prevent LDL oxidation and oxidative stress in

atherosclerotic plaque. The combined effects of vitamin C and vitamin E were not protective in improving peripheral endothelial function (97).

2.7.3. Vitamin D

Vitamin D is a fat-soluble vitamin that can be found in animal-derived foods, such as fish and eggs, in fortified foods such as milk and cereals, and can be synthesized in the skin via ultraviolet-B radiation. Vitamin D comes in two main forms: vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol), and is then converted to its active form 1,25 dihydroxyvitamin D3 (98). This vitamin has long been recognized for its role in facilitating calcium absorption and maintaining bone health. Recent evidence suggests vitamin D may play a role in the development of PAD. According to the National Health and Nutrition Examination Survey 2001 to 2004, the prevalence of PAD was 7.7% with serum levels of 25-hydroxyvitamin D (25(OH)D) <20 ng/ml (99). Vitamin D may play a role in vascular health as it regulates blood pressure (100) via the renin angiotensin system. Vitamin D has been shown to affect the growth of vascular smooth muscle cells (101). In one study, 60% of veterans with PAD had vitamin D deficiency (<20 ng/mL), which was associated with increased adiposity, triglyceride and glucose measures thus increasing the risk of amputation in PAD individuals (102). Vitamin D deficiency may also contribute to the high occurrence of PAD amongst African American adults: mean 25(OH)D levels were significantly lower compared to Caucasian adults (39.2 and 63.7 nmol/l, respectively), and prevalence of PAD was also greater in African Americans compared to Caucasians (103). It was previously shown that for each 10 ng/ml drop in 25(OH)D the prevalence of PAD increased by a ratio of 1.35 (104). Therefore, Vitamin D deficiency may play an even greater role in vascular health and integrity than was

previously thought. The beneficial effects of Vitamin D include lowering of smooth muscle proliferation, upregulation of anti-inflammatory cytokines, down regulation of pro-inflammatory cytokines and suppression of vascular calcification (105).

2.7.4. Folic Acid, Vitamin B_6 and Vitamin B_{12}

Folate is a generic term commonly used to describe folic acid (pteroylmonoglutamic acid) and related compounds having the similar biological activity as folic acid (63, 60). Sources of food folate include liver, yeast, leafy vegetables, fruits, pulses, nuts and foods enriched in folic acid (106). Folate plays an important role in single carbon transfer reactions, intermediary metabolic pathways and DNA synthesis (60). Folate supplementation has been suggested to play an important role in PAD (107). Dietary intake of folate and vitamin B_6 were found to be lower in PAD patients (above 50 years) and are independent predictors of PAD in men older than 50 yrs (108).

Homocysteine is a sulfuhydryl containing amino acid produced entirely from the methylation cycle and dietary methionine. The metabolism of homocysteine depends on two biochemical pathways; transsulfuration and the methionine cycle. Under fasting conditions, homocysteine is metabolized by a remethylation pathway forming methionine by methionine synthase enzyme this requires vitamin B_{12} a coenzyme and folate as a substrate. In the transsulfuration pathway homocysteine is catabolized to cysteine by cystathionine β synthase using vitamin B_6 as a cofactor (109). Studies have supported the association between atherosclerotic disease and elevated levels of homocysteine. The negative impact of homocysteine includes platelet aggregation (110) oxidative stress (111), vascular smooth muscle proliferation, decreased NO production (112), impaired endothelial function (113) and positive association with PAD (114) and CAD (115)

disease risk. Hyperhomocystenemia is considered as an independent risk factor for PAD (116). Deficiencies of folic acid and vitamin B_{12} can be detected through higher plasma homocysteine levels (60). High fasting total homocysteine (tHcy) has been shown in many studies to increase risk of developing PAD, varying from an odds ratio of 2.0 to 11.0 (109). Plasma homocysteine can be reduced with intake of folic acid, vitamin B_6 and B_{12} (109). An inverse correlation between plasma hcy level and folate, vitamin B_6 and vitamin B_{12} levels has been reported (117).

Furthermore, folate deficiency induces oxidative stress (118) and daily increased intake of folate and vitamin B_6 by 1 standard deviation decreases PAD risk by 46% with folate and 29% with vitamin B_6 (108). Weaker inverse associations have been found for vitamins B_6 and B_{12} with PAD risk. This could be due to some participants showing lower intake of these vitamins (119). This study has shown a 840 μg intake of folate resulted in a 33% PAD risk reduction in men (119). PAD patients who received 400 μg/day of folic acid or 5-methyltetrahydrofolate for 16 weeks experienced a significant drop in plasma tHyc, an improvement in vascular function and a significantly improvement in ABI in comparison to control patients (107). Similarly, another study found that folate supplementation (10 mg/day for 8 weeks) lowered homocysteine levels and significantly ($p<0.001$) improved vascular function (endothelial dilation) in PAD patients (113). Supplementation with a fortified dairy product (500ml/day) containing eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), oleic acid, folic acid, B6, Vit.A, E, D resulted in increased walking distance and ABI in PAD patients (120). Lowering homocysteine levels resulted in reduced (121), increased (122) and unchanged

effects (123) on cardiovascular events. The above mentioned studies demonstrate that the association between homocysteine and vascular disease risk remains controversial.

Vitamin B_{12} acts as a cofactor for methionine synthase, an enzyme that remethylates homocysteine to methionine by using 5-methyltetrahydrofolate as a methyl donor (109). Secondary folate deficiency can occur in vitamin B_{12} deficiency as vitamin B_{12} plays an important role in both the transport and storage of folate in cells (124). Deficiency of vitamin B_{12} or folate results in increased concentrations of serum total homocysteine (tHcy) which is associated with an increased risk of mortality in cardiovascular disease patients CVD patients (125). Further research is required to examine the effect of B vitamins in PAD patients. Due to a lack of clinical evidence, no recommendations have been established for Vitamin B, C, E and folic acid. (10).

2.7.5. Fibre

Fibre includes cellulose, hemicellulose, oligosaccharides, pectins, gums, waxes and indigestible non starch polysaccharides with each having specific physiological effects. Some important benefits of fibre include its ability to lower cholesterol, improve bowel motion and glycemic control, increase satiety and lower weight (126). High consumption of fibre containing foods such as whole grain cereals, fruits and vegetables are associated with a lower risk of CVD, hypertension, obesity and type-2 diabetes (127, 128). National Health and Nutrition Examination Survey (NHANES) data from 1999-2000 have shown an association between the highest quartile of fibre intake and CRP (OR= 0.64) (129). Also, an inverse association has been found between total dietary fibre (28.9 g/d) and CHD risk (RR=0.45) (130). Other studies have shown an inverse association between crude fibre intake and PAD (OR=0.33) (87) and (RR=0.49) at 10.3

g/day of cereal fiber intake (131). Participants with PAD tend to consume less fibre from whole grains and fruit and have a higher intake of saturated fat (132). PAD is significantly associated with low fibre and high fat (132). Fibre may play an important role in PAD partly by improving insulin sensitivity by slowing the absorption of nutrients from the gut (133) and reducing serum glucose levels (134). Furthermore, soluble fibre lowers LDL cholesterol (132). Additionally, soluble fibre intake of 10-20 g/d lowers total cholesterol and low-density lipoprotein (LDL) cholesterol in mild hypercholesterolemic patients (135). Higher cereal fibre intake may lead to a lowering of blood pressure, triacyglycerols and higher HDL cholesterol by improved glucose metabolism, thereby improving the risk profile for PAD (135). In a recent 8-week study conducted in PAD patients, a half cup serving daily of locally grown peas, beans and lentils lowered BMI, total cholesterol and LDL cholesterol. Consumption of pulses did not show any effect on triglycerides, HDL, cholesterol/HDL ratio and blood pressure. This study has also shown that a daily consumption of pulses may result in an improvement of blood vessel function in PAD patients. The beneficial impact of pulses are due to the presence of flavonoids that have anti-oxidant, platelet inhibitory, cholesterol lowering properties and also improve vascular reactivity. Three different flavonoids that are found in pulses with their functions are:-

a. Pterocarpans, which prevent vascular smooth cell proliferation.
b. Formononetin, which modifies arterial stiffness and reduces hypercholesterolemia

c. Anthocyanins which are isoflavones, stimulate adipocytes to secrete adiponectin, a cardioprotective hormone that improves cardiovascular health due to its anti-inflammatory properties in blood vessel cells (136).

Many studies have examined the role of fibre in weight loss but the role still remains inconclusive. However, studies have demonstrated that pulse consumers had lower body weight and reduced weight circumference as compared to non pulse consumers (137). Studies (138, 139) have explained the association of fibre on weight loss. Fibre increases the mastication time, creates a feeling of fullness and reduces the absorptive efficiency of the small intestine thereby regulating energy intake.

2.8.0. Macronutrients and PAD

2.8.1. Role of saturated and monounsaturated fatty acids

Replacing saturated fat (which increases LDL levels) with polyunsaturated fatty acids (PUFA) instead of replacing it with monounsaturated fatty acids (MUFA) or carbohydrates is protective against coronary heart disease (CHD) by reducing its risk among older men and women from a pooled analysis of 11 cohort studies (140). Decreasing energy intake from saturated fat by 5% and increasing energy intake from PUFA by 5% resulted in significant inverse association between PUFA and risk of coronary events (HR= 0.87) (140). A study conducted in Italian type-2 diabetic and control diabetic PAD patients consuming a traditional Mediterranean diet, depicted the preventive effect of healthy diet, independent of hypertension or diabetes duration (141). A healthy high dietary score (≥11) was associated with significantly decreased PAD risk (OR = 0.44). Further, the combination of healthy foods has beneficial effects in comparison to consumption of an individual food item. Saturated fat (OR=1.96), dietary

cholesterol (OR=6.07) and protein consumption (OR=2.86) have also been shown to be associated with increased PAD risk. PUFA and crude fibre decreased the PAD risk (OR =0.48 and 0.33 respectively) (87). Higher consumption of meat and meat products is significantly associated with lower ABI in males and females (54).

There are no specific dietary guidelines set up for PAD but the general guidelines for cardiovascular health are followed (142). Dietary recommendations for cardiovascular disease include consuming a diet high in fruits, vegetables and whole grains coupled with foods low in fat, saturated fat, sodium and cholesterol (143).

2.8.2. Role of Polyunsaturated Fatty Acids (PUFA)

From a dietary standpoint, the most widely recognized PUFAs are the ω-3 and ω-6 (n-6) series. With respect to potential impact on plasma lipids, lipoproteins, blood pressure and inflammatory markers, responses for the ω-6 fatty acids linoleic acid (LA; 18 C with 2 double bond) and arachidonic acid (AA; 20 carbons with 4 double bonds), are different from ω-3 fatty acids. The latter includes alpha-linolenic acid (ALA; 18 C with 3 double bond), eicosapentaenoic acid (EPA; 20 carbon with 5 double bonds) and docosahexaenoic acid (DHA; 22 C with 6 double bonds) (144). Dietary sources of ω-6 fats include poultry, eggs, vegetable oils, cereals, nuts and seeds (145). Dietary sources of ω-3 PUFA include fish, seafood (146), chicken, eggs, canola oil, soybean oil (147), flaxseed, hempseed oil and flaxseed oil (146) and in recent years from fortified foods such as bread, margarine, milk and eggs (66).

Many studies have reported an association between ALA levels and reduced cardiovascular events (148, 149). Regarding the impact of flaxseed consumption on triglyceride level, studies have shown a positive effect on reducing triglyceride levels (-

11% to -20%) (150, 151), whereas another study (152) did not report any changes in plasma triglyceride concentrations. In one of the studies (150) with younger participants, triglyceride concentrations decreased with flaxseed consumption (6 g/day of ALA from flaxseed) as opposed to older participants who did not show any decrease in blood triglyceride concentrations. One of the studies (152) has reported a significant reduction in plasma cholesterol levels with consumption of flaxseed at doses of 50 g/day while 25 g/day of flaxseed produced no reduction in cholesterol. Plasma ALA levels increased with flaxseed oil (6 g of ALA in oil) and milled flaxseed (30g) over a month period with no increase in plasma EPA or DHA (153). Milled flaxseed is the best form to increase plasma ALA levels significantly while creating no adverse gastrointestinal effect (153). Flaxseed supplementation in any of the three forms (oil, milled and whole seed) did not change plasma cholesterol or triglyceride levels (153). This study (153) has not found any anti-platelet aggregatory effect of a diet supplemented with flaxseed (ALA) as opposed to fish oil (EPA and DHA). In one of the studies with PAD patients consuming flaxseed, slight elevations of TC, LDL-C, TG and cholesterol/LDL ratio were recorded, but HDL-C and LDL/HDL ratio were found to be normal. This could be attributed to the fact that 74% of the patients were taking lipid lowering drugs (154).

Seafood is a source of the LC ω-3 PUFAs that have been beneficial in preventing cardiovascular events (155), often due to their anti-inflammatory actions (156) which decrease migration of monocytes into the intima of arteries and via reduced expression of adhesion molecules (98). PUFA are also known to have anticoagulant, anti-inflammatory and anti-aggregation effects and also positively influence endothelial

function and serum lipid profiles (157, 158). In vitro, fish oil consumption increased NO production by endothelial cells and reduced reactive oxygen species (159).

ω-3 PUFAs have been shown to be effective against CAD through effects on haemostatic function, inhibition of smooth muscle cell growth and changes in lipoprotein metabolism (160). An intake of 3 g/day of marine ω-3 PUFAs can lower fasting blood triglycerides by 30% (161). ω-3 PUFAs lower triglyceridemia, decrease the production of chemoattractants, growth factors, adhesion molecules, inflammatory eicosanoids and inflammatory cytokines. They also lower blood pressure, increase NO production, decrease thrombosis, endothelial relaxation and vascular compliance (162). The possibilities for similar beneficial effects of ω-3 fatty acids in PAD exist, as the underlying mechanism of pathogenesis is similar for PAD and coronary artery disease (163).

LC ω-3 PUFAs have generated substantial interest in relation to chronic disease risk. As these ω-3 PUFAs have a beneficial role in prevention of cardiovascular mortality these could be beneficial in PAD (41). However, ω-3 consumption did not show any beneficial effect on pain free and maximum walking distance in PAD study (164).

EPA and DHA can be protective against cardiovascular diseases due to their antiarrhythmic, antiatherogenic, antithrombotic effects (165) and they also decrease plasma TG (166, 167). DHA alone or with EPA and AA can change endothelial function and cause variability in heart rate (168). EPA and DHA increase erythrocyte deformability and reduce aggregation (169, 170). EPA and DHA (14g/day of fish oil for 6 weeks) supplementation decreased fibrinogen levels (171). DHA intake is associated with inhibition of oxidative stress by modulation of superoxide generation, anti-

inflammatory effects and a reduction in hypertension (172). Algal DHA supplementation in vegetarians at doses of 1.62 g/day for 6 weeks significantly increased DHA levels in serum PL's and decreased TG, TC/HDL ratio and LDL-C/ HDL-C (173). Studies have shown beneficial effects of ω-3 fatty acids (more than 3g/d) (174) in reducing blood pressure (175), with purified DHA (4g/d) being more beneficial than EPA in reducing blood pressure (176). One study has shown lowering of blood pressure with a lower dosage of DHA (0.7 g/day). When PAD patients were supplemented with 500 ml/d of an enriched-dairy product consisting of 2.1% EPA, 1.4% DHA, 54.4% oleic acid, folic acid (30 ng/100 ml) and other vitamins, there was a 3-fold increase in claudication distance, which directly correlates with plasma DHA concentrations ($r = 0.40$) (177).

In addition, it has been found that ω-3 PUFA intake was positively associated ($p=0.011$) with serum HDL cholesterol in Japanese men; this could be one of the reasons for the low mortality from CHD among Japanese men (178).

The above mentioned beneficial effects can be explained by the role of ω-3 and ω-6 PUFAs in the arachidonic acid pathway. Dietary lipid supply (ω-6 and ω-3) can alter prostaglandin and leukotriene production that can have pro and anti-inflammatory effects, respectively (179). EPA and DHA have anti-inflammatory effects (162) as they compete with AA for insertion into the sn-2 position of membrane phospholipids (41). These LC ω-3 PUFAs produce less potent eicosanoids than AA (41) and increase leucotriene B_5 levels and decrease leukotriene B_4 levels (180). Ω-3 fatty acids incorporate into plaque and have anti-inflammatory effects leading to stabilized atherosclerotic plaques and plaque repair (181) by decreased macrophage infiltration (182). LC ω-3 PUFA may help in retarding the growth of established atherosclerotic plaques, the development of new

plaques and they promote plaque stabilization (182, 183). LC ω-3 PUFAs may change the LDL particle size to one that is less atherogenic (146). Higher intakes of oleic acid, LA, ALA and total PUFA were associated with decreased risk of having PAD (OR= 0.47-0.57) (26). Benefits of PUFA intake are numerous as discussed in the above findings which may suggest a protective role of ω-3 PUFAs against PAD development.

Fish oil supplements reduce cardiovascular events (183) and have been supported by the FDA since 2006. In PAD patients, fish oil supplements (1g of EPA and 0.7g DHA) also improve ABI from 0.599 to 0.776 after 12 weeks of supplementation and also increased the time of walking before the onset of pain from 76.2 m to 140.6 m (184). ω-3 PUFAs are known to be beneficial by reducing blood viscosity and improving IC resulting in a small increase in LDL and total cholesterol (185). In individuals with PAD, dietary supplementation with canola oil (2.24 g/d of ALA) improved endothelial function and decreased total and LDL-cholesterol (186). ω-3 PUFAs improved endothelial dysfunction by improving brachial artery flow mediated dilatation and decreasing plasma soluble thrombomodulin in the group receiving an ω-3 PUFA supplements of 1g twice a day for three months (187). Other studies have also reported positive impact of ω-3 fatty acids on endothelial function (188, 189). EPA and DHA levels in erythrocyte membranes were correlated with an improved endothelial relaxation (significantly with EPA (r=0.71) and non significantly with DHA (r=0.536) (190). The possible mechanism behind the positive effect of ω-3 fatty acids on endothelial function could be due to changes in membrane fluidity of endothelial cells and promoting increased synthesis and release of NO, thus improving blood flow (190).

The mean intake of DHA in North America is 78 mg/day (191) and 47 mg/day or 0.02% energy among Canadian residents of a long-term facility (192). According to recent American dietary recommendations (2010), dietary saturated fat should be <7% of calories and replaced with MUFA and PUFA (193) that could reduce the TC:HDL ratio (194). The recommended nutrient intake of ω-3 PUFA for Canadians is 0.5% of energy or 1.1 gram of ω-3 PUFA per day for an individual consuming 2000 Kcal daily (195). According to the World Health Organization there should be regular fish consumption, 1-2 servings per week, with each serving contributing 200-500 mg of EPA and DHA per week, to protect against CAD. The scientific advisory committee on nutrition recommended 450 mg/day of EPA and DHA for the prevention of essential fatty acid deficiency and to reduce the risk of chronic diseases (196). Sufficient information is available to recommend increasing LC ω-3 PUFA intake in western diets.

According to the American Heart Association, dietary recommendations for CHD risk reduction include consumption of 1g/day of EPA and DHA. Adults should consume fatty fish at least twice a week (197). It is now known that high dietary saturated and trans fatty acids play a more important role than dietary cholesterol in increasing plasma cholesterol, a risk factor for CHD (198). Replacing saturated fat in the diet with PUFA has been shown to be effective in improving the fatty acid profile (reduction in LDL-C with HDL-C unchanged) (199), lowering total and LDL cholesterol (200) and reducing the risk of developing CHD.

2.9. DHA as a Biomarker

According to Willett (64), there are two important uses for biochemical indicators of dietary intake in epidemiological studies. The first one is as a surrogate for actual

dietary intake under conditions where measurement may be influenced by available food composition tables and within-food variation (storage, processing and preparation). A second use for biomarkers is to validate other forms of dietary assessments since biomarkers are generally used to measure true dietary intake. Biomarkers reflect actual dietary intake and nutrient metabolism (201), as well as correct biases reported in dietary assessment methods (202). In addition, random measurement errors from the biomarkers are independent of those from other dietary assessment methods since biomarkers are objective and do not rely on self-reports of food intake (203). Yet, biomarkers are difficult to interpret as they reflect endogenous and exogenous factors. The most optimal biomarker should be sensitive to small differences in dietary intake and reflect intake over the period of interest (206). In addition the biological material should be easily obtainable (202).

Biochemical measurements of particular nutrients in the blood can provide important information regarding nutrient intake (204). Dietary intake of a particular fatty acid and its level in the plasma or tissue lipid fraction depends on the range of intake of that fatty acid, the precision of the method used to measure intake, and the precision of the measurement of the plasma and tissue levels.

Saturated and monounsaturated fatty acids are synthesized endogenously and these fatty acid biomarkers have a poor correlation with diet as there is wide inter-individual and day-to-day variation (205). Polyunsaturated fatty acids of the ω-3 and n-6 series (due to reduced capacity of enzymes for elongation and desaturation) cannot be endogenously synthesized from carbohydrates (62). Therefore, these may be suitable for

use as biomarkers of dietary intake. Identification of an acceptable marker that could reflect increased LC ω-3 PUFA status in response to changes in dietary intake is lacking.

Membrane functions depend on the fatty acid composition of a membrane. This can easily be modified by diet as membranes respond to dietary fatty acids (ω-3 and n-6 fatty acids) by easily incorporating these dietary fatty acids. Their functions will be altered depending upon their fatty acid composition (206).

EPA and DHA are more biologically active than ALA (18-C fatty acid). In addition, DHA (22 C with 6 double bonds) contributes to the total bioactivity of ω-3 fatty acids. Approximately 5% to 10% of dietary ALA is converted to EPA and 2-5% is converted to DHA (207). Endogenous conversion of ALA to DHA is < 5% in humans (208). Due to limited conversion of ALA to DHA the major source of DHA intake is exogenous.

Biomarkers are required to define DHA status as the precise measurement of DHA intake is difficult (202). As DHA is an important fatty acid with a prime role in vascular health, it is important to study the dietary intake of DHA and its association with a valid biomarker in order to better understand the association between DHA intake and PAD.

In the literature, there are many known markers of dietary fat intake (e.g. cholesterol ester, phospholipid or triglyceride fractions of either serum, erythrocytes, platelets, adipose tissue, plasma or as free fatty acids) (64). Plasma fatty acid composition reflects the last 1-2 weeks of dietary intake, red blood cell fatty acids reflect the last 1-2 months and adipose tissue fatty acids reflect the intake of previous years (209, 210). Plasma PL fatty acids reflect intake over days (209) weeks or months (211).

Primarily DHA is carried in PLs rather than triacylglycerol and sterol esters as compared to EPA which is distributed equally in all three fractions (212). DHA concentration in serum, serum PL and erythrocyte membranes reflects short term intake or recent intake (days and months) on the basis of the assumption that individuals do not change their diet drastically in a short time frame (210). In determining DHA content, an adipose tissue biomarkers were preferably used for long term habitual fish consumption (213). Moreover, adipose tissue reflects dietary intake of the preceding 1-3 years of EPA and DHA concentrations, therefore, it is useful for studying the long term effects of dietary fat quality (213). Red blood cells (RBC) were found to be a good biomarker of EPA and DHA intake as they incorporate into RBC according to dose (66). Cholesterol ester and PL are independent of recent intake and can be used to study long term composition of fatty acid intake (214).

Biochemical measurements of DHA in the blood can provide important information regarding DHA intake. Biomarkers of DHA status that are often used are total lipid and PL-containing extracts of plasma and erythrocytes (202). Different supplementation studies have shown that doses ranging from 0.2 to 6 g of DHA/d (algal triacyglycerol sources or pure DHA ethyl esters) for 1-6 months results in an increase in plasma PL DHA concentrations in a dose dependent, saturable manner (212). However, DHA intake of doses up to 2 g/d reflects the proportionate increase in plasma DHA concentrations while doses above 2 g/d DHA result in a saturation of plasma DHA concentrations (212). In another study, combined daily supplementation of EPA and DHA (1.5, 3 and 6 g) caused a dose-dependent saturable increase (1.2 g/d) in plasma PL DHA concentrations (215). A study conducted to look at the kinetics of DHA

supplementation on plasma PLs and RBC DHA has shown that plasma PL DHA concentrations reach equilibrium within 1 month of the start of DHA supplementation. Conversely, RBC DHA concentrations reached equilibrium after 4-6 months as a result of a slower turnover of cells (212).

DHA concentration has been measured even at zero dietary DHA intake. Conversion of DHA from ALA prevents its complete absence from the blood. Further, an increase in DHA does not occur with ALA supplementation. Only DHA through diet increases DHA levels in blood and tissue (216). At lower levels of DHA intake, results in higher increase in plasma DHA (202) and higher increase in plasma PL (217). The higher the DHA intake, the lower is the proportional increase of DHA concentration in plasma PL has been reported (217). Single measurements of serum PLs reflect the habitual intake of EPA and DHA of marine origin in populations consuming higher and stable intakes of ω-3 PUFA (218).

Among Canadian long term care residents, LC ω-3 PUFA (PUFA: EPA + DHA + DPA) intake and ω-3 PUFA blood levels have been found to be significantly related. ω-3 PUFA levels of intake and their concentration in blood are lower than recommended (192).

In order to establish a link between dietary LC ω-3 PUFA and clinical outcomes, a biomarker for the assessment of LCPUFA status is required. Plasma concentrations of EPA and DHA derived from marine foods correlate positively with their relative dietary intake and, therefore, they may be useful biomarkers of relative ω-3 LCPUFA intakes irrespective of smoking, alcohol and habitual exercise (219). Plasma EPA and DHA have been reported as useful biomarkers for assessing the dietary intake of respective fatty

acids (220). Habitual intake of fish and cod liver oil was reflected in plasma PLs. EPA and DHA were positively associated with total fish intake (220). Plasma PL DHA and EPA increased with increased intake of fish and fish supplements (220).

The correlation coefficient between LC ω-3 PUFAs and plasma EPA and DHA was r = 0.566 and 0.574 in men and r = 0.602 and 0.303 in women, respectively (219). One study has reported a correlation between DHA intake from marine sources using FFQ. Analyzed by using the Canadian Nutrient File, and erythrocyte DHA (r = 0.4) (221). Another study reported r=0.42 between DHA intake from FFQ and plasma PL DHA (211). Sun et al., (222) also found a better correlation of DHA intake and erythrocyte DHA (0.56) rather than plasma DHA (0.48). The correlation coefficient between intake of DHA and serum PL DHA was found to be r = 0.53 (223). Taking into account the limited endogenous conversion of dietary ALA to EPA and DHA, several studies have reported DHA as a valid biomarker to study LCPUFA intake. These studies are summarized in Table 2.2.

Table 2.2 List of studies reporting the use of DHA as a biomarker for ω-3 intake from marine foods.

Author name, Year	**Title of study**	**Subject description**	**Biomarker reported/ dietary intake**	**Appears effective as a biomarker**
Sullivan, 2006(66)	Biomarker validation of long chain ω-3 polyunsaturated fatty acid food frequency questionnaire	Male=20 Age-38+/-12 yrs Female=33 Age-32+/-11 yrs	RBC and plasma/LC ω-3 PUFA(EPA, DHA) intakes	RBC and plasma EPA, DHA
Marckmann, 1995 (213)	Biomarkers of habitual fish intake in adipose	Men=17 Women=7 Age-20-29 yrs	Adipose tissue/fish and marine ω-3	Adipose tissue DHA is the best indicator

	tissue		PUFA intake	of fish intake.
Kobayashi, 2001(218)	Single measurement of serum phospholipid fatty acid as biomarkers of specific fatty acid intake in middle-aged Japanese men	87 male participants Age=57+/-5.0 yrs	Serum phospholipids /habitual intake of marine ω-3 PUFA	Serum phospholipids EPA,DHA
Kuriki, 2003 (219)	Plasma concentrations of ω-3 polyunsaturated fatty acids are good biomarkers of relative dietary fatty acid intake: A cross – sectional study	Men=15 Age-45.3+/-10.6 yrs Women=79 Age-47.2+/-8.1 yrs	Plasma EPA, DHA/marine food	Plasma EPA,DHA might be useful as biomarker
Sun Qi, 2007(222)	Comparison between plasma and erythrocyte fatty acid content as biomarkers of fatty acid intake in US women.	Women=306 Age=-43- 69	Plasma and erythrocyte /marine ω-3 PUFA	Erythrocyte and plasma DHA
Hjartaker,1997 (223)	Serum phospholipid fatty acid composition and habitual intake of marine foods registered by a semiquantitative food frequency questionnaire	234 subjects Age-40-42 yrs	Serum phospholipids /habitual intake of fish	Serum Phospholipids DHA
Hodge, 2007 (224)	Plasma phospholipids fatty acid composition as a	Men=2048 Age-55yrs Women=2391 Age-54.4yrs	Plasma phospholipids /fish intake, fish oil supplements	Plasma phospholipids DHA,EPA

	biomarker of habitual dietary fat intake in an ethnically diverse cohort			
Fuhrman, 2006 (225)	Erythrocyte membrane phospholipids composition as a biomarker of dietary fat	n=93, Pre-menopausal women Age-43.7 yrs n=104 Post-menopausal women age-57.1ys	Erythrocyte phospholipids (EPL) /fish and shellfish intake	Erythrocyte phospholipids EPA,DHA
McNaughton 2007 (226)	Validation of a FFQ to estimate the intake of PUFA using plasma phospholipids fatty acids and weighed food records	Men=18 Women=25 Mean Age for men-53.8, for women-49.5	Plasma Pl fatty acid /dietary intake	Plasma PL DHA

The serum PL fraction also reflects metabolism of fatty acids better than the cholesterol esters and triglyceride fractions (147). The fatty acid composition of serum PL fraction is similar to RBC membranes. RBC's are a good biomarker of total ω-3 PUFA and DHA intake, but they reflect the previous 120 days of intake of LC ω-3 PUFA. Serum reflects the recent intake of last 5-7 days. Plasma PL can also reflect the dietary intake of previous months. It can be concluded from the above mentioned studies that plasma DHA, plasma PL DHA and RBC membrane PL DHA can be a useful biomarkers for the assessment of short and long term DHA intake.

2.10. Factors Affecting DHA status, conversion of ALA to EPA, DPA and DHA

Smoking affects DHA status. There are products in cigarettes that inhibit the conversion of ALA to DHA (227). ALA is elongated to EPA, DPA and DHA at higher

rates in women as compared to men (228, 212). Dietary DHA and EPA can down regulate the conversion of ALA to DHA by 70% (229). Also, a high dietary intake of LA (ω-6) may affect conversion of ALA to EPA and DHA (230) through competition with elongase and desaturase enzymes (231, 232). ALA and LA conversion by delta-6 desaturase can also be inhibited by saturated fat, cholesterol and aging (233). Therefore, despite adequate intake of ALA or LA, conversion can be inhibited resulting in a lack of these nutrients in the body. Use of medications can also alter the conversion of ω-3 fatty acids into long chain PUFAs. Statins such as gemfibrozil can alter the association between dietary fatty acids and plasma PL (224). Aspirin inhibits cycloxygenase activity resulting in lesser production of the vasoconstricor thromboxane A2 and increasing amounts of prostacyclins (234). The factors affecting fat metabolism and serum DHA fatty acid concentration are listed in Table 2.3.

Table 2.3. List of factors affecting fat metabolism and serum DHA fatty acid concentration

Factor	Comment	References
Smoking	lowers plasma and serum DHA levels	64, 235
Genetic factors	alters lipoprotein levels	64
Alcohol intake, Trans fat	alters DHA level	236, 237
Age	EPA and DHA concentrations are greater in women as compared to men with similar intake of LCPUFA reflecting greater synthesis of DHA in women. Age affects delta-6 desaturase activity in postmenopausal women	225
Body mass index	negatively affects delta-9 desaturase activity of pre and postmenopausal women	225
Systemic diseases	chronic nephritis, cirrhosis of liver, cystic fibrosis, pancreatic disease	209 238, 239

	alter lipoprotein level, diabetes, Zellweger syndrome – decreases DHA synthesis, malabsorption of fat,	
Hormones	women taking oral contraceptives had higher conversion of ALA to as compared to non-consuming ones, upregulation of elongase and desaturase enzyme activity by oestrogen	228
Metalloenzymes deficiency	decreases fatty acid metabolism, genetic polymorphisms of elongase and desaturase enzymes	209, 202

All this aforementioned evidence supports the use of plasma, plasma PL and RBC membrane PL DHA as biomarkers for DHA intake. This requires further confirmation by the use of dietary assessment tools for DHA intake. To determine accurate habitual dietary ω-3 intake, a valid dietary assessment tool with the use of DHA as biomarker is required. The following section will focus on the 3DFR and FFQ as dietary assessment methods and DHA as a biomarker for estimating the ω-3 intake of PAD participants.

Further, biomarkers may be considered objective since they cannot be modified by the subject and recall of food items previously consumed is not needed (240). Also, biomarkers are not associated with portion size estimation and food composition databases (63).

2.11. Statement of Problem

Diet has a great impact on cardiovascular or metabolic co-morbidity (241, 242) especially high fat diets that impair endothelial function (243, 244). High fat diets could adversely affect the poor walking performance in patients with PAD and can also influence exercise performance (245). Despite the known benefits of a healthy diet on vascular health, little is known about the dietary pattern of individuals suffering from

PAD (10, 26). A high prevalence of PAD demands preventive measures so as to reduce the incidence of PAD among affected populations.

One direct cost-effective and non-invasive step towards the prevention of PAD is to examine the dietary patterns of PAD patients. Steps towards the prevention of PAD in terms of diet include gathering knowledge about the effect of diet, particularly fatty acid profiles, on PAD. The effect of the fatty acid profile on cardiovascular disease is known (140), but the effect of the fatty acid profile on PAD is not fully established. Therefore, in order to understand the relationship between dietary patterns and PAD, particularly the effect of fatty acid profile on PAD, we need to have detailed information about the fatty acid profile of PAD patients. Additionally, little information is available regarding the relationship between dietary patterns and PAD. Dietary advice in PAD patients is possible, but only if detailed information on their dietary profile is available. This purpose can be fulfilled with the collection of data on dietary fatty acid intake and fatty acid analysis.

In order to collect information about the patient's diet, it is critical to undertake dietary assessment methods. However, all current methods of dietary assessment have limitations and none of them are 100% reliable as a true measure of intake (246). Thus, a dietary assessment method that efficiently and accurately measures nutrient intakes in PAD patients is needed. Continuous educational interventions and nutritional observations, including dietary assessment and ongoing research endeavors, are required to reduce the risk of PAD among the affected population, which could be expected to result in reduced health care, societal costs and improved quality of life of PAD patients.

With all the aforementioned findings, the importance of diet and specific nutrients in the prevalence of PAD has been established. Therefore, a valid and precise dietary assessment tool may aid in establishing the nutrient intake of PAD patients, providing further information on key dietary risk factors. Considering the importance of LC ω-3 PUFAs in PAD, an accurate assessment method of their intake, especially DHA, could be useful in health care settings and in nutritional research.

In the past, FFQs have been used to estimate the intake of LC ω-3 PUFAs in comparison with 3DFR in a normal healthy population of Australian subjects (67, 70). To this end, no study has explored the use of the FFQ and 3-day food record as a dietary assessment method in PAD patients. Therefore, we propose to use the FFQ and 3DFR as a dietary assessment tool for estimating usual dietary intake in PAD patients.

In this study, two methods were employed: (a) 3DFR in which foods consumed for 2 weekdays and one weekend day were recorded on a form provided to the patients (instructions relating to the supplement type, food serving size, food consumed and memory aids for portion size estimates were provided) (b) FFQ with the aid of pictures of various foods that help in recalling the foods eaten.

In addition to the dietary assessment methods, biomarkers can be used as an alternative to dietary assessment methods to investigate the relationship between nutrient in blood which is a reflection of particular nutrient in diet (if that particular nutrient in the diet correlates with same nutrient in blood) and disease. Biomarkers measure true intake and they are used to validate dietary assessment methods against the specific nutrient in question. For the LC ω-3 PUFA, suitable biomarkers would be plasma, plasma PL and RBC membrane PL DHA concentrations.

With respect to the dietary lipids, dietary assessment methods using biomarkers of LC ω-3 PUFA that reflect intake of LC ω-3 PUFA are required. Therefore, this study used 3DFR and FFQ to estimate the DHA intakes of PAD patients and plasma, plasma PL and RBC membrane PL DHA as biomarkers to reflect DHA intake. Overall, these data will fill a significant gap in the literature. The use of dietary assessment methods also represents an important approach for the determination of nutrient intake of PAD patients (83), thus further helping to explore the important association between diet and PAD. This has implications in terms of morbidity and mortality rates for PAD patients.

The current literature provides information on the role of omega-3 fatty acids in CVD. However, information on the role of omega-3 fatty acids as well as the dietary habits of PAD patients is scarce. To gather this information, this study focused on the dietary habits of PAD patients with the aid of dietary assessment methods. Biomarkers of omega-3 intake were used which are independent of nutrient intake data gathered from the dietary assessment methods. Due to the importance of diet in the management of PAD, it is assumed that the presented research will lead to the development of data to understand the dietary habits of patients with PAD.

3.0 Hypothesis and Objectives

3.1. Purpose

The purpose of this study was to measure the habitual dietary intake, including macronutrients and ω-3 intake, of PAD patients using dietary assessment tools. To compare the dietary intake of PAD participants obtained using these dietary assessment tools with dietary recommendations of National Cholesterol Education Program (NCEP) and dietary reference intake values recommended by the Institute of Medicine (IOM) of the National Academy of Sciences. In addition, this study compared 3DFR and FFQ for LC ω-3 PUFA (DHA) intake with plasma, plasma PL and RBC DHA, respectively.

3.2. Hypotheses

We hypothesize that there exists an association between dietary DHA intake as measured with 3DFR and plasma, plasma PL and RBC membrane PL DHA concentrations in PAD patients. We also hypothesized that that there is an association between dietary DHA intake as obtained from FFQ and plasma, plasma PL and RBC membrane PL DHA concentrations. As stated earlier, plasma, plasma PL and RBC membrane PL DHA concentration may reflect the dietary LC ω-3 PUFA (DHA) intake. We also hypothesize that the PAD participants may not meet or exceed the dietary recommendations as given by IOM and NCEP. Based on these hypotheses, the objectives and sub-objectives of this study were:

1. To examine the association between plasma, plasma PL and RBC membrane PL DHA and 3DFR as a dietary assessment method for LC ω-3 (DHA) PUFA intake.
    a. To estimate the DHA intake using the 3DFR and compare with plasma, plasma PL and RBC membrane PL DHA.

b. To estimate the total dietary fat, fatty acids and cholesterol in PAD patients using 3DFR.

c. To quantify dietary lipid intake using 3DFR.

2. To examine the association between plasma, plasma PL and RBC membrane PL DHA and FFQ as a dietary assessment method for LC ω-3 (DHA) PUFA intake.

a. To estimate the DHA intake using the FFQ and compare with plasma, plasma PL and RBC membrane PL DHA.

b. To estimate the total dietary fat, fatty acids and cholesterol in PAD patients using FFQ.

c. To quantify dietary lipid intake using FFQ.

4.0. Materials and Methods

4.1. Study Design

This was an exploratory study of the nutritional status of PAD patients and used 3DFR and FFQ as dietary assessment tools for the assessment of ω-3 PUFA intake focused on the DHA intake of PAD participants. This was not an intervention study but a descriptive study that looked into the association between DHA intake of PAD patients and plasma, plasma PL and RBC DHA. This was a single site study designed to provide information on the dietary habits of PAD patients at the Asper Clinical Research Institute, St. Boniface Hospital Winnipeg, Manitoba. Participants were recruited from the vascular surgery clinic and via screening of vascular disease databases under the supervision of vascular surgeon Dr. Randy Guzman. All the participants met the inclusion and exclusion criteria and signed the consent form before participating in the study. A total of 30 PAD participants were recruited for this study. The participants were from a larger eight week egg intervention study (n=60) For this study, participants were asked to attend two in-person clinic visits over the 2-week duration as compared to four in person clinic visits for the 8 week study.

Participants did not receive direct compensation for participation in this study; however, they were reimbursed in the amount of $15.00 per in-person visit for costs associated with parking and transportation.

For this study, due to time and limited resources we have used an existing FFQ that has been developed and validated against for the four food records selecting non-consecutive days to estimate the usual long term food consumption among Montreal adults aged 18-82 years (71). FFQ is designed as a 8-1/2" by 11" booklet. This FFQ

consists of 75 foods categories and includes ancillary diet questions (that act as a quality filter of FFQ data collected) in addition to anthropometric, lifestyle and socio-demographic questions. In order to estimate portion sizes photos of similar food items are provided in the food guide. This FFQ has specific intervals such as 1-2 times per week, 1-3 times per month, once a day) for estimating frequency of particular food item consumed. This allows the patient to estimate food frequency in the various frequency ranges available. In addition, this FFQ has separate frequency and portion size ranges for beverages and food items thus differentiating food items and beverages more appropriately.

Participants underwent an FFQ interview which took 20-25 minutes with the interviewer. After finishing, the FFQ participants were given instructions to fill a 3-DFR at their first visit. Participants were asked to bring the filled 3-DFR at their next visit (visit 2), which was 1-2 weeks after the first visit (visit 1).

4.2 Recruitment of Participants

Recruitment efforts: The peripheral vascular disease database of the Asper Clinical Research Institute was screened for recruitment of PAD patients in 2010. In order to recruit patients (n=30) meeting the inclusion criteria for the study, approximately 700 patients were screened. This database included some patients having coronary artery disease. In this database, 60% of the patients were smokers and diabetic. Due to the strict inclusion criteria (no smokers and patients having controlled diabetes) very few patients met the inclusion criteria thus making the recruitment of the participants a challenging task. Participants that were involved in earlier studies conducted at the Asper Clinical Research Institute and had already given their consent for consideration for future studies

were also screened. As well, few patients who had recently diagnosed with PAD were also recruited from the ABI clinic of Dr. Randy Guzman at St. Boniface Hospital. Some patients who were ex-smokers (had quit smoking for at least 6 months) were also contacted; however, those who had become diabetic were recruited. Some patients who were consuming omega-3 supplements and flax seed were recruited after they had stopped consuming omega-3 supplements and flax seed for 3 months. Women patients taking post-menopausal hormones were also screened out. This highlights the fact that recruitment was a very challenging and slow process due to the strict inclusion criteria followed for this study. The patients were recruited from Manitoba and the surrounding areas, including the towns of: Libau, East Selkirk, Plumas, Lac Du Bonnet, Gimli.

Table 4.1 Criteria checklist (inclusion/exclusion) followed for the study

Inclusion	**Exclusion**
1) Presence of ABI <.90 at rest and reactive hyperemia or asymptomatic carotid stenosis of >50%. Newly or previously diagnosed with PAD or who had undergone surgical intervention.	1) Renal failure requiring dialysis
2) Male or female >50 years of age	2) Confirmed diabetes that requires diabetic medication. Participants with diabetes who do not require diabetic medication and have good glycemic control will be eligible for participation in the study. Participant with elevated fasting blood glucose levels and HbA1c above 'standard of care' at baseline will not proceed with the study
3) Willing to provide informed consent	3) Severe dyslipidemia. Hyperlipidemia as defined by a LDL-cholesterol of >4.1 mmol/L (as per the National Cholesterol Education Program Adult Treatment Panel III, (NCEP ATP III) guidelines
4) Willing to comply with protocol requirements	4) Current smoking or smoking within the last 6 months

5) Stable medication profile	5) Hormone replacement therapy
6) Participants having completed the Flax study are eligible to participate if their completion has been more than 3 months.	6) Inability to adhere to a regular diet
	7) Habitual egg intake of 5 or more eggs per week
	8) History of gastrointestinal reactions or allergies to eggs
	9) Daily consumption of omega-3 supplements

4.3. Study Procedures

The participants recruited for this study had moderate hyperlipidemia for PAD patients and comprised of both men and women. Participants followed the interview format described below in Table 4.2.

Table 4.2: Interview format followed in the study.

Interview Period	**Purpose**
Interview 1 (phone interview)	Information session
Interview 2 (Visit 1)	Consent form, FFQ (by interviewer, first dietary assessment method), ABI measured, instructions on 3DFR, instructed to fast prior to next visit
Interview 3 (Visit 2)	Blood sampling, 3DFR (filled by the subject, second dietary assessment method), supplement recorded

Participants were contacted by phone to ask if they were willing to participate in this study. General information about the study was given to the participants on the phone. In response to their interest in this study, they were asked to come to the research institute for screening.

Visit 1: Participants attended a detailed information session about the study. They were asked to sign the consent form prior to conducting any study-related procedures (Appendix 1). During this visit, PAD patients underwent a criteria checklist (Table 4.1) at the Asper Clinical Research Institute and St. Boniface Hospital to ensure eligibility. Eligible participants were interviewed at the Asper Clinical Research Institute, Winnipeg, MB. This information session and the consent form provided basic information on the nature of the research to the participants and what participation in the study involved. As well, it included permission to conduct analysis on the biological specimens that were collected and stored.

A medical history including age, sex, alcohol usage, exercise habits, current medical problems, medication intake and a family history of previous events (e.g. stroke, hypertension, diabetes and heart disease) was taken. Anthropometric measurements were also taken at this visit.

Anthropometric measurements

These measurements were taken during visit 1. The participants were wearing light clothing and no shoes during the measurement of weight and height. Body weight was measured with the weighing scale to the nearest 0.1 kg. Height was measured with a movable stadiometer to the nearest 0.1 cm. BMI was calculated using participants weight and height. Waist circumference was measured with a measuring tape midway between the lateral lower rib margins at the iliac crest in a standing position. The pulse was recorded by palpitating the radial artery for number of beats per 1 minute. Respiration

rate was calculated by observing at number of inhalations and exhalations during normal breathing in 1 minute. Blood pressure was determined by placing a pneumatic cuff on the left arm and blood pressure is raised. After releasing the pneumatic cuff the systolic and diastolic blood pressures were recorded using sphygmomanometer. Briefly, after 5 minutes of rest, the arterial blood pressures was measured in supine position from right and left arm brachial artery and posterior tibial or dorsalis pedis artery in the ankle by using Doppler 8-MHz ultrasound probe. Ankle brachial index (ABI) was calculated by dividing the systolic blood pressure measured at ankles by the systolic pressure measured at the arm. ABI was used to recruit the patients for the study and further analysis. Participants were interviewed during the morning or afternoon hours and the interviews lasted for about 2 hrs during visit 1 at Asper Clinical Research Institute, Winnipeg, Manitoba. Participants were recommended to adhere to their usual diet. During this visit, Physical Activity Levels (PAL) were recorded as levels 1, 2, 3 or 4. Physical activity levels were defined as 1= mainly sedentary (daily living activity include household tasks and walking to bus), 2=low active (30-60 minutes/day of moderate activity and daily activitity), 3= Active (daily living activity plus 60 minutes or more of moderate activity), 4=very active (daily living activity plus 120 minutes a day of moderate activity and 60 minutes of vigorous activity). Physical activity levels were used to determine the misreporting of energy intake in the study. In order to calculate the total energy expenditure in the participants following calculations were used (270):

1. Men 19 years and Older (BMI 18.5-25)
TEE = 662 - 9.53 × age (yr) + PA × [15.91 x Wt (kg) + 539.6 × Ht (m)]

2. Overweight and Obese Men 19 years and Older (BMI 25 +)
TEE = 1086 – 10.1 × age (yr) + PA × [13.7 x Wt (kg) + 416 × Ht (m)]

3. Women 19 years and Older (BMI 18.5-25)
TEE = 354 – 6.91×age (yr) + PA × [9.36 × Wt (kg) + 726×Ht (m)]

4. Overweight and Obese Women 19 years and Older (BMI 25 +)
TEE = 448 – 7.95 × age (yr) + PA × [11.4 x Wt (kg) + 619 ×Ht (m)]

Nutrient intake of all participants was assessed by using a dietary assessment tool: the FFQ. The FFQ was used to estimate the usual dietary patterns of participants over the last 12 months. The 3DFR forms were provided to all participants with detailed instructions from the interviewer on completing the form to determine food preferences. The 3DFR was recorded by the participants on two weekdays and one weekend day to capture variability in dietary intakes. During the visit, participants were also asked to bring any nutritional supplements and/ or medications they were currently taking to the next scheduled interview (visit 2) for the interviewer to record supplement usage by the patients. Medications were documented, as some medications interfere with nutrient (fat) absorption and/or cognitive status.

Visit 2: Venous blood samples were drawn at this visit following an overnight fast. Blood samples were placed on ice and transported within 2 hr to the laboratory for analyses. Plasma and RBC were separated and stored at -80°C. The samples were used to determine plasma, plasma PL, RBC membrane PL fatty acids (DHA) and plasma fasting lipid profiles (triglycerides, total cholesterol, LDL cholesterol and HDL cholesterol). Fasting triglycerides, HDL, total cholesterol, glucose and insulin were measured by radioimmunoassay using a Roche Cobas c501Clinical Chemistry Analyzer (Roche Biodiagnostics, Laval, QC). LDL cholesterol was calculated from plasma total cholesterol and HDL cholesterol. HbA1C was measured using the Roche Cobas Integra Clinical Chemistry Analyzer (Roche Biodiagnostics, Laval, QC).

During this visit, completed 3DFR forms were also collected from participants and reviewed for completeness and supplement usage. Data obtained in the 3DFR methods was

entered into the Food Choice Map (FCM) database. The FCM database has food consumption data from 1462 food items, including international and ethnic foods, therefore making it convenient for the measurement of food intake of various populations. Estimates of all the nutrients in the FCM database are from the Canadian Nutrient File database 2007b (247) and United States department of Agriculture database (USDA) values (248) to determine nutrient intakes. Copies of the FFQ were sent to Institut Universitaire de Gériatrie de Montréal for further analysis using DietSys. software (249).

4.4. Biochemical Analyses

Sample preparation

Following overnight fasting for 12 hours, i.e. from 8 p.m to 8 a.m, blood samples were obtained from the partcipants. About 4 ml of blood was centrifuged and spun at 2000g for 10 min at 4°C. The top layer containing the plasma was aliquoted to one 1 ml microcentrifuge tube and two 300µl microcentrifuge tubes and stored at -80°C until further analysis. The bottom layer containing RBCs was transferred to a 15 ml conical tube. In order to wash the RBC's, 2 ml of RBC was mixed with five times the volume of 0.9% normal saline (2ml RBC + 10 ml 0.9% normal saline). The RBCs were gently dispersed by mixing it with a plastic pipette. The RBCs were centrifuged at 650g for 10 min at 4°C. The saline was aspirated and discarded. The same process was repeated twice. The RBCs were resuspended in phosphate-buffer saline (PBS) double the volume of the RBCs, i.e. if there were 2 ml of RBC then 4 ml of PBS was used. One ml and two 300 µl aliquots of RBCs were created. RBC aliquots were stored at -80°C until further analysis.

Plasma total fatty acids

Plasma samples were removed from the freezer and fatty acid analysis was carried out by using the following method. Total plasma lipids were separated by 5 ml chloroform/methanol (2:1) with 0.01% butylated hydroxytoluene (BHT), using 200µl of C15:0 standard (0.64 mg/mL C15:0 as 1,2-dipentadecanoyl-sn-glycero-3-phosphocoline, Avanti Polar Lipids, Alabaster, AL) in 350 µl of plasma sample. After vortexing and centrifugation, the bottom layers were rinsed with chloroform:methanol:water in the ratio of 3:48:47. The bottom layer collected was evaporated to dryness under nitrogen in a 30°C water bath. In the extracted lipids, 3.5 ml of toluene with 0.01% BHT was added. One ml of the 3.5 ml solution was used for methylation of the extracted plasma lipids for plasma fatty acids, and the remaining 2.5 ml was used for thin layer chromatography and methylation for plasma phospholipids. The samples were then flushed with nitrogen and stored at -20°C or used to continue with the methylation steps.

The extracted lipids were methylated by adding 1.2 ml of methanolic HCl to 1 ml of extracted plasma sample. The samples were placed in a preheated 80°C oven for one hour, and then 1 ml deionized water was added to each tube. The tubes were capped, vortexed and centrifuged for 5 minutes at 2000g. The top layer was transferred to clean 8 ml tube and 1 ml petroleum ether was added to the bottom layer followed by vortexing and centrifugation. The top layer was collected and added to the previously collected top layer and the bottom layer was discarded. To the combined top layers, 2 ml deionized water was added followed by vortexing and centrifugation. The top layer was transferred to GC vials and evaporated under nitrogen in a warm water bath. Once the solvent was evaporated, 150 µl hexane was added to each GC vial and the samples stored in a -20°C freezer until GC analysis.

Plasma phospholipid fatty acid analysis

Plasma phospholipids were separated from the triglycerides by TLC using K6 Silica Gel 60 A plates (4860-820, Whatman, Richland, OH). The TLC plates were scored using a scalpel without a blade and the plates were placed in a preheated oven at 120°C for 30 minutes. The plates were removed after 30 minutes and cooled in a dessicator and spotted with 50µl samples using a Hamilton syringe. The mobile phase was prepared by using petroleum ether (80ml): ethyl ether anhydrous (20ml): glacial acetic acid (1ml) and the solvent was poured into the chromatography chamber tank. The plates were placed in the solvent chamber tank until the solvent reached a point 1 cm from the top of plate. The plates were sprayed with 0.1% 8 anilino-1-naptahline-sulfonic acid (Sigma-Aldrich, St Louis, MO) for the identification of the phospholipid band, visualized using U.V. light. The band corresponding to the phospholipid fraction was scraped with a razor blade onto weighing paper and transferred to individual test tubes. One ml toluene with 0.01% BHT was added to each test tube containing the phospholipid fraction. The tubes were flushed with nitrogen, capped and stored at -20°C, or continued directly to the methylation procedure, following the steps described above.

RBC fatty acid analysis

RBC samples were removed from the freezer and allowed to thaw at room temperature. About 400µl of the RBCs suspended in phosphate-buffer saline (PBS) were added into the 12 ml screw top test tubes and lipids were extracted by adding 80 µl of C15:0 as standard (0.64 mg/mL C15:0 as 1,2-dipentadecanoyl-sn-glycero-3-phosphocholine, Avanti Polar Lipids, Alabaster, AL) and the same procedure was followed as described above for plasma fatty acid analysis. In the extracted lipid, 2.5 ml

of toluene with 0.01% BHT was added, flushed with nitrogen and stored at -20°C or directed towards immediate methylation. The samples were evaporated to dryness and 50 µl 2:1 chloroform:methanol with 0.01% BHT added. All 50 µl of the sample was loaded on the chromatography plate and RBC membrane phospholipids were collected, using the same TLC procedures as described above for plasma phospholipids. The RBC membrane phospholipids were methylated using the same methylation steps as described above for plasma and plasma phospholipid fatty acids. In the last step, the solvent was evaporated, 50 µl hexane was added to each GC vial and the samples stored in a -20°C freezer until GC analysis.

The methanolic HCl (mHCl)-methylated lipids from plasma, plasma phospholipid and RBC were separated on a DB225MS column (30 m × 0.25 mm diameter and 0.25 µm film thickness; Agilent Technologies Canada Inc., Mississauga, Ontario) using a Varian 450 GC with flame ionization detection. The temperature program was 70°C for 2 min, then the temperature was raised to 180°C at 30°C/min, held for 1 min, raised to 200°C at 10°C/min, held for 2 min, and raised to 220°C at 2°C/min and held for 10 min. and finally raised to 240°C at 20°C/min and held for 5 min. Total run time was 36.67 min, and samples were run with a 10:1 split ratio and a 1.3 ml/min column flow. Hydrogen was used as the carrier gas for the method.

4.5. Statistical analysis

Statistical analysis was conducted using SPSS software (version18.0. Chicago: SPSS Inc.) with a significance level of 5%. Descriptive statistics (mean, standard deviations and ranges) were calculated for dietary intake data, anthropometric data, biochemical data and blood fatty acids (plasma, plasma PL and RBC membrane PL)

using SPSS. Associations between dietary DHA intake from 3DFR and plasma, plasma PL and RBC membrane PL DHA and also between dietary DHA intake from FFQ with plasma, plasma PL and RBC membrane PL DHA were assessed by Spearman correlation coefficients.

The ability of 3DFR and FFQ to correctly classify individuals was determined by assigning quartiles for DHA intake and biochemical indices (plasma, plasma PL and RBC DHA) of participants. Nutrient intake values from FFQ and 3DFR were assigned to quartiles. Similarly, quartiles were generated for plasma, plasma PL and RBC DHA. The ability of 3DFR and FFQ to correctly classify individuals into the same or adjacent quartiles was assessed by comparing the quartiles of DHA intake with quartiles of plasma, plasma PL and RBC membrane PL DHA. Under the hypothesis that there is no association between the quartile of actual biochemical values and the quartile based on nutrient intakes, it would be expected to obtain 25% of participants in the correctly classified category, 37.5% in the closely classified category and 37.5% to be in the misclassified category. The accuracy of the 3DFR and FFQ for correct classification of participants for DHA intake and biochemical indices was tested by comparing proportions of correctly, closely classified and misclassified participants using a Chi square goodness of fit test with 2 degrees of freedom. A model was created by including all of the variables that could affect DHA concentrations in plasma, plasma PL and RBC membrane PL DHA. Using multiple linear regression analysis, various independent variables (age, BMI, PAL, DHA intake, alcohol consumption, gender, HDL and LDL) were chosen to examine the association of these variables with dependent variables (plasma, plasma PL and RBC membrane PL DHA).

CHAPTER 5

RESULTS

Characteristics of the participants are presented in Table 5.1.

Table 5.1 : Characteristics of the PAD participants (n=30)

Patient Characteristics	Mean[1]	SD	Range (Min-Max)
Age (yrs)	72.6	6.5	51-81
Weight (Kg)	81.3	13.7	50.9-101.5
Height (cm)	170.2	8.5	152-188
BMI (Kg/m^2)	28.2	4.2	21.1-38.9
Waist circumferences (cm)	99.5	13.2	74-119
Pulse (P/min)	64	9.2	46-84
Respiration rate (RR/min)	19.9	0.91	16-22
Right ABI	0.9	0.2	0.41-1.43
Left ABI	0.9	0.2	0.31-1.33

[1]Arithmetic value

With respect to the activity levels (1, 2, 3 or 4) the percentage of sedentary (activity level 1), low active (activity level 2) and active participants (activity level 3), based on PAL, was 40%, 40% and 20% respectively. In total, 47% of participants consumed multivitamin supplements, with an average number of supplements consumed equivalent to 1.23 (SD =1.53; range = 0-5) supplements per day. Data derived from the FFQ indicated that 5% of the participants identified themselves as poor, 81% as adequate/self-sufficient, and 14% as well-off. With respect to highest level of education obtained, 5% of participants completed elementary school, 38% of participants completed 8^{th} through 11^{th} grade education, 10% of participants completed secondary V or grade 12, 29% completed CEGEP or trade school, 5 % completed an undergraduate degree, 5% completed a postgraduate degree and 10% of participants completed some years of university degree.

A large proportion of the PAD participants were on beta blockers, calcium channel blockers, angiotensin converting enzyme inhibitors, aspirin 81 mg and statins. Some participants were on medications such as alpha blockers, angiotensin receptor blockers, warfarin, antiarrthymic drugs, diuretics, hydrochlorothiazide, lasix, aspirin 321 mg, platelet inhibitors, cholesterol meds and fibrate.

The dietary absolute intakes of macronutrients and energy, presented as means, SD and ranges, are given in Table 5.2.

Table 5.2 : Dietary measures of participants with PAD using 3-DFR (n=30) and FFQ (n=21) and percentage of participants meeting IOM/NCEP recommendations

Dietary intake	3-DFR			FFQ				IOM[a,c]	NCEP[b,c]	%meeting IOM/NCEP recommendations			
										3-DFR		FFQ	
Variable	**Mean[1]**	**S.D**	**Min and Max**	**Mean[1]**	**S.D**	**Min and Max**	**Range**			**IOM**	**NCEP**	**IOM**	**NCEP**
Energy(Kcal)	2168.0	890.6	936.4-4428.5	1832.7	362.9	1151.7-2623.6	1471.9	N.D	N.D	N.D	N.D	N.D	N.D
Protein (g)	97.3	54.7	39.6-267.8	85.3	20.3	58.9-135	76.1	46-56	75	6.66	0	0	4.76
CHO (g)	254.6	88.8	102.3-494	195.9	41.3	106.4-280.1	174.4	130	250-300	0	0	0	23.08
Fat (g)	80.5	43.5	29.8-218.4	73.4	21.8	48-125	77.0	N.D	<55-78	N.D	66.66	N.D	61.9
Total saturated (g)	24.2	15.6	8.8-74.2	22.8	6.8	14.4-43.2	28.8	N.D	<15	N.D	40	N.D	14.28
Total monounsaturated (g)	29.9	17.2	12.2-79.5	29.1	9.8	17.8-54	36.1	N.D	≤ 44	N.D	86.66	N.D	90.47
Total Polyunsaturated (g)	15.4	7.7	5.2-41.7	14.6	5.5	6.8-28.3	21.4	N.D	≤ 22	N.D	0	N.D	90.47
Linoleic acid (18:2) (g)	13.4	6.8	4.2-37	12.4	4.9	5.9-29.3	19.3	11-14	N.D	16.66	N.D	23.8	N.D
Alpha linolenic acid (18:3n3) (mg)	1567.8	823.9	270-4420	1593.7	625.1	640-2560	1920.0	1.1-1.6	N.D	16.66	N.D	28.57	N.D
Eicosapentaenoic acid (20:5) (mg)	90.0	152.1	0-670	50.0	60.0	0-200	190.0	N.D	N.D	N.D	N.D	N.D	N.D
Docosapentaenoic acid (22:5) (mg)	18.7	16.7	0-80	N.D	N.D	N.D.	N.D.	N.D	N.D	N.D	N.D	N.D	N.D
Docosahexenoic acid (22:6) (mg)	128.5	140.6	0-570	110.0	79.0	10-300	290.0	N.D	N.D	N.D	N.D	N.D	N.D
Omega-6 (g)	13.6	6.9	4.4-37.2	N.D	N.D	N.D.	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Omega-3 (g)	1.81	0.9	.43-4.54	N.D	N.D	N.D.	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Sodium (mg)	4156.6	2852.3	1413.4-16245.6	2842.0	623.6	1777.3-4381.8	2604.4	1500	<1200-1300	0	3.33	0	0
Cholesterol (mg)	301.0	228.2	98.4-1073.9	248.8	73.0	129.3-443.8	314.5	N.D	<200	N.D	30	N.D	19.04
Alcohol consumption (g)	10.2	17.8	0-76.2	10.9	16.6	0-69.2	69.2	N.D	N.D	N.D	N.D	N.D	N.D
Fibre (g)	22.4	9.0	9.7-43.7	17.2	4.9	7.8-27.0	19.2	21-30	20-30	26.66	26.66	19.04	28.57
[a]IOM - Institute of Medicine recommendations	[b]NCEP-National Cholesterol Education Program recommendations												
[c]Recommendations based on 2000KCal/d Diet	N.D - Not defined												

[1]Arithmetic value

Results from the 3DFR showed that 16 participants did not consume fish whereas 14 participants did. On a sub-set of the data, based on the FFQ results, only 3 participants were not habitual fish consumers, while 18 participants consumed fish to varying degrees. Foods that contributed to DHA intake in this study were canned tuna, salmon, pickerel, soy beverage, shrimp, roasted soy nuts, pickled herring, canned sardines and crab. Foods that contributed to EPA intake in this study were pickled herring, salmon, sardine, tuna, pickerel, crab, shrimp. The mean dietary macronutrient distribution (% of dietary energy) as determined by 3DFR was 18% for protein, 33% for fat and 47% for carbohydrate. Corresponding data for the FFQ derived values were 19% for protein, 36% for fat and 43% for carbohydrate. Supplement usage for ω-3 is not reported as none of the participants reported consuming ω-3 supplements (according to the exclusion criteria established in a concurrent clinical study).

The energy misreporting (low energy and high energy reporters) was analyzed in 3-DFR (n=30) and FFQ data (n=21) by calculating the ratio of EI and TEE. Energy intakes of PAD participants were estimated from 3-DFR and FFQ.

The characteristics of low energy reporting participants from 3-DFR (n=30) and FFQ (n=21) are given in Table 5.3.

Table 5.3: Characteristics of low energy reporters (LER's) using 3-DFR (n=30) and FFQ (n=21) who participated in the PAD study.

Participant no.	3-DFR ratio of EI:TEE (n=30)	FFQ ratio of EI:TEE (n=21)	Age	Gender	BMI
4	0.77		78	M	28.7
7	0.75		61	F	28.8
10	0.67		76	M	31.3
11	0.76		76	M	30.6
13		0.72	74	M	27
15	0.76		72	M	25.9
16	0.60	0.53	51	F	32.6
20	0.41	0.75	74	M	33.8
21	0.61	0.66	79	M	27.9
26	0.64	0.71	76	F	38.9
28	0.65	0.66	74	M	29.2
29	0.72		67	M	25.9
31	0.40	0.50	63	M	31.8
34	0.71	0.75	77	M	23.8

N.B – Participant number 3, 5, 8 and 18 were excluded from the study due to personal reasons and non-compliance.

With respect to low energy reporters, 43% of participants in the 3-DFR and 38% participants in the FFQ method were low energy reporters. Most of the low energy reporters reported low energy intake in both methods.

The characteristics of high energy reporting participants from 3-DFR (n=30) and FFQ (n=21) are given in Table 5.4.

Table 5.4: Characteristics of high energy reporters (HER's) using 3-DFR (n=30) and FFQ (n=21) who participated in the study.

Participant	3-DFR ratio of EI:TEE (n=30)	FFQ ratio of EI:TEE (n=30)	Age	Gender	BMI
6	1.51		73	M	26
9	1.34		69	M	24.9
12	1.24		77	M	30.4
13	1.08		74	M	27
14	1.51		64	M	28.4
17	1.15		72	F	28.5
24	1.41		78	M	22.4
25	0.88	1.25	81	F	21.2
27	0.94	1.31	80	F	27.5
30	1.63		70	M	34.4
32	1.48		80	F	22
33	1.22		70	M	32.4

With respect to high energy reporters 30% of participants in 3-DFR and 9.5% of participants in FFQ method were high energy reporters. For participant number 1, 2, 19, 22 and 23 ratio of EI and TEE was in the range of 0.79-1.21.

The fatty acid profiles of plasma, plasma PL and RBC membrane PL are summarized in Table 5.5.

Table 5.5:- Plasma, plasma phospholipid (PL) and RBC fatty acid analysis of PAD participants (n=30) expressed as percentage of total fatty acids

Fatty acids	Plasma	Plasma PL	RBC
Fatty acids (%)	Mean[1] ± S.D	Mean[1] ±S.D	Mean[1] ± S.D
C14	1.07 ± 0.52	0.45 ± 0.14	0.40 ± 0.14
C 14:1	0.09 ± 0.04	.083 ± 0.04	0.19 ± 0.13
C16:0	21.94 ± 1.84	28.39 ± 1.84	22.62 ± 3.97
C16:1	2.52 ± 1.06	0.60 ± 0.27	0.41 ± 0.15
C 18:0	6.59 ± 0.87	14.95 ± 1.34	15.64 ± 1.12
C18:1	24.09 ± 3.26	9.22 ± 1.30	12.30 ± 1.16
C18:2	25.64 ± 4.01	17.44 ± 2.54	7.99 ± 1.18
C18:3n-3	0.84 ± 0.41	0.22 ± 0.10	0.32 ± 0.22
C 20:4	7.26 ± 1.59	11.40 ± 2.12	11.42 ± 3.02
C20:5n-3	7.26 ± 1.59	1.07 ± 0.34	0.55 ± 0.19
C 22:5 n-3	0.50 ± 0.12	0.90 ± 0.16	2.15 ± 0.53
C22:6 n-3	1.43 ± 0.44	2.94 ± 0.60	3.14 ± 1.07
Saturated	31.1 ± 2.30	47.7 ± 2.30	48.0 ± 5.6
Monounsaturated	30.1 ± 3.90	14.5 ± 1.70	20.8 ± 1.7
ω-3	3.6 ± 0.70	5.1 ± 0.70	6.2 ± 1.5
ω-6	35.2 ± 4.20	32.6 ± 2.3	25.1 ± 4.7
PUFA	38.8 ± 4.20	37.8 ± 2.3	31.3 ± 6.0

[1]Arithmeticvalue

Spearman correlation coefficient between dietary DHA intake calculated from 3DFR and FFQ with plasma, plasma PL and RBC DHA are summarized in Table 5.6.

Table 5.6: Spearman correlation coefficient (r_s) between DHA intake of participants as estimated from 3DFR (n=30) and FFQ (n=21) and plasma, plasma phospholipid (PL) and RBC membrane PL DHA

	Plasma DHA		Plasma PL DHA		RBC PL DHA	
DHA intake	**r_s**	**p**	**r_s**	**p**	**r_s**	**p**
3-DFR DHA	0.17	0.36	0.07	0.73	0.10	0.62
FFQ DHA	0.48	0.03	0.49	0.03	0.11	0.65

A p-value < 0.05 indicates a significant association between DHA intake and plasma, plasma PL and RBC membrane PL DHA concentrations.

Spearman correlation coefficient (r_s) between DHA intake from FFQ with plasma DHA (0.48) and plasma PL (0.49) is statistically significant ($p<0.05$).

A scatter plot of dietary DHA intake using 3DFR and FFQ with plasma, plasma PL and RBC membrane PL DHA concentrations are shown below in Figure 5.1, Figure 5.2, Figure 5.3, Figure 5.4, Figure 5.5 and Figure 5.6 respectively.

Figure 5.1: Correlation coefficient (r_S) of DHA intake and plasma DHA of PAD participants using 3-DFR (n=30)

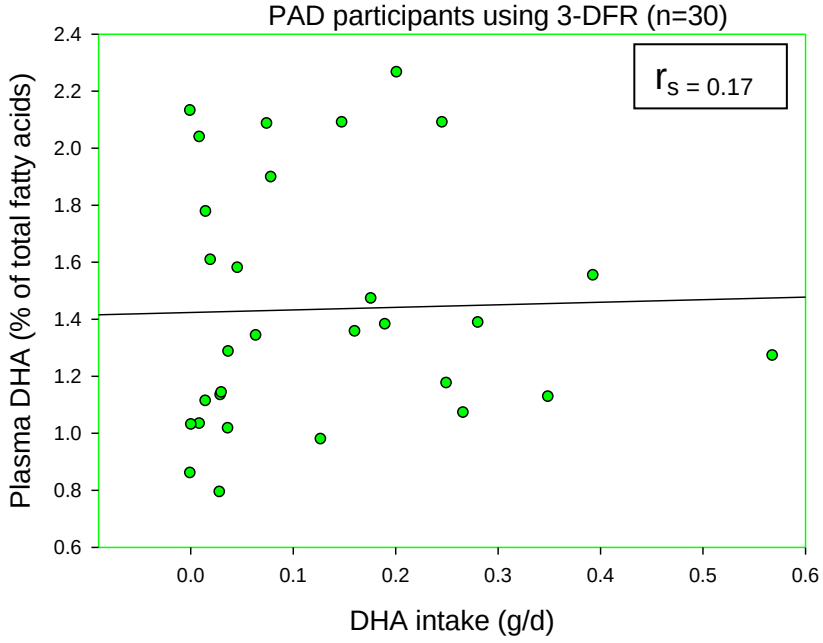


Figure 5.2 : Correlation coefficient (r_s) of DHA intake and plasma PL DHA of PAD participants using 3-DFR (n=30)

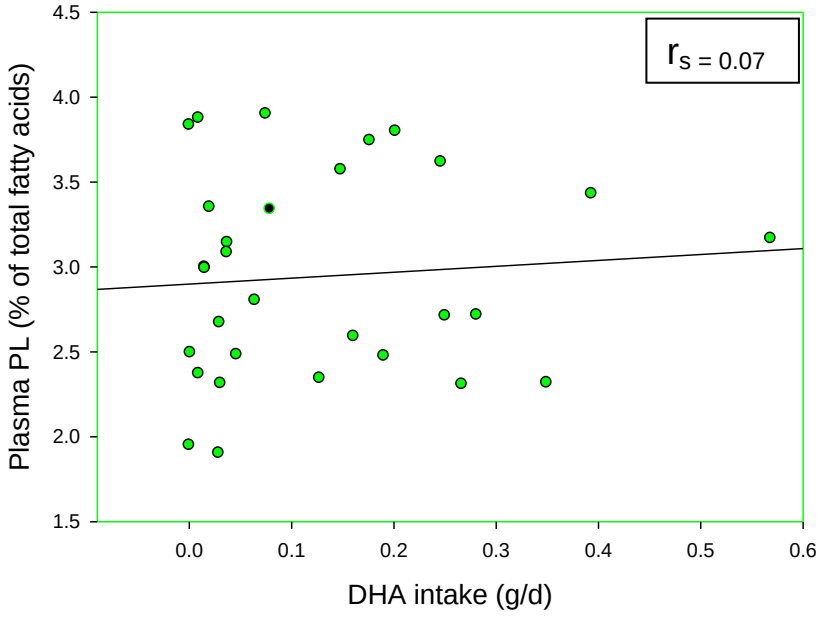


Figure 5.3: Correlation coefficient (r_S) of DHA intake and RBC membrane PL DHA of PAD participants using 3-DFR (n=30)

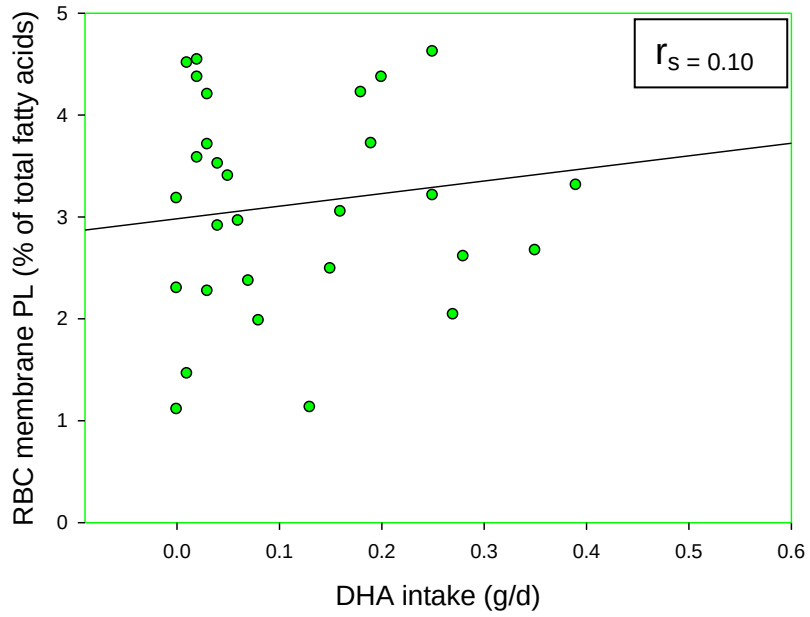


Figure 5.4: Correlation coefficient (r_S) of DHA intake and plasma DHA of PAD participants using FFQ (n=21)

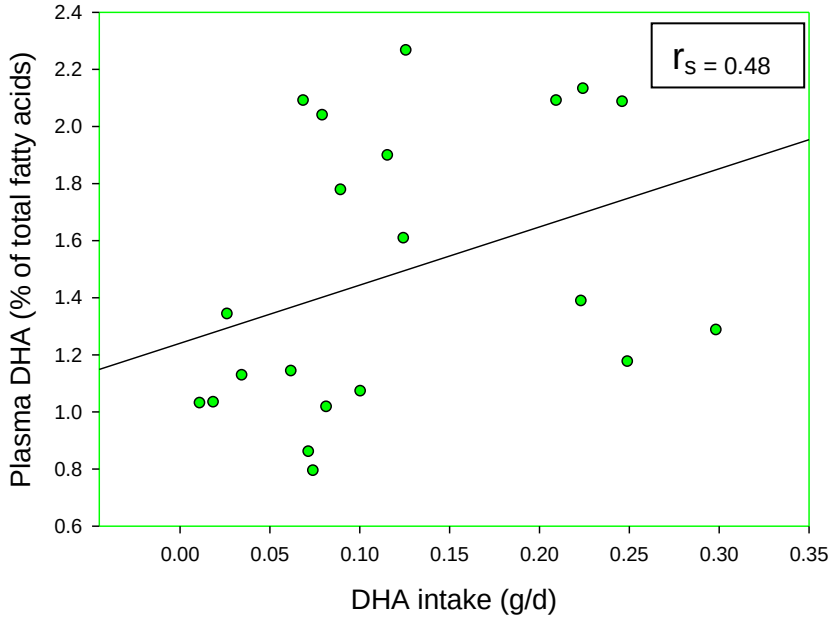


Figure 5.5: Correlation coefficient (r_s) of DHA intake and plasma PL DHA of PAD participnats using FFQ (n=21)

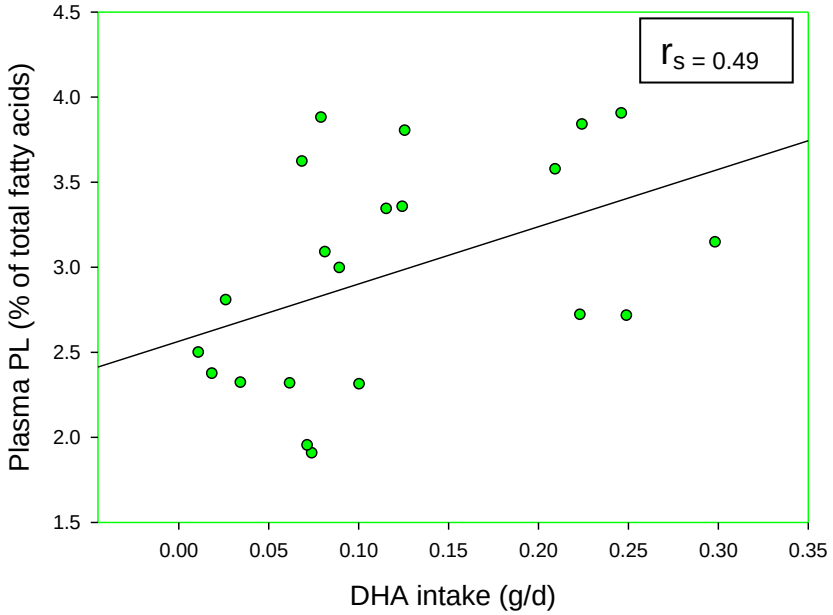
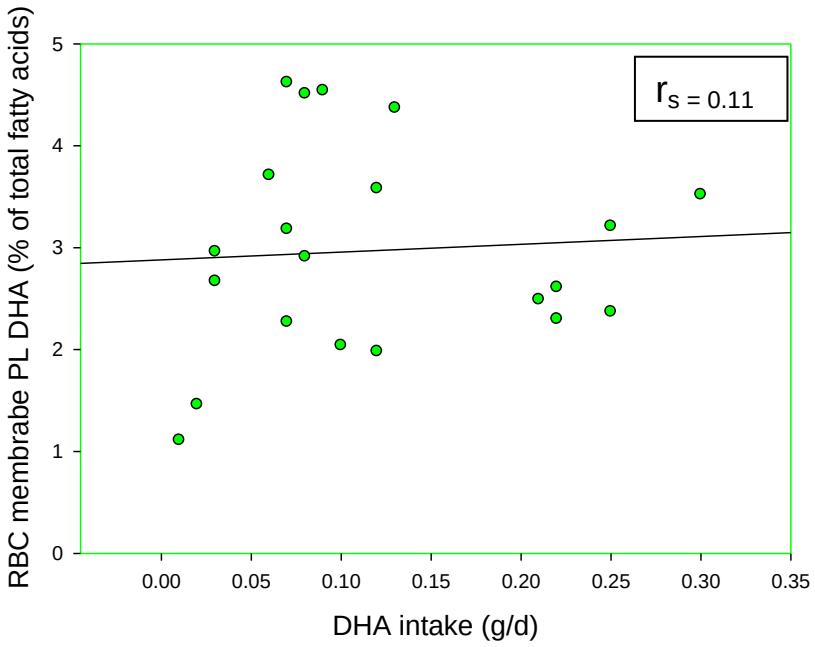


Figure 5.6: Correlation coefficient (r_s) of DHA intake and RBC membrane PL DHA of PAD participants using FFQ (n=21)



Mean, SD and the range for biochemical measurements are presented in Table 5.7.

Table 5.7 :- Mean, SD's and ranges of biochemical measurements of PAD participants (n=30)

Variable	Mean[1]	S.D.	Range	
			Min	Max
Insulin, pmol/l	79.6	49.3	25.0	243.0
Glucose, mmol/l	5.4	0.7	4.2	7.3
Cholesterol, mmol/l	4.4	1.1	2.4	6.4
Triglycerides, mmol/l	1.6	0.8	0.7	5.2
HDL cholesterol, mmol/l	1.4	0.5	0.7	2.6
LDL Cholesterol, mmol/l	2.3	0.9	0.8	4.2
Total cholesterol/HDL Cholesterol,	3.4	1.0	1.7	6.2
LDL Cholesterol/HDL Cholesterol,	1.8	0.9	0.6	4.2
Hemoglobin A1c,%	5.9	0.4	5.2	6.7

[1]Arithmetic value

Quartiles of dietary intake and biochemical indices were compared to see if the quartiles of DHA intake matched for the quartiles of blood levels of plasma, plasma PL and RBC DHA of the participants. If the participants quartile of DHA intake matched the respective quartiles of plasma DHA, the participants were considered as correctly classified. A similar approach was used for quartiles of DHA intake with quartiles of plasma PL DHA and quartiles of DHA intake with quartiles of RBC membrane PL DHA. Furthermore, if the participants quartiles of DHA intake corresponded to the adjacent quartile of plasma DHA the participants were considered as closely classified. A similar approach was used to compare quartiles of DHA intake with plasma PL and RBC membrane PL DHA. Participants were considered to be incorrectly classified or misclassified, if quartiles of DHA intake did not correspond to similar or adjacent quartile of plasma DHA. Similar approaches were used for incorrectly classified by comparing quartiles for DHA intake with plasma PL and RBC membrane PL DHA. The ability of the FFQ and 3DFR to classify individuals into the correct quartile of plasma, plasma PL

and RBC membrane PL DHA are given in Table 5.8. The ability of FFQ to classify individuals as correct and adjacent quartiles of plasma, plasma PL and RBC membrane PL DHA are 33%, 29%, 19% and 48%, 48%, 29% respectively. Actual numbers for plasma, plasma PL and RBC membrane PL DHA (70%, 64%, 66%) in correct and adjacent quartiles are close to the expected number in correct and adjacent quartiles combined together (62.5%) for plasma, plasma PL, RBC membrane PL as per 3DFR. Actual numbers for plasma, plasma PL and RBC membrane PL DHA (81%, 76%, 48%) in correct and adjacent quartiles are more than the expected number (62.5%) in correct and adjacent quartiles combined together for plasma and plasma PL and RBC membrane PL DHA as per FFQ.

Table 5.8 : Percentage of PAD participants correctly, closely or misclassified into quartiles of DHA intakes based on dietary intake methods FFQ (n = 21) and 3DFR (n = 30) compared with classification by plasma, plasma PL and RBC membrane DHA levels.

	3 Day Food Record (n=30)[4]				FFQ (n=21) [4]			
Variable	**Correctly Classified (%)**	**Closely Classified[1] (%)**	**Mis-Classified[2] (%)**	**p-value**	**Correctly Classified (%)**	**Closely Classified [1] (%)**	**Mis-Classified [2] (%)**	**p-value[3]**
Plasma DHA	43.33	26.67	30	0.06	33.33	47.62	19.05	0.21
Plasma PL DHA	26.67	36.67	36.67	0.97	28.57	47.62	23.81	0.42
RBC membrane DHA	30	33.33	36.67	0.80	19.05	28.57	52.38	0.37

[1]Percentage of participants misclassified by one quartile

[2]Percentage of participants misclassified by two or more quartiles

[3]A p-value of 0.05 indicated there is no significant association between DHA intake and plasma, plasma PL and RBC membrane PL DHA concentrations respectively.

[4]Number of participants

Results of multiple regressions on plasma, plasma PL and RBC membrane PL DHA concentrations as dependent variable and other independent variables (PAL, gender, age, BMI, DHA intake, alcohol consumption, LDL cholesterol and HDL cholesterol) using 3DFR are shown in Table 5.9, 6.0 and 6.1 respectively.

Table 5.9:- Multiple linear regression of participants independent variables (PAL, gender, age, BMI, DHA intake, alcohol consumption, LDL cholesterol, and HDL cholesterol) from 3DFR and plasma DHA (n=30) as dependent variable

Parameter Estimates

Dependent Variable:Plasma DHA

Parameter	B	Std. Error	t	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Intercept	-.086	1.084	-.079	.938	-2.355	2.183
[Physical_activity_level=1.00]	-.654	.238	-2.743	.013	-1.153	-.155
[Physical_activity_level=2.00]	-.201	.206	-.973	.343	-.633	.231
[Physical_activity_level=3.00]	0[a]	.	.	.	.	.
[GENDER=1.00]	.075	.184	.408	.688	-.310	.461
[GENDER=2.00]	0[a]	.	.	.	.	.
AGE	.022	.012	1.800	.088	-.004	.048
BMI	.016	.018	.877	.391	-.022	.053
HDL	.258	.199	1.297	.210	-.158	.675
LDL	-.195	.079	-2.478	.023	-.360	-.030
Docohexenoic_acid	-.245	.493	-.497	.625	-1.278	.788
Alcohol_consumption	-.012	.007	-1.835	.082	-.026	.002

a. This parameter is set to zero because it is redundant.

b. Adjusted R Squared=0.313

c. R Squared = 0.534

Table 6.0:- Multiple linear regression of participants independent variables (PAL, gender, age, BMI, DHA intake, alcohol consumption, LDL cholesterol, HDL cholesterol) from 3DFR and plasma PL DHA (n=30) as dependent variable.

Parameter Estimates

Dependent Variable:Plasma Phospholipid DHA

Parameter	B	Std. Error	t	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Intercept	.670	1.458	.459	.651	-2.381	3.720
[Physical_activity_level=1.00]	-.483	.321	-1.508	.148	-1.155	.188
[Physical_activity_level=2.00]	.149	.278	.538	.597	-.432	.730
[Physical_activity_level=3.00]	0[a]	.	.	.	.	.
[GENDER=1.00]	.379	.248	1.531	.142	-.139	.898
[GENDER=2.00]	0[a]	.	.	.	.	.
AGE	.030	.017	1.791	.089	-.005	.064
BMI	.020	.024	.843	.410	-.030	.071
HDL	.323	.268	1.205	.243	-.238	.883
LDL	-.383	.106	-3.609	.002	-.605	-.161
Docohexenoic_acid	.036	.664	.055	.957	-1.353	1.425
Alcohol_consumption	-.018	.009	-2.007	.059	-.036	.001

a. This parameter is set to zero because it is redundant. b. Adjusted R Squared = 0.371 c. R Squared = 0.574

Table 6.1:- Multiple linear regression of participants independent variables (PAL, gender, age, BMI, DHA intake, alcohol consumption, LDL cholesterol, HDL cholesterol) from 3DFR and RBC membrane PL (n=30) as dependent variable.

Parameter Estimates

Dependent Variable:RBC DHA

Parameter	B	Std. Error	t	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Intercept	-.104	2.967	-.035	.972	-6.314	6.105
[Physical_activity_level=1.00]	-.580	.653	-.889	.385	-1.946	.786
[Physical_activity_level=2.00]	.152	.565	.269	.791	-1.031	1.334
[Physical_activity_level=3.00]	0[a]	.	.	.	.	.
[GENDER=1.00]	-.601	.504	-1.193	.248	-1.656	.454
[GENDER=2.00]	0[a]	.	.	.	.	.
AGE	.064	.034	1.908	.072	-.006	.135
BMI	-.008	.049	-.162	.873	-.111	.095
HDL	-.032	.545	-.059	.954	-1.172	1.108
LDL	-.297	.216	-1.376	.185	-.749	.155
Docohexenoic_acid	.776	1.350	.575	.572	-2.051	3.603
Alcohol_consumption	-.003	.018	-.162	.873	-.041	.035

a. This parameter is set to zero because it is redundant. b. Adjusted R Squared = 0.095 c. R Squared = 0.386

Multiple regression results from 3DFR shows that sedentary participants (PAL-1) have lesser plasma DHA in comparison to low active participants (PAL-2). Low active participants (PAL-2) have lesser plasma DHA in comparison to active participants (PAL-3). Male participants showed lower DHA concentration in plasma and RBC membrane PL as comparison to females. LDL cholesterol (P = .02) and sedentary participants (PAL-1) (P = .01) were significantly associated with plasma DHA. LDL cholesterol was significantly (P = 0.002) associated with plasma PL DHA. In addition to that, alcohol consumption was not significantly (P = 0.05) associated with plasma PL DHA. Age was not significantly (P = 0.08) associated with plasma PL DHA. DHA intake was not significantly associated with plasma (P = 0.62), plasma PL (P = 0.95) and RBC DHA (0.57). None of the variables recorded in this study are significantly associated with RBC membrane PL DHA. Results of multiple regressions on plasma, plasma PL and RBC membrane PL DHA concentrations as dependent variable and other independent variables (PAL, gender, age, BMI, DHA intake, alcohol consumption, LDL cholesterol and HDL cholesterol) using FFQ are shown in Table 6.2, 6.3 and 6.4 respectively.

Table 6.2:- Multiple linear regression of participants independent variables (PAL, gender, age, BMI, DHA intake, alcohol consumption, LDL cholesterol, HDL cholesterol) from FFQ and plasma DHA (n = 21) as dependent variable.

Parameter Estimates

Dependent Variable:Plasma_DHA

Parameter	B	Std. Error	t	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Intercept	.162	1.376	.117	.909	-2.867	3.191
[Physical_activity=1.00]	-.838	.293	-2.860	.016	-1.484	-.193
[Physical_activity=2.00]	-.366	.285	-1.282	.226	-.994	.262
[Physical_activity=3.00]	0[a]	.	.	.	.	.
[GENDER=1.00]	-.030	.228	-.133	.897	-.531	.471
[GENDER=2.00]	0[a]	.	.	.	.	.
AGE	.021	.015	1.434	.179	-.011	.054
BMI	.019	.021	.885	.395	-.028	.066
HDL	.049	.221	.223	.828	-.436	.535
LDL	-.177	.147	-1.211	.251	-.500	.145
DOCOSAHEXENOIC	2.436	1.662	1.465	.171	-1.223	6.095
ALCOHOL	-.013	.008	-1.649	.127	-.029	.004

a. This parameter is set to zero because it is redundant. b. Adjusted R squared = 0.435 c. R Squared = 0.689

Table 6.3:- Multiple linear regression of participants independent variables (PAL, gender, age, BMI, DHA intake, alcohol consumption, LDL cholesterol, HDL cholesterol) from FFQ and plasma PL DHA (n = 21) as dependent variable.

Parameter Estimates

Dependent Variable:Plasma phospholipid_DHA

Parameter	B	Std. Error	t	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Intercept	1.397	1.953	.716	.489	-2.900	5.695
[Physical_activity=1.00]	-.823	.416	-1.978	.073	-1.738	.093
[Physical_activity=2.00]	-.265	.405	-.653	.527	-1.156	.627
[Physical_activity=3.00]	0[a]	.	.	.	.	.
[GENDER=1.00]	.114	.323	.352	.732	-.597	.824
[GENDER=2.00]	0[a]	.	.	.	.	.
AGE	.026	.021	1.218	.249	-.021	.072
BMI	.021	.030	.707	.494	-.045	.088
HDL	.044	.313	.141	.890	-.645	.733
LDL	-.330	.208	-1.585	.141	-.787	.128
DOCOSAHEXENOIC	3.137	2.359	1.330	.210	-2.055	8.329
ALCOHOL	-.013	.011	-1.207	.253	-.037	.011

a. This parameter is set to zero because it is redundant.

b. Adjusted R Squared = 0.356

c. R Squared = 0.646

Table 6.4:- Multiple linear regression of participants independent variables (PAL, gender, age, BMI, DHA intake, alcohol consumption, LDL cholesterol, HDL cholesterol) from FFQ and RBC membrane PL DHA (n= 21) as dependent variable.

Parameter Estimates

Dependent Variable:RBC_DHA

Parameter	B	Std. Error	t	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Intercept	-.288	3.902	-.074	.943	-8.875	8.300
[Physical_activity=1.00]	-.030	.831	-.036	.972	-1.859	1.799
[Physical_activity=2.00]	.226	.809	.279	.786	-1.555	2.006
[Physical_activity=3.00]	0[a]	.	.	.	.	.
[GENDER=1.00]	-.759	.645	-1.176	.265	-2.179	.662
[GENDER=2.00]	0[a]	.	.	.	.	.
AGE	.046	.042	1.105	.293	-.046	.138
BMI	5.475E-5	.061	.001	.999	-.133	.133
HDL	.314	.626	.502	.626	-1.063	1.691
LDL	-.067	.415	-.161	.875	-.981	.847
DOCOSAHEXENOIC	-1.094	4.713	-.232	.821	-11.468	9.279
ALCOHOL	.015	.022	.688	.506	-.033	.063

a. This parameter is set to zero because it is redundant.

b. Adjusted R Squared = -0.076

c. R Squared = 0.408

Multiple regression results from FFQ demonstrated that sedentary (PAL 1) participants were significantly (P = .016) associated with plasma. Sedentary (PAL 1) participants were not significantly (P = .073) associated with plasma PL DHA. DHA intake was not significantly associated with plasma (P = 0.17) and plasma PL (P = 0.21) and RBC DHA (P = 0.82). There was no evidence of associations of plasma, plasma PL and RBC membrane PL DHA with other covariates when other models were employed.

Chapter 6

DISCUSSION

Despite the high prevalence of PAD, it is an under-diagnosed, under-recognized and an under-treated condition (1). Diet is a risk factor for many chronic diseases and poor nutrition is implicated in the prevalence of CVD (CAD and PAD) and other chronic diseases. The National Health and Nutrition Examination Survey (10) found that nutrition has a significant impact on the factors associated with PAD disease risk and, hence, diet can be an important modifiable risk factor in PAD. Recently, the role of LC ω-3 PUFAs in particular, has generated substantial interest in relation to chronic disease risk. As these LC ω-3 PUFAs have a beneficial role in the prevention of CAD mortality and morbidity (41), the possibilities for similar beneficial effects of LC ω-3 PUFAs in PAD exist particularly EPA and DHA, as the underlying mechanism of pathogenesis is similar for PAD and CAD. Therefore, it is essential to understand the dietary intake of PAD patients so that dietary interventions can be developed to reduce the dietary risk factors associated with PAD.

To understand the relationship between nutritional status and disease in PAD population groups, it is important to obtain information on their dietary intake using appropriate dietary assessment methods such as 3DFR and FFQ, and then correlate the collected information to anthropometric measures as well as biochemical and clinical indices, and dietary recommendations through clinical trials. However, the estimation of LC ω-3 PUFAs dietary intake using 3DFR and FFQ has not been studied specifically for PAD patients. Considering the importance of LC ω-3 PUFAs in PAD, it becomes important to obtain the information regarding the dietary intake of LC ω-3 PUFAs for PAD patients and correlate it with appropriate biomarkers in order to fill significant gaps in the

literature. In this study, we used 3DFR and FFQ as dietary assessment tools to determine nutrient intakes, with a special focus on ω-3 intakes, of PAD patients and compared these to plasma, plasma PL and RBC membrane PL DHA as potential biomarker of LC ω-3 PUFA intake.

PAD was diagnosed as ABI< 0.9. Study participants had ABI values ranging from 0.31-1.43. ABI>0.9 witnessed in some participants was a result of the surgical intervention made in these participants to remove coronary artery blockages in the past. The physical activity levels of 80% of the participants were classes as either sedentary or low active. This could be attributed to the fact that PAD affects lower extremity function leading to less walking capacity. Overall, PAD participants consumed 61% higher saturated fat than NCEP recommendations and 177% and 246% higher sodium intake than IOM and NCEP recommendations, respectively, using 3DFR. These results are similar to the findings reported by Gardner and others (245) in PAD and claudication patients, where more than three-quarters of the participants exceeded recommendations for saturated fat intake. These findings highlight the fact that these participants are less motivated about their health and need more awareness about the role of nutrition in health.

Higher BMI is associated with obesity which is further associated with cardiovascular risk. These participants were in the normal to obese (18.5 to 30 or greater) weight category. The range of BMI recorded in this study was 21.1-38.9 with an average of 28.3 (overweight category). 47% of the participants were in the overweight category, 34% of the participants in the obese category and the rest (19%) fell into the normal category. Hence, these findings highlight an important link between obesity and PAD risk.

The participants in this study were adequate in terms of their financial situation. Eight participants had completed their education between grades 8 and 11. With respect to the highest level of education, one participant completed elementary school, two participants completed secondary or grade 12, six participants completed trade school, one participant completed post-graduation, two participants completed a few years in university, and one participant completed undergraduate studies. High socioeconomic status is associated with higher consumption of fish. The participants in this study had an average socioeconomic status, and this may be related to their reporting low or no fish intake.

Mean daily energy intake, as calculated from 3DFR was 2168 Kcal and 1833 Kcal from the FFQ. By comparison, the reported daily energy intake was 2279 Kcal, as determined by 3DFR, for 15 participants aged 79-98 years recruited from a Canadian long term care facility (192) in Ontario. The NHANES survey data (1999-2004) yielded daily calorie intakes of PAD individuals as 1742 ± 44.3 Kcal (10). Yet another study (245), using a 7DFR design, reported daily energy intakes of 46 PAD individuals as 1918 ± 422.0 Kcal, values that are somewhat lower than those reported in the current study (2168 Kcal) with 3DFR. This potential difference in reported energy intakes may be due to the differences in assessment methods employed (ie: 7 day weighed record to estimate the dietary intake of participants (245) as compared to the 3DFR used in the current study). In the current study, 53% of the participants consumed calories below the recommended daily reference value of 2000 Kcal (250) for men aged 51 years and older, as determined by the 3DFR, while 81% of participants consumed less than 2000 Kcal/d when the FFQ method was employed. These results are similar to the results of another study (245) which

reported participants consumed less than 2000 Kcal/d for men aged 51 years and older. Energy intake as calculated from the FFQ was lower than that obtained with the 3DFR in our study, and this may be due to the under-reporting of energy intake associated with the use of FFQ (249). Further, underreporting of energy has been reported to be a major issue in dietary assessment (60).

Carbohydrate, protein, fat and PUFA intakes of 46 PAD participants using 7DFR were 245 ± 65, 81 ± 21, 65 ± 23 and 11 ± 4.0 g/day (245), while they were 254.6 ± 88.8, 97.3 ± 54.7, 80.5 ± 43.5 and 15.4 ± 7.7 g/day in the current study. Using FFQ carbohydrate, protein, fat and PUFA intakes were 195.9 ± 41.3 g/day, 85.3 ± 20.3 g/day, 73.4 ± 21.8 g/day and 14.6 ± 5.5 g/day in our study. Data from the NHANES survey (10) provides estimates of reported PUFA intakes of PAD participants as 13.8 ± 0.45 g/day, using a 24 hr recall method. The current study showed that 47.0%, 17.9% and 33.4% of energy is contributed by carbohydrate, protein and fat using 3 DFR, respectively, which is similar to the results (51%, 17% and 30%) reported by Gardner and others (245) using the weighed 7-DFR approach. These data provide evidence that the results of the current study are in general agreement with those previously reported (245).

There is a gap in the literature regarding LC ω-3 PUFA intake and status of PAD participants. One of the novel findings of this study was provision of an estimated daily intake of LC ω-3 PUFA (EPA and DHA) in PAD participants. We recorded a combined EPA and DHA intake of 218.5 mg/d from 3DFR and 160 mg/d from FFQ. These data compare well with daily dietary EPA and DHA intakes of elderly adults (below 400 mg/day) (251) and older Canadian individuals in long term care (70 mg/day) (192). Lower intakes of EPA and DHA in long term care older people may be due to the lack of choice

and somewhat restricted food offerings in the facility in question. A DHA intake (independent of EPA intake) of 47 mg/day has been reported in older Canadians residing in long term care, and 180 mg/day in the Framingham Heart study (252). Data from the current study provides an estimated DHA intake of 128 mg/day in PAD patients. In our study none of the PAD participants met the AHA recommendations (1 g/day) for EPA and DHA intake combined together.

In the current study, approximately 43% of the participants were classified correctly in similar quartiles of plasma DHA, as compared to 27% and 30% for plasma PL and RBC membrane PL, respectively, using 3DFR. The corresponding percentages were 33% (plasma), 29% (plasma PL) and 19% (RBC membrane PL) using the FFQ results. These results provide evidence that employing the results from the 3DFR yielded data to correctly classify individuals into the same quartiles of DHA intake and plasma DHA in a more accurate fashion, as compared to the FFQ. The results show a tendency for an association between the 3DFR DHA intake and plasma DHA ($p=0.06$). One explanation for this could be that plasma reflects intake over a shorter time period of time (few weeks) and the 3DFR is likely a better reflection of short term intake, as compared to the FFQ, which reflects intake over months. The latter data are likely better reflected by the biomarkers associated with plasma PL and RBC membrane PL (253). The FFQ did not correctly classify more participants in RBC membrane PL as compared to 3DFR. This may be due to a lower number of participants in FFQ (21 participants) as compared to the 3DFR (30 participants). However, the FFQ classified more participants in adjacent quartiles as compared to the 3DFR. We could not see a better classification with a higher percentage of participants in the correct quartiles because of the large variability in the dietary intake of

the participants and individual physiological variation and metabolic efficiency. Increasing the sample size of participants may help improve this classification.

Our study did not show a good correlation between DHA intake and plasma, plasma PL and RBC membrane PL DHA from the 3DFR results. Higher variability in DHA intake (0-570 mg using 3DFR and 10-300 mg using FFQ) as well as with some participants demonstrating higher proportions of DHA in plasma, plasma PL and RBC membrane PL with zero intake of DHA may help explain these results. Moreover, most of the studies (213, 218, 219, 222) in the literature have used DHA intake from 7 day weighed records or FFQ which are more accurate for portion size estimation, thus giving better correlations with DHA intake or nutrient of interest and biomarker (plasma, plasma PL, RBC DHA).

We observed a significant correlation of DHA intake and plasma (0.48) and plasma PL (0.48) using FFQ while it was 0.10 with RBC membrane PL DHA using FFQ. The poor correlation between DHA intake and RBC membrane PL DHA could be due to the inaccuracies associated with FFQ such as underestimation or overestimation of portion sizes, as portion size is a big challenge in dietary assessment (60). In FFQ, fractional choices are not available, and this consequently lead to higher or lower portion size estimations, as compared to 3DFR in which portion size can be recorded as a half, quarter or one third. Also, the accuracy of a FFQ is dependent upon accurate recall of the food consumed, portion size and food composition databases (253).

An additional factor for consideration relates to the potential for comorbidities in these participants, which may impact metabolic pathways associated with fatty acids, leading to a poor correlation between dietary nutrient intake and biomarkers. Also, fatty

acid concentrations could be altered by metabolism, absorption, genetic and lifestyle factors (253). Alcohol consumption, the consumption of trans-fats and smoking can lead to decreases in the intake of DHA, as well as increased excretion, lower rates of absorption, increased turnover and loss of DHA in tissues (202). Given the role of FADS1 and FADS2 enzymes on the accumulation of LC ω-3 PUFAs in plasma PLs and erythrocyte membranes, genetic variations within the respective genes may affect DHA intake and its status (254). One study has reported a correlation between DHA intake from 3DFR and RBC DHA as 0.10 and 0.16 using FFQ DHA and RBC DHA, respectively (255). Evidence exists to show that with low dietary DHA intakes, plasma DHA may be a better reflection of DHA intake, whereas plasma PL may better reflect DHA status at higher DHA intakes (202). Higher ω-3 PUFA levels with lower intakes of EPA and DHA among non-fish eaters and vegetarians, as compared to fish eaters, has also been reported (256). The latter observation may be due to a higher conversion of ALA to DHA in non-fish consumers and vegetarians. Additionally, plasma PL EPA and DHA correlate with the consumption of non-fried fish as compared to fried, shellfish or fish in mixed dishes (257), and plasma levels tend to be better correlated with the intake of fatty fish as compared to lean fish.

An additional factor to consider when linking dietary records with biomarkers relates to the nature of the dietary assessment employed: Within the 3DFR methodology, intakes of the participants are measured on three particular days and it is a possibility that they might not have consumed fish on those three days, but might have consumed fish just outside of this time window, thus altering plasma DHA results. The plasma reflects the DHA intake of the past 1-2 weeks (209). With the FFQ, there is a lower chance to introduce such errors, as the FFQ uses questions about the participants usual intake over

the past year and the recording of foods eaten on unrecorded eating days is highly unlikely and only reflects their usually eaten foods. Interpretation of the results and their applicability may be limited with smaller sample sizes. Increasing the sample size could help to better explain the correlations between DHA intake and plasma, plasma PL and RBC membrane PL, and the ability of FFQ and 3DFR to classify individuals in to different quartiles. Furthermore, the potential exists for a lack of complete information and inaccuracies associated with food composition databases in relation to LC ω-3 PUFA, and this fact cannot be understated. These potential inaccuracies in food composition databases bring difficulties in reporting accurate DHA intake by the study participants, but this is a challenge with all dietary assessment methods.

In the current study, 71.4% of the participants had fibre intakes below the recommended NCEP guidelines. Fibre plays an important role in inflammation as there is an association between higher fibre intake and lower CRP levels. Also soluble fiber lowers LDL cholesterol. High consumption of fibre-containing foods such as whole grain cereals, fruits and vegetables are associated with a lower risk of CVD, hypertension, obesity and type-2 diabetes (41). A dose-dependent beneficial effect of fiber was seen in the NHANES survey, as PAD risk decreased with each quartile increase in fiber intake. A study conducted in men found that ABI increases with increasing cereal fibre intake, with a resultant lower prevalence of PAD in men (54). These findings emphasize the importance of fibre intake and PAD risk.

Multiple linear regression analysis was conducted by keeping all independent variables (age, BMI, alcohol intake, gender, LDL and HDL cholesterol) constant in all

regression models and changing dependent variables as plasma DHA, plasma PL DHA, RBC membrane PL DHA.

In this study, sedentary participants are significantly ($p<0.05$) and negatively associated with plasma DHA using 3DFR and FFQ. Higher physical activity levels, age and BMI are associated with ω-3 consumption and can affect DHA levels. LDL levels are significantly ($p<0.05$) negatively associated with plasma DHA and plasma PL. Again unhealthy dietary habits with high saturated fat, trans-fats and low ω-3 intake are associated with higher LDL levels. Higher intake of PUFA is an indicator of healthy diet in men and women (44). With respect to simple correlations, in the current study, alcohol consumption and age were not significantly ($p>0.05$) associated with plasma DHA, plasma PL and RBC membrane PL DHA, when using either the 3DFR or FFQ data. Physical activity levels, age and BMI are associated with ω-3 consumption and can affect DHA levels. However, another study (218) has reported no association between age, BMI, alcohol consumption or smoking with marine ω-3 PUFA composition of serum PLs.

By comparing the dietary intake results of participants in the current study with NCEP and IOM guidelines, the following observations were made:

- 3-DFR results indicate that PAD participants had a higher intake of saturated fat and sodium, by 60% and 96.6%, respectively, when compared to NCEP guidelines. 53.3% of PAD participants had fiber intake below the recommended NCEP guidelines.
- As per the FFQ results, PAD participants exceeded the recommended NCEP intake for saturated fat and sodium by 85.7% and 100%, respectively. 71.4% of PAD participants had fiber intake below the recommended NCEP guidelines.

- 3-DFR results demonstrate that PAD participants had a higher intake of sodium by 96.6% as compared to IOM guidelines. 53.3% of PAD participants had fiber intake below the recommended IOM guidelines.
- As per the FFQ results, PAD participants had a higher intake of sodium by 100% as compare to IOM guidelines. 81% of the PAD participants had fiber intake below the recommended IOM guidelines.
- None of the participants meet the AHA recommendations for EPA and DHA intakes (1 g) combined together as per the 3-DFR and FFQ data.

Energy misreporting is a major problem in dietary assessment studies. The results of this study demonstrated that 43% of participants in the 3-DFR analysis and 38% of participants in the FFQ analysis were energy misreporters. Underreporting of energy could be due to the perceived need to meet dietary expectations thus impacting reported usual intakes. A lower number of females were LER's and HER's as compared to males. This may be due to the lower number of female participants (n=9) in the study as compared to males (n=21). In the 3-DFR analysis, 30% of the participants were high energy reporters and 9.5% participants were HER's in FFQ analysis. This could be due to the higher consumption of foods on any particular days or higher energy intake may represent their usual eating habits. New dietary assessment methods should be explored to accurately measure the dietary intake of nutrients in this population.

6.1. Conclusions

Results of this study show that PAD participants consume a diet which is high in saturated fat and sodium and exceeds the daily recommendations of both NCEP and IOM.

Also, the participants did not meet the daily recommendations of NCEP and IOM for fiber intake. EPA and DHA have a beneficial role in the prevention of CAD mortality and morbidity. PAD participants consumed on an average 218.5 mg/day of EPA and DHA combined together using 3DFR and 160 mg/day from FFQ. None of the participants met the AHA recommendations (1g/d) for EPA and DHA intake in established cardiovascular disease. These results show poor nutrition in these participants. Data from the FFQ reflected DHA intake in plasma ($r_s = 0.48$) and plasma PL DHA ($r_s = 0.48$) as compared to the 3DFR, which did not show a correlation between DHA intake and plasma, plasma PL and RBC membrane PL DHA. The FFQ was more sensitive in reflecting DHA concentration in plasma and plasma PL in comparison to 3-DFR. The ability of the 3DFR to correctly classify participants into the same quartiles of DHA intake and plasma DHA was better than the FFQ. However, the FFQ classified more participants into the adjacent quartile as compared to 3DFR. Multiple regression results showed sedentary PAL, alcohol consumption and LDL levels were negatively associated with plasma and plasma PL DHA.

6.2. Limitations

Recruitment of PAD participants meeting the strict criteria (non diabetic and non smokers) was itself a very challenging task as smoking and diabetes are common in PAD individuals. This group of participants does not include ω-3 supplement users that might have underestimated their dietary ω-3 and DHA intake. Lack of complete information and inaccuracies associated with food composition databases in relation to LC ω-3 PUFAs cannot be understated. The potential incompleteness of food composition

databases brings difficulties in reporting accurate DHA intake of the study participants. Underestimation or overestimation of portion sizes is a big challenge in dietary assessment. Due to the small sample size it becomes difficult to interpret the collected data and to find good associations that further distort the ability of dietary assessment methods to reflect intake in plasma, plasma PL and RBC membrane PL DHA. Finally, the small sample size used in the current study may limit the power of study. Participants were selected from a large egg study intervention trial in PAD patients which were non smoker and non diabetic. As such dietary habits of participants of this study may differ from smoker and diabetic PAD patients.

6.3. Future implications

This research provides information on the dietary habits of PAD participants. With a limited sample size, the results indicate the need to educate subjects with PAD on beneficial dietary habits, including consultation to consume diets low in saturated fat and sodium, and higher in fiber, in order to improve risk factors associated with PAD. This study highlights the importance of increasing the daily intake of EPA and DHA of PAD participants coupled together in order to meet the IOM and NCEP recommendations of 1g/d. This may result in a beneficial effect on PAD risk as the mechanism of pathogenesis is similar in PAD and CVD.

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Appendix 1

3 Day Food Record

Instructions: To complete the 3-Day Food Record please write down all the foods you eat and drink for 3 complete days that include 2 weekdays and 1 weekend days on the sheets provided. Choose days which represent your ***usual*** eating patterns (not days when you have a family barbeque or wedding). **The 3 days do NOT have to be consecutive.** For example, you can record all foods consumed on Thursday, Friday and Saturday, or Monday, Tuesday and Saturday. Begin each day's record by recording the date and your initials. **Before you** begin your **own record please use the sample food record as an example** and to make sure your food record is as accurate as possible. **For accuracy it is best to record each meal or** snack eaten **during a 24 hour period as soon as you finish eating and as soon as you wake up in the morning on each of three days.** Look over the ***List of Reminders for Completion of the 3-Day Food Record*** to make sure you have included the necessary details about your food intake. **Please ensure that you complete your 3-Day Food Record before the next scheduled visit.**

We would like to know the following:

1) Food item: Include all foods consumed throughout the day including condiments, snacks and beverages consumed with or between meals. Be sure to include coffee, tea, soda, etc. Include all additions to the food at the table such as salt, sugar, or milk. Please include any additions on salads such as dressings and toppings (e.g. nuts, cheese, dried fruit, etc.).
2) Description, Amount and Method of Preparation: Be specific about every food item indicating amount and detailed description. For example, instead of just recording "large bowl of cereal", record the type of cereal, amount, and what type of milk you

used (l%, whole, skim, ω-3 enriched milk). The *list of reminders* can help with providing detail. Include exact amounts for *everything* (e.g. sugar, jelly, creamer, salt, butter, oil, etc.).You can record the amount using standard household measures such as cups, teaspoons, tablespoons or, you can describe the size of the amount consumed, such as size of

thumb or fist, deck of cards, slice of bread, etc. For example, if you eat cheese, please describe the type of cheese, i.e. cheese, cheddar, 1cube = ½ x ½ x ½ inch. For food that requires preparation, please describe the method you used to prepare it, i.e. steak, barbequed, broiled, breaded, sautéed. If you are taking vitamin/mineral/fish oil supplements, please write down what you take, brand name and amount of the nutrient taken. Examples of supplements are: multi-vitamin, iron, calcium; or any herbal and dietary supplements such as Echinacea, or Ginseng, ω-3 supplements, algal oil supplements, flaxseed (linseed) oil supplements, cod liver oil or fish oil capsules.

3) Place: Record the place where foods and/or supplements are consumed throughout the day such as at home, restaurant, friend's house, work, etc. If you are eating at a restaurant, please indicate which one and the specific food item eaten.

4) If you have any questions do not hesitate to call the Study Coordinator, Wendy Weighell at 235-3916.

Food Record (Example)

Date: August 19, 2009 **Initials:** A.B.C.

Time of Day	List Food Item, Description and Method of Preparation	Amount	Place
7:30 a.m.	cold cereal, Corn Flakes	1 1/2 cups	Home
7:30 a.m.	1% milk	1 cup	Home
7:30 a.m.	multi-vitamin, Centrum Select 50+	1 tablet	Home
10:00 a.m.	coffee (with cream and sugar, see below)	1 cup	Home
10.00 a.m.	half and half cream	1 tablespoon	Work
10.00 a.m.	white sugar	1 teaspoon	Work
10:00 a.m.	toast, 60% whole wheat	1 slice	Work
10:00 a.m.	margarine, Becel	thumb tip	Work
10:00 a.m.	strawberry jam	1 tablespoon	Work
12:00 p.m.	tuna sandwich (described on next lines)	1 sandwich	Work
12:00 p.m.	white bread	2 slices	Work
12:00 p.m.	tuna, flaked, canned in water	1/2 can	Work
12:00 p.m.	mayonnaise, regular, Hellman's	2 tablespoons	Work
12:00 p.m.	salt	dash	Work
12:00 p.m.	pepper	dash	Work
12:00 p.m.	Sprite, diet	one can, 355ml	Work
2:00 p.m.	Granny Smith apple, raw	small size	Work
2:00 p.m.	cheese, cheddar, 1 cube = ½ x ½ x ½ inch	5 cubes	Work
4:00 p.m.	herbal tea, lemon	1 cup	friend's place
4:00 p.m.	Shortbread cookies, homemade	2, width of golf ball	friend's place
6:30 p.m.	steak barbequed	2 deck of cards	Home
6:30 p.m.	barbeque sauce, Kraft's chicken & rib	2 tablespoons	Home
6:30 p.m.	red potato, sliced and fried in canola oil	3/4 cup	Home
6:30 p.m.	mixed vegetables (corn, beans, peas) boiled	1 cup	Home
6:30 p.m.	Coca-cola, regular	1 cup	Home
8:00 p.m.	chocolate bar, Snickers (58 grams)	1 bar	Home
10:00 p.m.	chocolate milk, 2%	3/4 cup	Home

A List of Reminders for Completion of the 3-Day Food Record

Please **review** this checklist to ensure you include all the necessary details for the 3-Day Food Record

Type of Food	**Did you specify...**
All	Amount eaten? Use cup, tablespoon, ounce, deck of cards, and slice of bread or other common measures. Use size (dimensions such as length, width, thickness). Record quantity or weight.
Cereal	Serving size? Brand name? Instant or ready-to-eat? Remember to include additions such as fruit, sugar, milk, or nuts.
Baked Goods	Homemade or commercial? From scratch or mix? Brand? Frosting or topping? Include size and/or weight.
Beverages/Drinks	Brand? Amount? Sweetened or diet? If juice, fresh, canned, or frozen? Is drink 100% fruit juice or a juice drink? Remember to include drinks with alcohol.
Eggs, Fish	Type of eggs (ω-3, normal eggs), type of fish you consume (fried, non fried such as broiled, baked, grilled, steamed, raw fish and non fried shell fish), fatty fish (canned tuna fish, dark meat fish e.g. mackerel, salmon, sardines, bluefish and sword fish), lean fish (shrimp, lobsters, scallops or other fish), white fish (cod, haddock, frozen fish). Water or oil packed tuna? Size of amount eaten (3 oz =size of a deck of playing cards)? How prepared? Any fat/oil added? Size?
Fats and Oils	Brand? Type (canola, olive, hempseed, flaxseed oil, butter, ω-3 enriched margarine, antioxidant-enriched margarine, shortening, etc.)? Stick, tub, diet, whipped, squeeze, or liquid margarine?
Fruits, Vegetables	Fresh, canned, frozen, or dried? Serving size (small, medium, large)? Fresh, frozen, canned? Cooked? Sauces or other additions? If lettuce, what kind?
Meat, Poultry	How cooked? Added fat? Type of cut? Regular, lean, extra lean? With or without fat or skin? Cooked weight or size of amount eaten (3 oz =size of a deck of playing cards)?

Milk, Cheese, Yogurt	Amount? Skim, whole, or low-fat milk? Percent fat? Liquid or powdered? Type of cheese? Added fruit or flavour to yogurt? Milk substitute?
Mixed Dishes	Homemade, frozen, or restaurant-prepared? Brand? Cooking method? Added fat? Did you list individual ingredients such as meat, noodles, cheese, etc. and amounts?
Restaurant Meals	Refer to "Mixed Dishes". Name of restaurant? Include nutrient information if available.
Sandwiches	Type and amount of bread, meat, cheese? Amount and type of condiments (ketchup, mayonnaise, mustard, etc.)? Lettuce, tomato, onion pickles, etc.
Snacks	Brand name of snack? Size, weight, number eaten?

Day 1 Food Record

Date: _____ **Initials:_____**

Time of Day	List Food Item, Description and Method of Preparation	Amount	Place

Day 2 Food Record

Date: _____ **Initials: _____**

Time of Day	List Food Item, Description and Method of Preparation	Amount	Place

Day 3 Food Record

Date: _____ **Initials:_____**

Time of Day	List Food Item, Description and Method of Preparation	Amount	Place

APPENDIX 2

RESEARCH SUBJECT INFORMATION AND CONSENT FORM FOR COLLECTION OF BIOLOGICAL MATERIAL FOR RESEARCH

Title of Study:	**The Impact of Egg Consumption on Indices of Vascular Health in Individuals with Peripheral Arterial Disease**
Investigator:	Dr. James House PhD Head, Department of Human Nutritional Sciences University of Manitoba Telephone: 204-474-6837
Co-Investigator:	Dr. Peter Zahradka PhD Professor, Department of Physiology, University of Manitoba Team Leader, Canadian Centre for Agri-food Research in Health & Medicine, St. Boniface Hospital Research Centre Telephone: 204-235-3507
Funding:	Provided by the Egg Farmers of Canada and the Agri-food Research and Development Initiative (ARDI)

You have been asked to participate in a research study because you have been diagnosed with Peripheral Arterial Disease (PAD). This part asks for your consent to use a small sample of your blood to study your genes and the physical makeup of your cells. Blood cells contain genes. Genes provide instructions for processes in the body and for traits such as eye colour. Everyone's genes are a little different and there is evidence that these differences can influence whether you are resistant or susceptible to conditions such as PAD. Information about these differences among people can help researchers understand how to best use drugs to treat disease. All information that the sample came from you will be removed from it. Please take your time to review this Information and Consent Form for Collection of Biological Material for Research and discuss any questions you may have with the study staff. You may take your time to make your decision about participating in this study and you may discuss it with your regular doctor, friends, and family. This consent form may contain words that you do not understand. Please ask the study doctor or study staff to explain any words or information that you do not clearly understand. This study is being funded by the Egg Farmers of Canada and the Agri-food Research and Development Initiative (ARDI).

The Impact of Egg Consumption on Indices of Vascular Health in Individuals with Peripheral Arterial Disease

Purpose of Study

The purpose of this research is to collect, store, and use your samples to study genes and determine if there is a relationship to PAD and whether eating eggs or not will change this pattern. A blood sample will be used to profile the RNA present in you blood cells and to measure the compounds that are released into your blood following a meal. If changes are found, we would also like to look at the DNA present in your blood cells. This information about your genes may also be compared with information from the genes of other people, including subjects with similar conditions.

Procedures

Approximately 15 ml or 1 tablespoon of the blood that will be taken at the baseline visit will be used for genetic testing. The sample will be stored locally in the study researcher, Dr. Zahradka's lab, at the St. Boniface General Hospital Research Centre for approximately 5 years and then it will be discarded. This will allow the researchers involved in the study enough time to conduct the procedures necessary to complete this portion of the research. The analysis will be performed by Dr. Zahradka and his research team. Samples may also be sent to the University Health Network Microarray Centre in Toronto, Ontario, and the University of Alberta, which are services that provide assistance to obtain certain pieces of information. The data collected will be sent to research collaborators situated at Crosslinks in the Netherlands and will also remain with Dr. Zahradka at the St. Boniface General Hospital Research Centre.

Risks and Discomforts

The risks of blood sampling include possible discomfort and pain, infection, minor bruising, and swelling at the site of the needle stick. There is also the possibility of perforation or penetration of the needle through the vein.

There is a potential for additional but rare risks associated with conducting genetic research, however, these risks are rare due to the extra protections in place to assure your confidentiality. When you donate your blood or tissue for genetic testing or research, you are sharing genetic information, not only about yourself, but also about biological (blood) relatives who share your genes or DNA.

There is a risk that information gained from genetic research could eventually be linked to you. This potential re- identification of the information (e.g., to an employer or insurer) could lead to loss of privacy and to possible future discrimination in employment or insurance, against you or your biological relatives. However, these risks are quite remote since appropriate confidentiality measures will be taken to protect any information about your health that is revealed by your DNA sample. The genetic material collected for the purposes of this study will not be used for diagnosis of a disease condition.

You should be aware that genetic information cannot be protected from disclosure by court order. Due to the rapid pace of technological advances, the potential future use of genetic information is unknown and therefore the potential future risks also are unknown.

Benefits

You may not benefit from participation in this research; however, the knowledge gained from this research may benefit others.

Confidentiality

All medical records and research materials in which you are identified will be kept confidential in accordance with the Personal Health Information Act of Manitoba and will not be made available except to the study doctor and the study coordinator involved in your care at the St. Boniface General Hospital. The Biomedical Research Ethics Board at the University of Manitoba and St. Boniface General Hospital may also review your research-related records for quality assurance purposes. If the results of the study are published, no one will know you were a part of the study since your identity will remain confidential. Personal information such as your name, address, telephone number, and/or any other identifying information will not leave St. Boniface General Hospital. Information identifying you will not be included with your blood sample for this study. Initially when you begin the study, you will be given a study number. The sample will be labeled with this number. After the samples are received by the researchers who will conduct the genetic testing, a new code will be given to the sample. The link between the first code and the new code will be held in a password-protected computer system with access from only those who are authorized. Once all of the samples have been collected and prepared, the link is permanently deleted. At this point the sample is considered "anonymized" and cannot be traced back to you personally. Therefore, your identity will never be revealed to anyone or be connected with genetic information from your sample. Neither you, your legal representative, nor your physician, will be able to know the results of any genetic analyses performed on your sample.

Sample Ownership

The researchers involved in this study will not sell or transfer ownership of the samples to other parties. The samples will be used only by the researchers associated with this study. The sponsoring organizations of this study will not have access to your samples. The findings from this study in general may be used by the researchers to conduct future studies.

Medical Care for Injury Related to the Study

In the event of an injury that occurs to you as a result of undergoing this procedure, you will receive the necessary medical treatment. In the event that you suffer injury as a direct result of participating in this research, necessary medical treatment not covered by

provincial health care insurance will be available at no additional cost to you. You are not waiving any of your legal rights by signing this consent form, or releasing the investigator or the sponsor from their legal and professional responsibilities. If you are injured because of taking part in this study, medical care will be provided to you by the study doctor, Dr. Guzman.

Compensation

There is no direct compensation for participation is this part of the research. There will be no cost to you for the procedures performed as part of this research. By signing the attached Informed Consent Form you consent to direct access to your medical records.

Withdrawal From This Research

If you change you mind about participating, you can withdraw your samples by making a request to the study doctor. However, once your identity has been removed from the sample, it will not be possible for you to withdraw it.

Questions

Before you sign this form, you should ask questions about anything that you do not understand. You are free to ask any questions that you may have about your treatment and your rights as a research subject:

Investigator:	Dr. James House	Tel No.	204-474-6837
Investigator:	Dr. Peter Zahradka	Tel No.	204-235-3507
Study Coordinator:	Mary-Lynn Wilson	Tel No.	204-258-1319

If any questions come up during or after the study or if you have a research-related injury, you may contact the study doctor and the study staff. For questions about your rights as a research subject, you may contact: The Biomedical Research Ethics Board, University of Manitoba at 789-
3389.

Do not sign this consent form unless you have had a chance to ask questions and have received satisfactory answers to all of your questions.

The Impact of Egg Consumption on Indices of Vascular Health in Individuals with Peripheral Arterial Disease

Consent

1. I have read and understood this Information and Consent Form for Collection of Biological Material for Research, and I freely and voluntarily agree to take part in this research.
2. I understand that I will be given a copy of the signed and dated Information and Consent Form for Collection of Biological Material for Research. I have received an explanation of the purpose and duration of the research, and the potential risks and benefits that I might expect. I was given sufficient time and opportunity to ask questions and to reflect back my understanding of the study to study personnel. My questions were answered to my satisfaction.
3. I agree to cooperate fully with the study doctor and will tell him if I experience any side effects, symptoms, or changes in my health as a result of the procedures involved.
4. I am free to withdraw my sample before my identity has been removed without prejudice to my future medical treatment.
5. I understand that representatives of the hospital and other regulatory authorities may wish to review my medical records. I have been assured that my name, address and telephone number will be kept confidential to the extent permitted by applicable laws and/or regulations. By signing this document, I give permission for such review and data collection, and grant direct access to my medical records.
6. By signing and dating this document, I am aware that none of my legal rights are being waived.

Signature:_____ Date/Time: _____

Printed name of above:_____

I confirm that I have explained the purpose of this research as well as any potential risks and benefits, to the subject whose name and signature appears above. I confirm that I believe that the subject has understood and has knowingly given their consent to participate by his/her personally dated signature.

Signature:_____Date/Time: _____

Printed name of above:____Study role:__

Appendix 3

Procedures

Screening Visit

The inclusion and exclusion criteria will be reviewed to ensure eligibility. Participants will be required to provide written informed consent prior to conducting any study related procedures. They will be assigned a study number to ensure anonymity. A medical history including age, sex, alcohol usage, exercise habits, current medical problems, medication intake, and a family history of previous events (e.g. stroke, hypertension, diabetes, heart disease) will be recorded. Participants will complete the FFQ at the screening visit. Administration of the FFQ takes pproximately 25 minutes to complete. Participants will have the option of taking the FFQ home to complete and return it at the baseline visit. Participants will be requested to complete 3-day dietary food record to determine food preferences and usual consumption of eggs prior to the baseline visit. Randomization will follow and participants will be assigned to one of the three assignments of the study. All participants will be advised to follow their habitual diet. Participants assigned to the eggs groups will receive nutritional information on eggs as well as serving suggestions and recipes for eggs. Participants will be reminded to fast (for 12 hours) prior to their next clinic visit for blood sampling.

Baseline Visit

At the baseline visit, participants will be asked to provide a urine sample and fasting blood samples (50 mL) for assessment of the biochemical analyses. A physical exam will be conducted to determine height, weight, body mass index, waist circumference and blood pressure. Baseline (pre-egg consumption) determinations of arterial compliance will also be assessed. Participants assigned to the egg consumption schedules will be given a 2-week allotment of eggs.

Assessments for Week 2, 4, and 6

After 2 weeks into their study schedule, participants will attend an interim in-person visit to the clinic for assessment. The semi-structured interview will be conducted in-person.

A fasting blood sample (20 mL) will be drawn for assessment of lipids, glucose, and glycated hemoglobin at Week 2 (during the in-person visit) and at Week 4 (lab only visit). Participants who experience elevated LDL-cholesterol with a result of 1.5 times greater than the accepted range of the upper normal limit (UNL) or elevated fasting glucose or glycated hemoglobin that exceed the standard of care at these time points in the study schedule will be asked to undergo a repeat sample one week later. Participants who experience a persistent elevation of LDL-cholesterol, fasting glucose, or glycated hemoglobin after having undergone repeat sampling will be asked to stop egg

consumption and they will be withdrawn from the study. If participants are withdrawn at any time during the study schedule, a (final) in-person visit will be conducted.

Telephone follow-up and a brief semi-structured interview will be conducted by telephone at the end of Week 4, and Week 6 for assessment of compliance, general side effects, changes in medication intake and medical condition, tolerability, recommendations, and continuation. Participants will be reminded to complete a 3-day food record at Week 6, prior to attending the final visit. Participants will be reminded to fast prior to the final (Week 8) visit. Participants assigned to the egg consumption schedules will be given their eggs every 2 weeks for the duration of the study schedule and asked to keep a record of their egg consumption.

Final Visit (Week 8)

At the end of the 8 week intervention phase, participants will report to the clinic in a fasted state, and urine and blood samples will be obtained. A final semi- structured interview will be conducted (in-person) for assessment of compliance, general side effects, changes in medication intake and medical condition, tolerability, and recommendations. Measurements for arterial compliance and the physical assessment (height, weight, body mass index, waist circumference and blood pressure) will be repeated.

Details of Study Variables

Anthropometrics

Purpose: To establish indirect measures of body composition at baseline and study completion.

- Body weight
- Height
- Waist circumference
- BMI

Dietary Assessment

Purpose: To assess dietary patterns and nutrient intake of participants with PAD.

- Food Frequency Questionnaire (FFQ)
- 3-day dietary food record

Biochemical Analyses.

Purpose: To establish concentrations of biomarkers of cardiovascular health at baseline, interim visits (lipids, glucose, and glycated hemoglobin only) and study completion.

- Fasting plasma lipid profile (i.e., triglycerides, total cholesterol, LDL-cholesterol, HDL-cholesterol);
- Pro- and anti-inflammatory markers including high-sensitivity C-reactive protein, interleukin-6, interleukin 10;
- Plasma homocysteine;
- Nutrient levels (i.e., serum folate, vitamin B12, and vitamin B6);
- Serum Adipokines (i.e., adiponectin, leptin);
- Markers for endothelial dysfunction (i.e., soluble adhesion molecules, including E-selectin, vascular cell adhesion molecule-1, intercellular adhesion

molecule-1, endothelial microparticles);

- Oxidative stress (i.e., serum TBARS (Thiobarbituric Acid Reactive Substances), oxLDL, nitrate/nitrite, nitrosylation, 8OH-dG, dityrosine, MDA, paraoxonase, glutathione);
- Urinary isoprostanes (marker of oxidative stress);
- Markers for diabetes (i.e., glucose, insulin, HbA1c);
- Plasma and red blood cell phospholipid fatty acid composition.

Expression Profiling: RNA, DNA and Metabolomic Testing

Purpose: To profile RNA expression and metabolite levels, and if changes are detected to examine DNA sequence, modification and allelic differences, in PAD participants who have/have not consumed eggs.

- RNA from blood samples at the baseline visit will be sent for analysis to the University Health Network Microarray Centre in Toronto, Ontario. As these data will help identify differences due to omega-3 egg consumption, it will be necessary to compare the results with both the baseline and normal egg group. Data generated will be sent to our collaborators at Crosslinks (Netherlands) and Agriculture and Agri-Food Canada (Winnipeg) to verify relevant phenotypic changes. DNA will be isolated from blood samples and banked until the RNA profiling data has been analyzed. Blood samples will also be collected for metabolomics testing at the University of Alberta via FT-mass spectrometry and proton- NMR instrumentation.

Arterial Compliance.

Purpose: To directly measure arterial health in patients with PAD at baseline and study completion.

- Ankle-brachial index (ratio of blood pressures of leg and arm): This is the standard assessment used in the physician's office.
- Arterial stiffness (measurement of pulse wave velocity, augmentation pressure and augmentation index)
- A non-invasive instrument will be used to measure the velocity of the blood pressure waveform between two different superficial artery sites. Analysis of these pulse waveforms (PWA) will be used to derive the central aortic pressure pulse waveform and calculate a range of central indices of ventricular-vascular interaction (i.e. augmentation pressure, augmentation index, ejection fracture, and Buckberg ratio).
- Arterial vasodilation (measured with an ENDO-Pat 2000) - This modern non-invasive device uses two probes that hook up to each index finger to measure blood flow. It is designed to evaluate endothelial cells which line blood vessels that regulate blood flow.
- Advanced Glycation Endproduct (AGE) reader - This diagnostic devices can non-invasively diagnose and assess the risk of diabetes and its complications by detecting fluorescence of AGEs in tissue. AGEs are essential biomarkers of metabolic and glycemic stress and have been implicated as causative factors in the progression of a host of age- related diseases, such as atherosclerosis, diabetes, and renal failure.

Semi-Structured Interview

Purpose: To illicit participants' perceptions of compliance, general side effects, changes in medication intake and medical condition, tolerability, recommendations, and continuation.