# THE EFFECTS OF VITAMIN D DEFICIENCY ON ATHEROSCLEROSIS STATUS IN SAUDI ARABIA DWELLERS

**WEDAD AZHAR**

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**WEDAD AZHAR**

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## Abstract

*Background:* Vitamin D has been shown to play a critical role in several systems other than the skeleton system. This study investigates the role of vitamin D in the development and progression of cardiovascular disease, particularly atherosclerosis.

*Hypothesis:* Adequate blood concentrations of vitamin D have a role in preventing atherosclerosis disease and reducing atherosclerosis risk factors.

*Methods:* Participants n=97 aged (Mean±SD) 48.2±8.4 years from both sexes segregated into n=33 controls, n=30 at-risk, and n=34 diagnosed with atherosclerosis. Background characteristics and vitamin D status were assessed from serum 25(OH)D, diet, supplements, and sun exposure. Atherosclerosis status and risk factors were assessed using a combination of clinical records, fasted blood (to ascertain C-reactive protein (CRP), blood glucose (FBG), and lipids profiles) and vascular structural and functional characteristics (including carotid-radial pulse wave velocity (PWV), central-blood pressure (cBP), peripheral-blood pressure (pBP), carotid intima-media thickness (IMT), and carotid inter-adventitial diameter (IAD)).

*Results:* Amongst the participants 51.5% were vitamin D deficient, 25(OH)D <20 ng/mL and 28.9% were insufficient 25(OH)D < 30 ng/mL. 25(OH)D levels were associated with all of the sources of the vitamin ($P<0.01$). Participants health status displayed distinct values for age, waist-to-hip ratio, CRP, FBG, low-density lipoprotein, triglycerides, pBP, cBP, IMT, and IAD ($P<0.05$). No association was observed between 25(OH)D concentrations and measured atherosclerosis characteristics in the pooled sample. Indeed, when vitamin D supplements were used 25(OH)D was negatively associated with CRP in the at-risk group and pBP in those diagnosed with atherosclerosis. When supplements were not used 25(OH)D was negatively associated with IMT in the control group ($P<0.05$).

*Conclusion:* The current study supports the previous reports on the incidence of vitamin D deficiency in Saudi Arabia. However, the current data do not show any link between 25(OH)D and critical to markers of atherosclerosis. Further Longitudinal experimental research is needed to clarify the association.

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# Declaration

I declare that this thesis is my own work, and no portion of the work has been submitted in support of an application for another degree or qualification of this university or any other learning institution. To the best of my knowledge, this thesis contains no material written or distributed previously by any other parties, apart from where I have otherwise stated.

# List of publication and conference presentations

- Wedad Azhar, Bartek Buczkowski, Gladys Onambele-Pearson, Christopher Smith (2015). The association between vitamin D deficiency and endothelial function. The 8th Manchester Metropolitan University postgraduate research conference, Manchester, UK, (5 November 2015)
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# List of contents

# List of tables

# List of figures

# List of abbreviations

| Abbreviations | Description |
|---|---|
| **$1,25(OH)_2D$** | 1,25-dihydroxyvitamin D (calcitriol) |
| **25(OH)D** | 25-hydroxyvitamin D (calcidiol) |
| **BFI** | Blood flow integral |
| **BMI** | Body mass index($kg/m^2$) |
| **B-mode** | brightness-mode |
| **CABG** | Coronary artery bypass grafting |
| **CAC** | coronary artery calcification |
| **cBP** | Central blood pressure |
| **CCA** | Common carotid artery |
| **CHD** | Coronary heart disease |
| **CP** | Central pressure |
| **CPBA** | Competitive electro-chemiluminescent protein binding assay |
| **CRP** | C-reactive protein |
| **cSBP** | Central blood pressure |
| **CT** | Computerised tomography |
| **CV** | Coefficient of variation |
| **CVD** | Cardiovascular diseases |
| **DBP** | Diastolic blood pressure |
| **DBP** | Diastolic blood pressure |
| **DI-VitD** | Dietary intake of vitamin D |
| **DSA** | Digital subtraction angiography |
| **ECG** | electrocardiogram |
| **ECLIA** | Electrochemiluminescence immunoassay |
| **eNOS** | Endothelial nitric oxide synthase |
| **FBG** | Fasting blood Glucose |
| **FFQ** | Food frequency questionnaire |
| **FMD** | Flow mediated dilation |
| **GBP** | Great British Pound |
| **HDL** | High density lipoprotein |
| **HR** | Heart Rate |
| **IAD** | Inter-adventitial diameter |
| **IMT** | Intima-media thickness |
| **LC-MS** | liquid chromatography in tandem with mass spectrometry |
| **LDL** | Low density lipoprotein |
| **NO** | Nitric oxide |
| **pBP** | Peripheral blood pressure |

| | |
|---|---|
| **PMCO** | The Prince Mutaib Chair for Biomarkers of Osteoporosis |
| **PP** | Pulse pressure |
| **pSBP** | Peripheral blood pressure |
| **PTH** | Parathyroid hormone |
| **PWV** | Pulse wave velocity |
| **RI** | Resistive index |
| **RIA** | Radioimmunoassay |
| **SBP** | Systolic blood pressure |
| **SCEBHC** | The Saudi Centre for Evidence Based Health Care |
| **SLE** | Systemic lupus erythematosus |
| **SPF** | Sun protection factor |
| **SR** | Saudi Riyal |
| **TC** | Total cholesterol |
| **TG** | Triglycerides |
| **TI-VitD** | Total intake of vitamin D |
| **TSE** | Total sun exposure |
| **UVB** | Ultraviolet B |
| **VDBP** | Vitamin D binding protein |
| **VDR** | Vitamin D receptor |
| **WHR** | Waist-to-hip ratio |

# Chapter 1 - Introduction

## 1.1. Background

Like all living beings, humans require nutrients to live and function. These nutrients are obtained mainly from food and drinks except for some exceptions, such as the sunlight to obtain vitamin D. Essential nutrients include protein, carbohydrates, fat, vitamins, and minerals and trace elements. Each nutrient has specific functions in the body, and as such, each nutrient is a key requirement for overall health.

In the past, observations of clinical disease like Scurvy, Beriberi, and Rickets led to the discovery of many nutrient deficiencies. Today, researchers are attempting to discover the further implication of nutrients-associated physiological dysfunction ranging from severe or moderate. Also, key, is the identification of nutrients impact not just on the easily recognisable characteristic symptoms but also on the development of certain diseases, including at the early stages of said diseases. Moreover, nutrient-related research is important in order to distinguish the risks of developing a disease.

This body of work went beyond research into the effects of vitamin D deficiency on bone health and systematically investigated the impact of vitamin D in the cardiovascular system, in particular, the incidence and development of atherosclerosis. Special models had to be designed to analyse the sources of the vitamin from diet and sun exposure to investigate these data in a country that has an abundant source of the sun-derived vitamin, however, most of its dwellers are reportedly deficient. In addition, assessments of atherosclerosis status were performed using current and new techniques to ensure a clear and accurate assessment. Moreover, to have a complete understanding of the situation, investigation of the factors that have an impact on both vitamin D and atherosclerosis disease, as well as the inter-relationship between these parameters were considered. Furthermore, to discover the association between the nutrient and the disease, analyses were to be carried on participants with different health statuses.

## 1.2. Structure of the thesis

This PhD thesis focuses on finding the association between serum 25 (OH)D concentration and other factors and markers that affect vitamin D status and atherosclerosis disease. Therefore, after the introduction in chapter 1, chapter 2 provides a literature review, which is an overview of vitamin D and atherosclerosis disease as reported in existing peer-reviewed journal articles on any link between vitamin D and atherosclerosis disease. After that, Chapter 3 clarifies the study aims and objectives.

Chapters 4 to 7 provide methods, results, and discussions of each section of the studies. Briefly, chapter 4 analyses and discusses quantification of the vitamin D status from circulating 25(OH)D levels, vitamin D supplements, dietary intake, and sun exposure. Chapter 5 analyses and discusses how lifestyle factors (i.e. gender, age, education level, occupation, socioeconomic status, smoking, body mass index (BMI) and waist-to-hip ratio (WHR)) affect circulating 25(OH)D levels. Chapter 6 analyses and discusses the relationships between circulating 25(OH)D concentrations and other blood markers of health (including C-reactive protein (CRP) , fasting blood glucose (FBG), total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL), and triglycerides (TG) as indicators of the markers discussed above). Chapter 7 analyses and discusses the association between circulating 25(OH)D levels and vascular structural and functional characteristics (i.e. carotid-radial pulse wave velocity (PWV), central blood pressure (cBP), peripheral blood pressure (pBP) carotid intima-media thickness (IMT), carotid artery inter-adventitial diameter (IAD)). Finally, chapter 8 provides a general discussion of the whole PhD study, the study limitations and recommendations for future work.

# Chapter 2 - Literature review

## 2.1. Vitamin D

### *2.1.1. Introduction*

In recent years, vitamin D deficiency or insufficiency has been shown to be a common international problem (Holick, 2017). Humans and other mammals mainly produce the 'sunshine vitamin' (vitamin D) when exposed to ultraviolet B (UVB) radiation, which is provided by sunlight (Lehmann and Meurer, 2010; Lips, 2010). Vitamin D is a hormone produced by sterols in the body, through the photolytic action of ultraviolet light in the skin (Holick, 1995; Combs, 2012). However, many factors diminish that process, such as the seasons, latitude, skin pigmentation, aging, sunscreen use, total body coverage, obesity, and smoking (Valle et al., 2011). Furthermore, small amounts of vitamin D are present in certain food: oily fish, liver, egg yolk and portobello mushrooms (Ross et al., 2011). Nevertheless, typically, dietary vitamin D represents 10 to 20 % of the circulating level of vitamin D (Dalan et al., 2014). However, this could be increased by eating fortified food or taking supplements (Combs, 2012). Apart from the well-known role of vitamin D in calcium homeostasis and bone metabolism, recent studies have suggested a diverse range of biological actions for the various forms of vitamin D, such as in cell growth and differentiation, the immune system, and hormonal system (Giovannucci, 2009; Adamczak, 2017; Bostock et al., 2017). However, those actions and roles are under investigation and studies are needed to confirm or deny their effectiveness (Lertratanakul et al., 2014; Faridi et al., 2017; Holick, 2017).

### *2.1.2. Vitamin D metabolism*

Vitamin D is an essential fat-soluble vitamin. The most important forms of the vitamin in humans are, Ergocalciferol ($D_2$) and Cholecalciferol ($D_3$) (Lehmann and Meurer, 2010). The human body can convert 7-dehydrocholesterol present in the skin to pre-vitamin $D_3$ when exposed to UV radiation (280-320 nanometres (nm)); this

compound can then be converted into vitamin $D_3$ (Valle et al., 2011). Other sources of vitamin D include foods that contain or are fortified with vitamin D. Vitamin $D_2$ is produced from ergosterol by yeast when it is exposed to ultraviolet radiation (Combs, 2012). Vitamin $D_2$ is manufactured by the food industry from lanolin, via exposure to ultraviolet radiation (Holick, 2009). Both vitamin $D_2$ and $D_3$ are commercially available as supplements. In addition, both types of vitamin D can be stored in and released from fat cells (Holick, 2007).

In the liver, vitamin D is converted to calcidiol [25-hydroxycholecalciferol, 25-hydroxy vitamin D, 25(OH)D], which is the major circulating form of vitamin D (Lehmann and Meurer, 2010). During circulation in the blood, vitamin D binds to vitamin D binding protein (VDBP) for transport to the targeted cells (Adriana et al., 2005). 25(OH)D is a biologically inactive form of the vitamin thus it must be transported to the kidneys and converted to calcitriol (1,25-dihydoroxyvitamin D) by 25-hydroxyvitamin D-1α–hydroxylase (CYP27B1) (Holick, 2009). Calcitriol [1,25-$(OH)_2$ D] is the most active form of vitamin D in the body, however, it has a half-life of 2 to 3 weeks compared to 4 to 6 weeks for 25(OH)D (Combs, 2012). Once the active form of vitamin D has reached the desired cell, it is released from its binding protein and attaches to the vitamin D receptor (VDR) which is available in most of the body tissues and on most cells (Holick and Chen, 2008; Quraishi and Camargo, 2012) (figure 2-1).

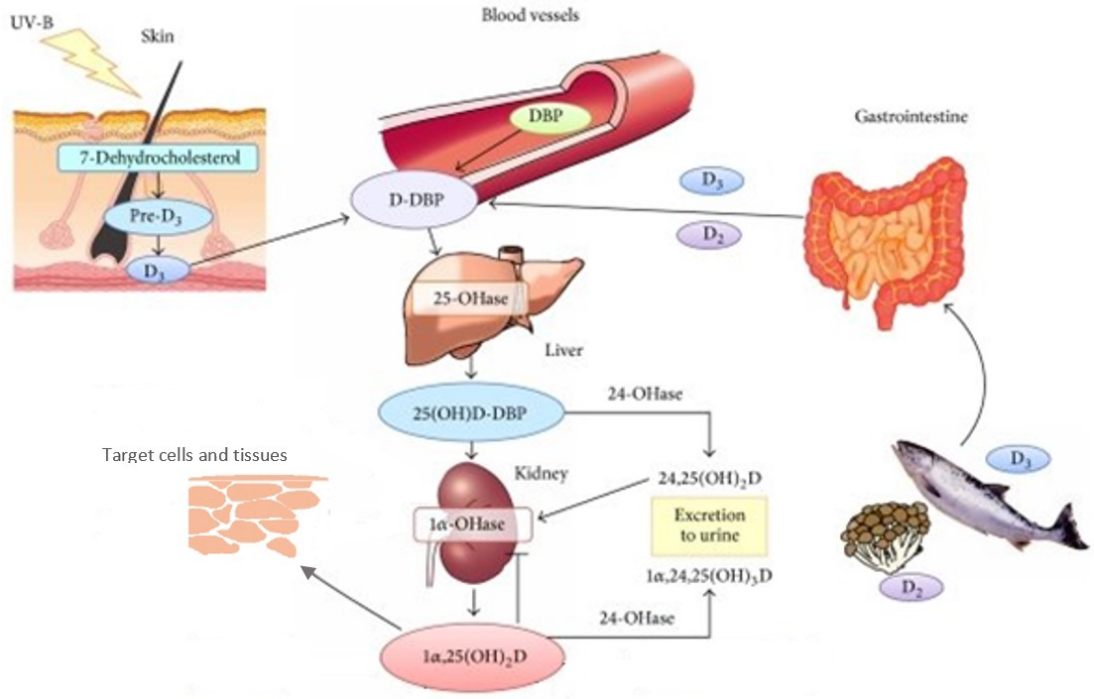


*Figure 2-1 Vitamin D metabolism in the body (Obi et al., 2015)*

### *2.1.3. Factors that influence circulating and bound levels of vitamin D*

Vitamin D levels are affected by a variety of factors for instance, distance from the equator, skin tone, and some diseases (Holick et al., 2011). The UV levels are lower at higher latitudes (above 35°) because of the filtering of sunlight by the atmospheric thereby influencing the body's ability to synthesise vitamin D (Binkley et al., 2007; Holick, 2007 Mithal et al., 2009). It has also been demonstrated that cloth, window glass, and the use of sunscreen all prevent the absorption of this radiation by more than 95% (Holick et al., 2011; Aljefree et al., 2017a). Moreover, people with dark tone skin have some natural sun protection and require more time, three to five times longer, than people with light coloured skin when exposing their skin to the sunlight for effective vitamin D levels to be reached (Holick et al., 2011; Combs, 2012; Al-Daghri et al., 2016). Ageing also magnifies the reduction of 7-dehydrocholesterol in the skin and thereby further decreases the ability to produce and metabolise vitamin D in the body (Arain et al., 2015). In addition, obesity decreases the bioavailability of vitamin D, as the vitamin is sequestered in fat cells (Ardawi et al., 2012). Patients with fat malabsorption syndromes, bariatric patients, and those with nephrotic syndrome are often unable to absorb vitamin D (Holick et al., 2011). Some medications also

reduce or prevent the absorption or the ability to metabolise vitamin D such as anticonvulsants and HIV medications (Lehmann and Meurer, 2010). Some diseases also reduce the body's ability to absorb or produce vitamin D, i.e. liver or kidney diseases (as hepatic or renal disease), metabolic bone disease (e.g. osteoporosis), cancer, or hypercortisolism (Holick and Chen, 2008; Holick et al., 2011; van Schoor and Lips, 2011).

In Saudi Arabia, there are additional factors that influence circulating levels of vitamin D, as a direct result of the climate and lifestyle. Saudis usually avoid spending time outdoors to shelter from the scorching sun, which is imperative as temperatures rise up to above 48°C during the summer in most of the regions, particularly in the centre, west, and south of the country (Sadat-Ali et al., 2014; Al-Daghri et al., 2016). Indeed, sunstroke is a common illness in Saudi Arabia for those who fail to avoid the sun (Ministry of Health, 2017). On the other hand, skin cancer is not a common illness in Saudi Arabia when compared to other countries at the same latitude (Al-Daghri et al., 2016; Al-Dawsari and Amra, 2016). It is the ninth most common malignancy and represents approximately 3.2% of all newly diagnosed cases of cancer in Saudi Arabia (Al-Dawsari and Amra, 2016). Outdoor clothing style is another factor that limits sun exposure. Men usually wear a Thoup, which appears as a dress with long sleeves (usually white), as well as a Shomak on their head, which is a triangular piece of cloth and functions as protection from the excessive sun (Al-Agha et al., 2016; Aljefree et al., 2017b). Contrastingly, women mostly wear dark veils that cover their entire bodies. They also wear a scarf to cover their head; in addition, some women use a Niqab that covers their face, for cultural and religious reasons (Ardawi et al., 2012; Sadat-Ali et al., 2014).

### *2.1.4. Measurement of vitamin D*

Determination of serum 25(OH)D levels is considered to provide an accurate and reliable reflection of vitamin D status other than the 1,25$(OH)_2$D (Ford, 2013; Holick, 2017). Serum 25(OH)D reflects both oral intake and subcutaneous vitamin D production. Currently, three different methods of measuring 25(OH)D have been

defined: liquid chromatography in tandem with mass spectrometry (LC-MS), radioimmunoassay (RIA), and a competitive protein binding assay (CPBA) (Holick, 2009; Sadat-Ali et al., 2014). In general, LC-MS is considered the gold standard, even though it is a very complex technique (Ford, 2013). Whilst, the RIA is a fast and robust measurement, it can be affected by the presence of other substances which contribute 10-15 % of the 25(OH)D status (Holick, 2009; Alyami et al., 2014). The CPBA technique is a reliable technique for measuring 25(OH)D in serum or plasma (van den Ouweland et al., 2013). The assay uses vitamin D binding protein (VDBP) as a capture protein. In fact, the CPBA has a 93% concordance rate when compared to the gold standard and has 100% cross-reactivity with 25(OH)D (Lensmeyer et al., 2006; Reis et al., 2009; Abdel-Wareth et al., 2013).

A consensus has not been reached at the present time as to what should be considered to be a deficiency of vitamin D (Ford, 2013). The current study considering guidelines from the Saudi Ministry of Health and findings from pioneer studies in classing serum concentrations as follows; 25(OH)D < 20 nanogram per millilitre (ng/mL) considered deficient, 20 ≤ 30 ng/mL is insufficient, and > 30 ng/mL is adequate vitamin D serum levels (Alyami et al., 2014; Dalan et al., 2014; Sadat-Ali et al., 2014; Panel., 2014; Al-Daghri et al., 2016).

### *2.1.5. Vitamin D status worldwide*

Hypovitaminosis D (vitamin D deficiency) occurs in 30 to 50 % of people, even in places where there is adequate sunshine all year round (Menezes et al., 2014). Vitamin D deficiency remains prevalent in Middle-Eastern countries and South Asia mainly due to clothing styles, skin tone and lack of outdoor activities (mean 25(OH)D in males 17.6 ng/mL and 11.2 ng/mL in females) (Okano, 2003; Mithal et al., 2009). Similarly, in Africa low serum 25(OH)D is a common issue, which is probably caused by dark skin colour and a tradition of fully-covered clothing in some parts of the continent (mean 25(OH)D in males 20.5 ng/mL and 12.5 ng/mL in females) (Lips, 2010). Conversely, in Europe, people in Nordic countries show a higher vitamin D status than those in the Mediterranean countries, which is arguably due to the lighter

skin colour and the high consumption of cod liver oil. However, in winter months, 25(OH)D concentrations decrease by up to 15ng/ml below summer month levels (van Schoor and Lips, 2011). Furthermore, the United States and Canada generally have populations with sufficient 25(OH)D concentrations (mean = 26.4 ng/mL) probably as a result of milk supplementation with vitamin D and the common use of supplements (Arain et al., 2015). Similarly, populations in Latin America have levels comparable to those in North America due to the habit of doing lots of outdoor activities and extensive exposure to sunlight (mean 25(OH)D 27.2 ng/mL) (van Schoor and Lips, 2011). In contrast, vitamin D deficiency is prevalent in Australia and New Zealand (mean 25(OH)D = 16.7 ng/mL) (Mithal et al., 2009).

### *2.1.6. Vitamin D status in Saudi Arabia*

Saudi Arabia has ample sunlight for adequate vitamin D production, and yet there is a high prevalence of vitamin D deficiency across all age groups (mean 25(OH)D = 12.7 ng/mL in males and 6.3 ng/mL in females) (van Schoor and Lips, 2011). Saudi Arabia is located in the South West of Asia between latitude (between 16° N and 33° N) (figure 2-2). During the summer months, the temperature can rise to 48°C, and it is mostly sunny all year round (AccuWeather, 2015). However, the extreme high temperature, lifestyle and other factors decrease the advantages gained from an abundance of sunshine. Many studies have examined the vitamin D status and confirmed the prevalence of vitamin D deficiency in the Saudi population (Ardawi et al., 2012; Aljefree et al., 2016; Kaddam et al., 2017). Table 2-1 shows studies measuring 25(OH)D status in different regions of Saudi Arabia.


*Figure 2-2 Map of Saudi Arabia showing the geographical location of the country on the world map (inset) and its regions and main cities.*

*Table 2-1 Studies determining vitamin D status in Saudi Arabia*

| Study | Subjects | Sample size | Vitamin D deficient* | Vitamin D** sufficient |
|---|---|---|---|---|
| (Kaddam et al., 2017) | Saudi school students (50.4% males, 49.6% females) aged 6-19 and Saudi school employees (50.9% males, 49.1% females) aged 20-62 In Central, Western and Eastern regions- different cities | 4000 students and 2075 employees | 95.4% students<br>89.1% employees | 4.6% *** students<br>10.9%*** employees |
| (Aljefree et al., 2016) | Coronary heart disease patients and control adults In Western region- Jeddah city | 325 | 57.5 % | 42.5 %*** |
| (Abulkhair et al., 2016) | Newly diagnosed breast cancer patients aged 42-57 In Central region- Riyadh city | 406 | 71 % | 29 % *** |
| (Al-Saleh et al., 2015) | School children aged 15.1 ± 2.2 In Central region- Riyadh city | 1188 boys 1038 girls | 79 % boys 92.5 % girls | 17.2 % boys 4.7 % girls |
| (Ardawi et al., 2012) | Men aged 20-74 In Western region – Jeddah city | 834 | 51.5 % | 38.8 % |
| (Azhar, 2009) | Women aged 20-60 (pregnant and non-pregnant) In Western region- Makkah city | 118 | 78.9 % | 17.8 % |
| (Siddiqui and Kamfar, 2007) | Girls aged 12-15 In Western region- Jeddah city | 433 | 40 % | 41 % |
| (Al Faraj and Al Mutairi, 2003) | People with chronic low back pain aged 15-52 In Central region- Riyadh city | 360 (90% women, 10% men) | 83% | 12% |
| (Serenius et al., 1984) | Pregnant women In Central region- Riyadh city | 119 | 25.2% < 4ng/mL and 36.1% > 4 - 25 ng/mL | 38.7% ≥ 25 ng/mL |

- *Deficient at 25(OH)D < 20 ng/mL
- **Sufficient at 20≤ 25(OH)D > 30 ng/mL
- *** 25(OH)D > 20 ng/mL
- 1 ng/mL (nanogram per millilitre) = 2.496 nmol/L (nanomole per litre)

### *2.1.7. Guidelines for vitamin D in Saudi Arabia*

In response to the local need to investigate and address the challenge of vitamin D deficiency, the local authority developed guidelines targeting Saudi dwellers, clinicians, and policy makers. The two main guidelines were the osteoporosis clinical practice guidelines on the role of vitamin D, calcium, and exercise in fracture prevention in the elderly, which is run by the Saudi Centre for Evidence Based Health Care (SCEBHC), Ministry of Health (Saudi Expert Panel., 2014). The second guideline is the Prince Mutaib Chair for Biomarkers of Osteoporosis (PMCO), in King Saud University, Riyadh, KSA. Local pioneers and international experts worked together to generate the guidelines for vitamin D based on the local circumstances not just in Saudi Arabia but also throughout the Middle-East (Al-Daghri et al., 2016). Both guidelines proved that total 25(OH)D is the gold standard biomarker for vitamin D status as has been proved internationally (Holick et al., 2011). The cut-offs points of 25(OH)D levels are the same as those used in the current study section 2.1.4. Table 2-2 illustrates the PMCO list of people at high risk of vitamin D deficiency and those who are eligible for screening, which is also similar to the guidelines used internationally (Ross et al., 2011; Holick, 2017).

*Table 2-2 The PMCO list of people at a high risk of vitamin D deficiency in Saudi Arabia (Al-Daghri et al., 2016)*

- Pregnant and lactating mothers
- Infants and children
- Obese or morbidly obese persons
- People with dark skin tone
- Elderly individuals
- People with low sun exposure habits

Clinical conditions:

- Patients with malabsorption syndromes
- Patients diagnosed or suspected with, osteopenia, osteoporosis, ostemalacia, or rickets
- Patients with renal or liver disease
- Patients with musculoskeletal disease
- Post bariatric surgery patients

Based on the weather in Saudi Arabia, just recently the PMCO generated specific recommendations for sun exposure to obtain sufficient amounts of vitamin D and to prevent other complications from excessive sun exposure such as sunstroke and skin cancer. The sun exposure recommendations are as follows:

- During summer, sun exposure should be three to four times a week from 9:00 to 10:30 am in the morning and from 2:00 to 3:00 pm in the afternoon.
- During winter, the times should be from 10:00 am to 2:00 pm.
- At least 20% of the body should be directly exposed to sunlight, especially the hands and legs. Direct exposure means without sunscreen and not through glass, windows, or an umbrella.
- People with darker skin tone may need more time for adequate sunlight exposure (Saudi Expert Panel., 2014; Al-Daghri et al., 2016).

The Saudi Ministry of Health recommends a dietary intake of vitamin D for adults up to 70 years old for males and females of 600 IU/day (Saudi Expert Panel., 2014; Ministry of Health, 2017). In regard to the use of supplements, the PMCO and SCEBHC have provided specific guidelines for Saudi dwellers. People at high risk of vitamin D deficiency are advised to take a maintenance dose of vitamin D supplements (table 2-3). The PMCO established guidelines for treatment doses and the length of the treatment for people in different age groups and with different health conditions. In addition, vitamin D supplementation is advised for people who have serum 25(OH)D levels <20 ng/mL until they reach a sufficient level of 30 ng/mL, and may need a maintenance dose afterwards (Saudi Expert Panel., 2014; Al-Daghri et al., 2016).

*Table 2-3 The PMCO recommendation for vitamin D supplements maintenance dose (Al-Daghri et al., 2016)*

| **People recommended to take vitamin D supplements** | **Recommended maintenance dose IU/day** |
|---|---|
| Premature infants | 400-800 |
| Infants 0-6 months | 400 |
| Infants 6-12 months | 400-600 |
| Children aged 1-10 years | 600-1000 |
| Adolescents aged 11-17 years | 600-1000 |
| Obese children* | 1200-2000 |
| Pregnant/ lactating women | 1000-2000 |
| Elderly patients | 1000-2000 |
| Postmenopausal women | 800-1000 |
| Obese postmenopausal women* | 3000-6000 |
| * Patients who are obese, have morbid obesity, malabsorption syndromes, hepatic, renal diseases, or have had post bariatric surgery. | |

## 2.2. Atherosclerosis

Atherosclerosis (arteriosclerotic vascular disease) is the most common type of heart disease, which makes it the most common cause of death worldwide (Joseph, 2011; Lertratanakul et al., 2014). It is a condition that thickens the artery wall due to the accumulation of white blood cells and fatty substances (fatty sticks) (Libby, 2006) (figure 2-3). Intima and media are the main layers in the human arterial wall. The inner side of the wall is the intima; it contains a layer of cells called the endothelium and then the internal elastic lamina (George and Johnson, 2010; Adams et al., 2017). Between the internal elastic lamina and the external elastic lamina is the media layer (Joseph, 2011). The media consist of vascular smooth muscle cells (VSMCs) surrounded by the basement membrane (figure 2-3) (George and Johnson, 2010). Dysfunction of the endothelial cells causes the formation of plaques or atheroma (British Heart Foundation , 2017). Plaques form slowly and consist of: multiple cell types, cholesterol, triglycerides, calcium, proteoglycans, and extensive connective tissue (Anderson et al., 2012). Consequently, the plaque formation hardens and narrows the arteries, which restricts blood flow in the circulatory system (Menezes et al., 2014). Moreover, if the plaque ruptures, it will cause a blood clot that can block the artery. Depending on the location of the clot in the arterial tree, the organ that is supplied by the artery will be damaged.

Over time, atherosclerosis will lead to one or more of the following conditions:

- Angina: which is a discomfort or pain in the chest area and that is due to a low level of blood and oxygen going to the heart muscle.
- Heart attack: a condition when the plaque or the blood clot completely blocks the artery that supplies the heart.
- Ischaemic stroke: a state when the block occurs in the arteries supplying the brain.
- Peripheral arterial disease (PAD): a situation which occurs when not enough blood reaches the leg muscles (Giovannucci, 2009; BHF, 2017).

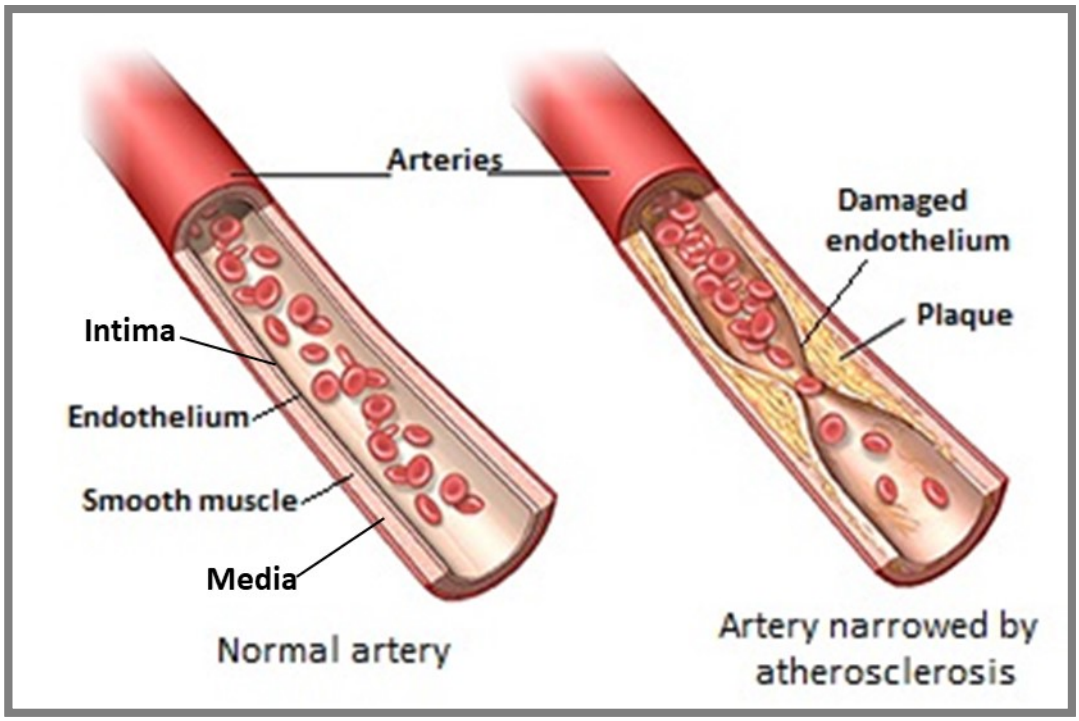


*Figure 2-3 Illustration of a normal artery and one damaged by atherosclerosis (UNIVERSITY OF ROCHESTER MEDICAL CENTER, 2018)*

### *2.2.1. Atherosclerosis risk factors*

Many risk factors can increase an individual's chances of developing atherosclerosis. The unmodifiable risk factors are stated as: advanced age, maleness, and inheritance of premature atherosclerotic disease (Beckman et al., 2002) whereas, modifiable risk factors are: hypercholesterolemia, hypertension, hyperglycaemia, smoking, and obesity (Anderson et al., 2012). Lifestyle can modulate the development and the progression of atherosclerosis, including physical inactivity, smoking, poor diet, and stress (Doran et al., 2008).

### *2.2.2. Assessment of atherosclerosis*

Atherosclerosis is initially an asymptomatic condition until patients show signs of angina or any of the other complications of the disease. Otherwise, usually, it can be indicated by the presence of risk factors such as hyperglycaemia and hypertension and/or inflammatory markers, such as hyperlipidaemia and high C-reactive protein (CRP) concentrations, that are associated with the disease (Sibley et al., 2014).

Further assessment tests such as: electrocardiogram (ECG), computerised tomography (CT) scan, exercise stress tests, and ultrasound as non-invasive techniques could be used when patients have signs of atherosclerosis (Libby, 2006). Other examples of invasive tests that are used for assessment and treatment of atherosclerosis are digital subtraction angiography (DSA) and coronary artery bypass grafting (CABG) (Kim et al., 2008).

Using the above assessment methods, the arteries' function, resistance, and stiffness can be examined. The measurement of the carotid artery is considered the most commonly assessed artery for atherosclerosis especially in non-invasive methods (Polak et al., 2013; Adams et al., 2017). The artery can be tested for intima media thickness (IMT), Inter-adventitial Diameter (IAD), resistive index (RI), and blood flow integral (BFI) (Adams et al., 2017; Awad and Abbas, 2017). Other methods can be used to measure the resistance or the blood flow of the arteries using the flow speed between two arteries such as the carotid-femoral, brachial-ankle, or carotid-radical (Libby, 2006). Additional methods can measure blood flow, vessel diameter or artery stiffness such as coronary microvascular vasoreactivity, flow-mediated dilation (FMD), or pulse wave velocity (PWV) (Kim et al., 2008). PWV is emerging as the gold standard for the measurement of arterial stiffness (Collaboration, 2010; Pereira et al., 2014).

### *2.2.3. Endothelium*

The endothelium is a thin layer of simple cells that line the inner walls of the vessels and provide critical homeostatic functions in the cardiovascular system (Giovannucci, 2009). Endothelial cells are exposed to a variety of blood-borne signals and intravascular stressors, which regulate vascular tone, nutrient delivery, platelet activity, leukocyte adhesion, and angiogenesis (Libby, 2006; Arrebola-Moreno et al., 2012). Moreover, the endothelium synthesises important substances, including antiproliferative, antithrombotic molecules, nitric oxide (NO) and prostacyclin (Joseph, 2011). Specifically, the limitation of NO secretion is the key manifestation of endothelial dysfunction, which promotes atherosclerosis (Widlansky et al., 2003).

#### *2.2.3.1. Nitric oxide (NO)*

Nitric Oxide is a simple molecule that consists of a single oxygen atom bonded to one nitrogen atom (Ignarro, 2009). It has been proven that NO has many essential roles in the human body such as being a mediator in cell-to-cell communication (Napoli and Ignarro, 2001). In addition to its influence in the vascular system, it has an impact on the immune system, enzyme synthesis, autoimmune diabetes, and liver inflammation and infection (Ignarro, 2009).

Nitric oxide is a very important substance in the cardiovascular system. It controls vascular relaxation by potently dilating vessels (Bryan et al., 2008). It also prevents platelet aggregation, monocyte adhesion to endothelial cells, and abnormal smooth muscle cell proliferation (Anderson et al., 2012). The human body can produce NO by converting nitrates found in plants, such as beets, spinach, and leafy green vegetables (Hord et al., 2009). When these forms of food are chewed and mixed with saliva, commensal bacteria convert nitrates into nitrites (Lancaster, 1996). Subsequently, in the stomach, gastric juices convert nitrites to NO (Hord et al., 2009), which is then absorbed into the bloodstream (Bryan et al., 2008). However, as the human body ages, this process becomes less effective, which is one of the reasons endothelial dysfunction develops with age (Ignarro, 2009).

#### *2.2.3.2. Endothelial dysfunction*

Endothelial dysfunction is an early event in atherosclerosis, which is involved in plaque progression and the recurrence of atherosclerotic complications (Hadi et al., 2005). The inadequacy of NO bioavailability is the main indicator of endothelial dysfunction, in addition to vasodilators and an increase of endothelium contracting factors (Jablonski et al., 2011). Endothelial nitric oxide synthase (eNOS) is an endogenous inhibitor that contributes to the development of diabetes mellitus, hypertension, renal failure, and other diseases (Joseph, 2011). In addition to endothelium vasodilation weakness, endothelial dysfunction also involves endothelial activation, which influences all stages of atherosclerosis (Anderson, 1999). In fact, all of the modifiable and unmodifiable atherosclerosis risk factors are

associated with variations in endothelial function (Hadi et al., 2005). Recent studies have also associated endothelial dysfunction with elevated C-RP, chronic systemic infection, air pollution, and vitamin D deficiency (Alyami et al., 2014; Joseph, 2011; Yiu et al., 2011).

## 2.3. The role of vitamin D in atherosclerosis

Over the previous decades, vitamin D has been shown to have preventive and disruptive effects against the development of atherosclerosis (Giovannucci, 2009; Menezes et al., 2014). Vitamin D has a vascular-protective effect by regulating vascular smooth muscle cells (VSMC) against endothelial dysfunction, and by managing the inflammatory and immune processes (Doran et al., 2008; Lertratanakul et al., 2014; Menezes et al., 2014; Faridi et al., 2017).

Endothelial cells are found to express the vitamin D receptor (VDR), which has a protective effect on the incidence and progression of endothelial dysfunction (Menezes et al., 2014). The endothelial cells host 1-α hydroxylase, which converts 25(OH)D to 1,25$(OH)_2$D, the biologically active form of the vitamin (de Boer et al., 2009). Moreover, calcitriol is the transcriptional regulator of NO synthase, which is the critical vasodilator (Menezes et al., 2014). Consequently, vitamin D deficiency is associated with a lack of NO synthesis, which causes endothelial dysfunction (Yiu et al., 2011). Similarly, vitamin D has an effect on the phosphatidylinositol 3 kinase enzyme in endothelial cells, as the enzyme is also responsible for the synthesis of NO (Alyami et al., 2014). It was observed by Aoshima et al. (2012) that, interactions between vitamin D and the VDR cause phosphorylation, which leads to eNOS activation and an increase in NO formation. Nitric oxide can prevent early atherosclerosis development by inhibiting platelets and leukocytes from aggregation and adhesion (Jablonski et al., 2011).

Vitamin D has a role in protecting endothelial cells from oxidative stress, as it can oppose superoxide anion generation, which restrains reactive species and counteracts apoptosis (Wong et al., 2008). Menezes et al. (2014) has suggested a mechanism of 25(OH)D that influences muscular-vascular tone through an influx of

calcium into the endothelial cells that causes activation of calcium-dependent phospholipids. Furthermore, vitamin D has anti-proliferative effects on VSMC, which is a layer of cells in the vascular wall (Jablonski et al., 2011). VSMC produce molecules called the 'extracellular matrix', which modifies the lipid content and the formation of plaques in the vascular wall (Doran et al., 2008). In addition, VSMC contributes several inflammatory molecules that help to limit the development of atherosclerosis (Doran et al., 2008). The vitamin D response elements interact with gene promoter regions, which are genetic sequences related to arterial wall functions such as vascular endothelial growth factors, myosin, and type 1 collagen (Nibbelink et al., 2007).

### *2.3.1. Studies on the effects of vitamin D on atherosclerosis and the endothelium*

Many studies have investigated the association between vitamin D status and CVD, atherosclerosis, and endothelial function (Jablonski et al., 2011; Alyami et al., 2014; Aljefree et al., 2017b). Jablonski et al. (2011) observed a significant correlation between endothelial function and serum consentration of 25(OH)D in middle aged and older adults (50-79 years). Results showed that FMD was lower ($P<0.01$) when the 25(OH)D level < 20 ng/mL versus 25(OH)D level > 20 ng/mL. Additionally, the VDR and 1-α hydroxylase were associated positively with FMD ($P<0.05$). Tarcin et al. (2009) also found a positive correlation between 25(OH)D levels and FMD ($r=0.45$; $P<0.05$) when the participants presented as deficient (25(OH)D concentration < 25 ng/mL), and after treatment with vitamin $D_3$ supplements.

Another study monitored participants over 4-5 years, and indicated a significant correlation between the development of CVD and low vitamin D (25(OH)D ≤ 15 ng/mL) $P<0.001$ (Wong et al., 2008). Kilkkinen et al. (2009) examined the impact of vitamin D concentration on the development of, and mortality from, CVD in healthy persons over a period of 25 years. The study found that, based on their 25(OH)D serum levels, individuals within the upper 20% (20 ng/mL) had less than half the risk of death from CVD compared to those with serum levels of 25(OH)D in the bottom

20%. In addition, a large-scale study observed the association between vitamin D levels and hypertension, which is one of the atherosclerosis risk factors. This study followed 613 men and 1198 women for 12 ± 3 years. The results indicated an inverse association between the risk of hypertension and 25(OH)D concentration ($P<0.001$) (Forman et al., 2007). A study in Scotland (Sugden et al.2008) investigated the impact of vitamin D levels on endothelial function in patients with type 2 diabetes. The diabetic patients with 25(OH)D serum levels < 20 ng/mL consumed a single dose of 100 000 IU vitamin $D_2$ and their endothelial function was re-examined after 8 months. The supplements significantly improved the participants FMD status by 2.3% ($P<0.05$). Table 2-4 illustrates additional studies that show the impact of vitamin D on atherosclerosis and atherosclerosis risk factors.

*Table 2-4 List of previous studies on vitamin D and atherosclerosis and atherosclerosis risk factors*

| Study | Subjects | Sample size and age | Location | Study design | Main measurements | Results |
|---|---|---|---|---|---|---|
| **(de Boer et al., 2009)** | 53% of participants with a prevalent of CAC[1] at baseline and the rest were healthy controls | N=1370 45-84 years | United States of America | Cohort study | 25(OH)D, 1,52(OH)2D, CAC using CT scan | 21% of participants with CAC at baseline developed an incidence of CAC and had a lower 25(OH)D after 3 years' follow up |
| **(Lertratanakul et al., 2014)** | SLE[2] Patients participating in inception cohort from 8 countries. Patient follow-up after 15 months of first diagnosis | N=890 Adults | Canada, Iceland, Korea, Spain, Switzerland ,United Kingdom, and United States of America | inception cohort study | 25(OH)D, SLE assessments, CVD[3] risk factors and events | Patients with lower 25(OH)D at baseline had higher incidence of CVD[3] risk factors. Patients with higher 25(OH)D were less likely to have hypertension, hyperlipidaemia, CVD events and more likely to have lower CRP, systemic lupus erythematosus disease activity. |
| **(Targher et al., 2006)** | Type 2 diabetic adults and healthy control adults | N= 780 (390 diabetic & 390 nondiabetic) | Arzignano, Italy | Randomised control trial | IMT in CCA 25(OH)D | Diabetic patients had lower 25(OH)D concentrations than non-diabetics P<0.001. Diabetic patients with low 25(OH)D had higher IMT when compared with sufficient 25(OH)D patients P<0.001. They also had higher CRP concentrations. |
| **(Faridi et al., 2017)** | Participants in a high-risk community for atherosclerosis | N= 13,039 Baseline age 57 ± 6 | United States of America | Longitudinal community-based study | 25(OH)D and lipid profiles were measured 3 times, each time | Deficient concentrations of 25(OH)D were associated with low TC and high HDL concentrations. |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | was ~ 3 years apart. | |
| **(Reis et al., 2009)** | Older adults without a history or incidence of CVD[3] | N= 654<br>55-96 years | United States of America | Cross-sectional study | 25(OH)D, $1,25(OH)_2D$, IMT, BP | There was no association between $1,25(OH)_2D$ and IMT in total sample.<br>In a subgroup of hypertensive there was an inverse association between $1,25(OH)_2D$ and IMT P=0.036 |
| **(Zittermann et al., 2009)** | Healthy overweight participants with mean 25(OH)D =12 ng/mL receiving vitamin D supplements or placebo | N= 200<br>18-70 years | United States of America | A double blind study | 25(OH)D, $1,25(OH)_2D$, | Significant improvement of vitamin D status in supplement group.<br>A positive improvement in cardiovascular markers. |
| **(Aljefree et al., 2016)** | Participants were CHD[4] and age-sex matched controls | N=130 CHD & 195 controls<br>20-70 year | Saudi Arabia | Case-control study | 25(OH)D, FBG, lipids profile, | 46% of CHD and 3% of controls had 25(OH)D <20 ng/mL (P=0.001) |
| **(Al Mheid et al., 2011)** | Participants free of any acute illness | N=554<br>20-79 years | Australia | Community-based asymptomatic population | 25(OH)D, FMD, PWV , RI, lipid profiles | Vitamin D deficiency associated with arterial stiffness, endothelial dysfunction and CVD risk markers.<br>25(OH)D associated with FMD (P=0.03), RI (P=<0.001), and PWV, (P=0.04). |
| **(Taskiran et al., 2017)** | Type 1 diabetic patients | N-93<br>20-45 years | Turkey | Observational | 25(OH)D, HbA1c, IMT, Lipid profile | 78% had 25(OH)D < 20 ng/mL<br>No association was observed between 25(OH)D and IMT |

1- Coronary artery calcification (CAC)
2- Systemic lupus erythematosus (SLE)
3- Cardiovascular disease (CVD)
4- Coronary heart disease (CHD)

## 2.4. Effect of vitamin D on atherosclerosis risk factors

Studies have suggested a substantial role for vitamin D in modifying many of the atherosclerosis risk factors both modifiable and non-modifiable (Lertratanakul et al., 2014; Menezes et al., 2014; Arora and Rehan, 2015).

### *2.4.1. Effect of vitamin D on lipid profiles*

Many studies have demonstrated the impact of vitamin D deficiency in dyslipidaemia. Low concentrations of vitamin D have been associated with increasing the blood concentrations of TC, LDL, TG and decreasing the concentrations of HDL (Giovannucci, 2009; Faridi et al., 2017). Researchers suggest that vitamin D increases the absorption of intestinal calcium, which reduces the amount of lipids absorbed in the gut, which will reduce serum concentrations of LDL and TG (van den Ouweland et al., 2013; Faridi et al., 2017).

A large cross-sectional, prospective study by Faridi et al., (2017) examined the impact of vitamin D on the lipid profiles of 13,039 participants. Measurements were taken at baseline and followed up twice each visit was approximately 3 years apart. Vitamin D deficiency was associated with lower TC and higher HDL levels. Another double-blind randomised control study by Qadhi, (2016) investigated the impact of vitamin D supplements for a period of 16 weeks. The significant impact of the supplementations were shown in the changes in the serum LDL (P=0.001) and TC (P=0.028).

### *2.4.2. Effect of vitamin D on glucose levels and insulin resistance*

There is some evidence correlating vitamin D concentration to the risk and development of diabetes (Lee et al., 2008; Witham et al., 2010; Taskiran et al., 2017). β-cells, which synthesise and secrete insulin, in the pancreas express the VDR and vitamin D activating enzyme, 1α–hydroxylase (Lee et al., 2008; Holick, 2017). Other studies have suggested a role for vitamin D in insulin production and glucose homeostasis (Talaei et al., 2013;Al-Daghri, 2016).

In a double-blind randomised controlled trial, 60 type 2 diabetes patients were given vitamin D fortified yogurt daily for 12 weeks. There was a positive, significant change in FBG (P=0.016) and in HBA1c (P=0.001) (Neyestani et al., 2012). Another study by Talaei et al., (2013) examined the use of vitamin D supplements in 100 type 2 diabetic patients for 2 months. The study found a positive impact of using the supplements on the participant's serum insulin (P=0.02) and FBG (P=0.02).

### *2.4.3. Effect of vitamin D on blood pressure*

The positive impact of vitamin D concentration on hypertension has been shown in many studies (Holick and Chen, 2008; Berry, 2012). Vitamin D deficiency is associated with high parathyroid hormone (PTH) concentrations and elevated blood pressure (Reis et al., 2009). Experts have suggested that one role of $1,25(OH)_2D$ is to suppress renin enzyme expression and VSMC proliferation, which influences blood pressure (Forman et al., 2007; Aljefree et al., 2016). The influence of vitamin D on endothelial function is the primary controlling tool of blood pressure (Forman et al., 2007; Menezes et al., 2014).

Two prospective cohort studies examined the risk and incidence of hypertension associated with the concentration of 25(OH)D over 4-8 years. The studies included 613 males in the first one and 1198 females in the second one. The relative risk of the incidence of hypertension was 6.13 times greater in deficient males compared to participants with an adequate concentration of 25(OH)D. In the females study, the relative risk was 3.18 times greater (Forman et al., 2007). Another study investigated the impact of sun exposure three times a week for three months on patients with hypertension. Eventually, their 25(OH)D increased by 180% and both systolic and diastolic blood pressure became normal (Holick, 2007).

### *2.4.4. Effect of vitamin D on body weight*

Obesity is considered one of the major risk factors for cardiovascular disease. Many studies have found an association between the increase of CVD events in obese subjects and have suggested a role for vitamin D deficiency in an increased risk of being overweight or obese (Holick, 2007; Konradsen et al., 2008; Zittermann et al., 2009). A study by Shirazi et al. (2013) indicated that women with an optimal concentration of vitamin D in their serum are less likely to be overweight or obese. One study calculated that for each 10% increase in BMI there would be a 4.2% decrease in vitamin D serum levels (Vimaleswaran et al., 2013).

One of the explanations for the correlation between vitamin D levels and obesity is that vitamin D is a fat-soluble vitamin, adipose tissue, that contains VDR, sequesters the vitamin (Holick et al., 2011).

### *2.4.5. Effect of vitamin D on aging*

Studies have suggested an association between aging and vitamin D deficiency (Lips, 2010; Holick et al., 2011). When people reach 70 years of age, the reduction in 7-dehydrocholesterol in the skin reduces the production of vitamin D by up to 75% (Holick, 2007). Furthermore, older adults are a high-risk group for falls and osteopathic fracture for many reasons one of which is muscle weakness. Researchers suggest that vitamin D deficiency causes muscle weakness (Al-Daghri et al., 2016).

## 2.5. Summary

Vitamin D deficiency is a common problem worldwide, including Saudi Arabia. This prevalence of vitamin D deficiency may be linked to wider biological effects. Some studies suggest an association between vitamin D deficiency and atherosclerosis. Some suggest a direct association and some observed an association with the disease's risk factors. However, there is a gap in the research which this thesis attempts to address by identifying the relationships clearly and identifying the factors that might have an impact on them, specifically in Saudi Arabia. A better

understanding of this potential association is needed, starting from analysing all of the sources of the vitamin, and the factors affecting the status of the vitamin, to the biomarkers of atherosclerosis disease and its risk factors. A comprehensive diagnosis of the disease and its risk factors are needed through blood tests and non-invasive modern assessment devices. In order to study the relationship between the disease and the vitamin, three different groups, (sub-clinical) healthy, at risk and previously diagnosed, should be studied. Data collection models should be created specifically to fit the target group considering the climate, culture, and habits. Subjects should be free of additional interventions that could affect the study results. Regardless of whether an association is discovered, this observational investigation will provide a comprehensive dataset that will assist in understanding the relationship between vitamin D and atherosclerosis, especially in a country that has ample sunshine such as Saudi Arabia.

# Chapter 3 - Thesis aims and objectives

## 3.1. Hypothesis

The research hypothesis is that an adequate concentration of vitamin D plays a role in preventing atherosclerosis and atherosclerosis risk factors in a population of middle age adults living in Saudi Arabia.

## 3.2. Aims

- To identify the vitamin D status in three Saudi Arabian populations: 1) a healthy population i.e. having no known disease; 2) a population of patients considered by independent clinicians to be at risk of developing atherosclerosis; 3) a population of patients with diagnosed atherosclerosis.
- To establish if there are any relationships between the vitamin D status of the study groups and the potential development of, or known incidence of, atherosclerosis in a sample of Saudi Arabia dwellers.
- To apply a cross-sectional examination of the impact of a background of vitamin D supplement usage on the atherosclerosis status of both healthy and clinical populations.

## 3.3. Objectives

- To collect questionnaire data on health, lifestyle factors, sun exposure, dietary intake and determine their association with circulating 25(OH)D.
- To directly assess circulating 25(OH)D and other biochemical parameters in the three study groups of interest.
- To determine if there is a relationship between circulating 25(OH)D levels and vascular structural and functional characteristics.
- To study the association between vitamin D status and the development/incidence of atherosclerosis.
- To examine the strength of associations between circulating 25(OH)D levels and quantitative and qualitative markers of cardiovascular health.

# Chapter 4 - Quantification of the vitamin D status from circulating 25(OH)D concentration, vitamin D supplements, dietary intake, and sun exposure in a sample of Saudi Arabian volunteers

## 4.1. Introduction

The gold standard to determine vitamin D status is the serum concentration of 25(OH)D (Holick et al., 2011). The sources of vitamin D are confined to diet, exposure to sunlight, and the use of vitamin D supplements (Ford, 2013). The status of these sources and their measurements, in addition to the associations between them, will be investigated. The measurements were determined on the pooled study sample and in each of the sub-groups; (control group (CG), at-risk group (ARG), and participants diagnosed with atherosclerosis group (DAG)) which are called the clinical groups, and between the sexes. Analysis based on the clinical groups was an indicator of the association between the measurement and the participants' clinical condition. Analysis between sexes determined if there is any gender variation in each of the measurements. This chapter will give an introductory overview of the data collection phase and the methods used during data collection.

### *4.1.1 Chapter aim*

To identify circulating 25(OH)D concentrations and determine any association with diet, sun exposure and usage of vitamin D supplements in three populations: 1) a healthy population i.e. having no known disease; 2) a population of patients considered by independent clinicians to be at risk of developing atherosclerosis; 3) a population of patients with diagnosed atherosclerosis.

### *4.1.2. Chapter objectives*

1- To determine circulating 25(OH)D concentrations in the pooled study sample and in each of the study sub-groups (clinical group, gender, and vitamin D supplementation group).
2- To assess the dietary intake of vitamin D in the pooled sample and in the sub-groups and to correlate that with circulating 25(OH)D concentrations.
3- To investigate the exposure to sunlight in the pooled sample and in the sub-groups and correlate that with circulating 25(OH)D concentrations.
4- To compare the magnitude of the associations between vitamin D and modulators.

## 4.2. General methods

### *4.2.1. Ethical approval*

Prior to initiating data collection for the current study, ethical approval was obtained from both the Faculty of Research Degrees Committee at Manchester Metropolitan University -HOLL151601- and from the Committee of Medical Ethics at Al-Noor Specialist Hospital in Saudi Arabia -019337- (Appendices 1 and 2). Both committees requested ethics forms to be filled in with details of the methods, equipment, and facilities that would be used for the study. Additionally, risk assessment forms for the blood sample collection, the use of ultrasound, and the pulse wave velocity device were provided. Information sheets, consent forms, and a copy of the questionnaire were submitted for approval. All of the documents were submitted to both committees in two languages, namely Arabic and English.

Ultimately, the study participant information sheet, study participant consent form, and questionnaire were translated into Arabic and then checked for accuracy to ensure no loss of meaning had occurred during translation, as Arabic is the native language used in Saudi Arabia. The final versions of the English and Arabic documents are presented in Appendices 3 to 8. All the study measurements and examination were performed using the same protocol and the same operator to ensure reliability.

All the information obtained during this study (hard and soft copies) was kept confidential in a locked cabinet placed in a locked office that only the researcher had access to. Participants' names were not associated with the data. All participants were assigned a code number instead of their names in the coded documents. Data were collected in accordance with the Data Protection Act (1998) guidelines. For the blood samples, the code numbers were written on the relevant sample collection tubes and the blood samples were kept in a separate secure refrigerator until data collection was completed. Storage of samples was in accordance with Saudi Ministry of Health guidelines, and in line with the Human Tissue Act and Manchester Metropolitan University guidelines.

### *4.2.2. Study design*

The study took place at Al-Noor specialist hospital in Makkah city, Saudi Arabia. Details of study participants' identification and selection are given below. With ethical approvals in place, a total of 97 volunteers participated in the study. Participants were of both sexes (82 females, and 15 males) aged 35 to 60 years (48.2 ± 8.36) were recruited representing the three health condition being studied. A dedicated research room, with a suitable bed, adjacent to an ultrasound unit was used for the study. The research room was equipped with an electronic scale, blood pressure monitor, and a PC with access to the patient's medical files. The collected data included blood samples, anthropometric measurements, ultrasound measurements, central pulse wave velocity measurements, and lifestyle questionnaires.

#### *4.2.2.1. Inclusion and exclusion criteria*

The inclusion criterion for all participants was being an adult aged 35 to 60. The exclusion criteria for all of the participants were a medical condition that could affect vitamin D metabolism, including liver or kidney diseases (as hepatic or renal disease), metabolic bone disease (e.g. osteoporosis), cancer, or hypercortisolism. Participants were also excluded if they had undergone bypass surgery, were currently pregnant or breast-feeding.

#### *4.2.2.2. Participants and recruitment*

The participants were divided into three groups. The control group (CG) consisted of healthy participants [n=33], who had never been diagnosed with any systemic disease. The second group was of patients at risk of developing atherosclerosis (ARG) [n=30], i.e. having at least one of the major risk factor diseases (hypertension, diabetes, and/or hyperlipidaemia). The third group was of patients with established, formally diagnosed atherosclerosis (DAG) [n=34].

Candidates from all three groups were invited to participate in the study using one of three approaches. Most of the cardiovascular patients were invited to participate

during their routine visit to the cardiovascular clinic at the hospital, however, not all of the participants from this approach were in the DAG as some were in the other two groups. With ethical approval in place, the health files of the patients who had appointments at Al-Noor Hospital were checked and patients fitting the recruitment criteria were approached. Out of 370 outpatients' files reviewed, 186 met the criteria and 38 agreed to participate (Figure 4-1).

The second recruitment approach was through the local hospital staff and/or relatives who had attended the hospital with patients as companions. The subjects were asked to participate in the study if they met the inclusion criteria. Twenty participants were invited using this approach and 14 agreed to participate in the study (Figure 4-1). Participants form this approach were in either the CG or ARG.

The third recruitment approach was through a text message sent to hospital patients, asking them to participate if they fitted the criteria (Appendix 9). Thereafter, if patients replied, a visit was arranged for them at the hospital. Out of 72 text messages sent, 45 participants replied and were included in the study (Figure 4 1). Most of the participants from this approach in the ARG and CG and some were in the DAG.

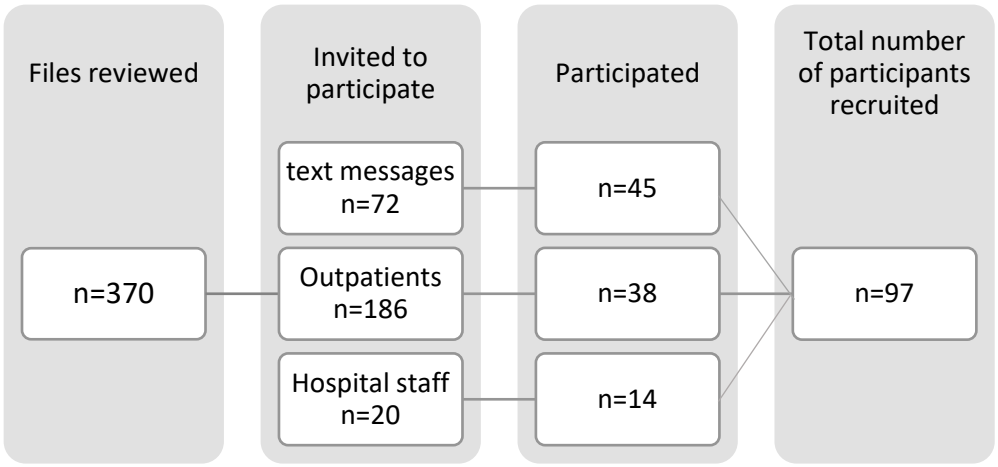


*Figure 4-1 Recruitment of the participants for the study data collection at Al-Noor Specialist Hospital*

#### *4.2.2.2. Process during the participants' visit*

During the visit, a scheduled process was followed to avoid any confusion or any steps being missed. The first step was to invite the participants to join the study. Volunteers were provided with details of the study, their personal details and clinical status were checked, and, if they conformed to the study inclusion requirements and agreed, they were invited to join the study. Participants were issued with an information sheet (pre-approved by the ethics board) to read and were asked to sign a consent form, if they were happy to take part in the study. If participants could not read or had difficulty reading the information sheet, the documents were read out-loud and explained to them verbally. If a participant was struggling to read and had a companion (e.g. friend or family member) with them, the companion was asked to read the information sheet on the participant's behalf and the consent form was then signed by the participant.

Potential participants recruited using the second and third approaches were given temporary file numbers if they did not have one at the hospital. Blood sample requests, which were required for the study, were printed from the hospital database system and given to the participants to use when giving the blood sample. If the participant had already fasted for at least 10 hours, a blood sample was taken at the hospital laboratory. If they had not fasted, another appointment was arranged to collect blood samples. To maximise the number participants returning to the hospital from the previously non-fasted participant groups, volunteers were contacted, via either text message or a phone call depending on the participant's preference, a day prior to their planned return visit as a further reminder.

The first step before starting any of the measurements, the participants were informed as to what exactly would be done and what they might feel. Furthermore, the participants were asked to inform the operator if they felt uncomfortable at any stage of the study.

After the blood sample had been taken or arranged to be taken, the participants' anthropometric measurements (height, mass, waist and hip circumference) were

taken and recorded in the testing room. The testing room was equipped with all of the equipment and devices needed for the data collection. All of the measurements were recorded concurrently in a notebook. Then, the measurements were re-recorded in the participant's questionnaire and the pulse wave analysis device (Complior software, v1.9.4, Vincennes, France). The participants were then offered sweet dates (individually wrapped) and orange juice (freshly squeezed) if they had given a blood sample. The participants were asked to remove any clothing or jewellery from their neck and wrists (testing points). After the anthropometric measurements were completed, the participants were asked to lie flat on the test bed and allowed to rest for 10 minutes.

After the rest period, the following measurements were taken; blood pressure was measured and recorded; then the ultrasound measurements of the carotid artery were taken and recorded. Before applying the gel for the ultrasound, the participants were informed that the gel might be slightly cold. Clean tissues were positioned in order to protect the participants' clothes. After completion of the test, the gel was carefully removed. Finally, the pulse wave velocity test was performed. The pulse wave velocity apparatus software recorded all of the measurements. Then, after the examination, the measurements were copied to an Excel spreadsheet. All of the measurements were repeated at least three times to ensure internal validity.

During the appointment, the participants were asked to fill in a questionnaire. A participant's companion or the operator could do this if the participant could not do this/preferred this support. When they reached the food frequency questionnaire (FFQ) section, plastic examples of the food items and cans were shown in different sizes and amounts of possible consumption to the participants to simplify identification of the amounts consumed (figure 4-2).


*Figure 4-2 Plastic food samples that were used to help the participant determine the amount of food consumed*

Finally, at the end of the meeting, further explanation was provided about vitamin D benefits and sources. The participants were informed that the blood test results would be ready within 5 working days. The participants were asked if they wanted a copy of their blood test result, and how they wanted it to be sent to them. No medical information, based on the results obtained, was provided to the participants. However, participants were advised to contact their physician if they had any medical query about their results. Figure 4-3 illustrate a diagram of the process used during the participant's visit.

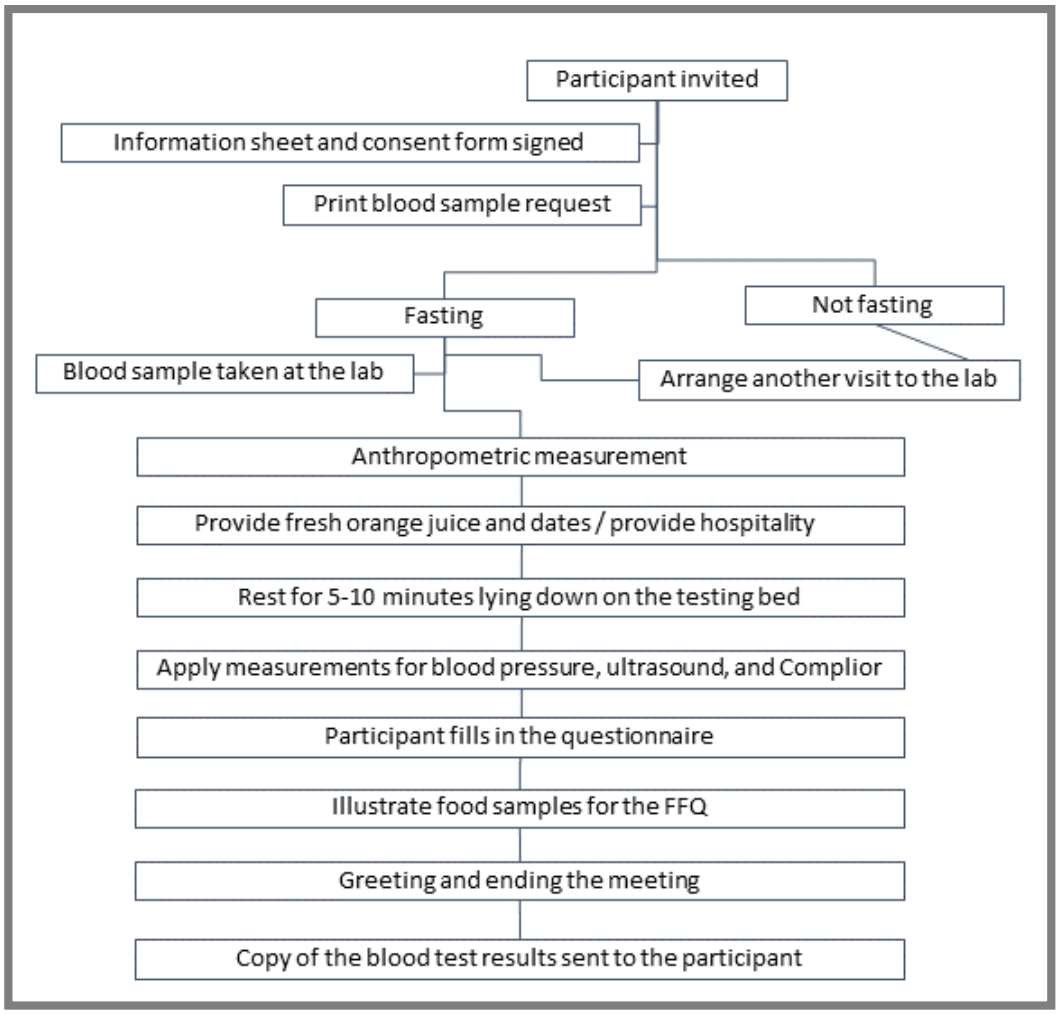


Figure 4-3 The flow diagram of the participant's visit plan

### *4.2.3. Questionnaire design*

A questionnaire was designed based on a review of the literature (McCarty, 2008; Holick et al., 2011; Hatfield et al., 2014).The questions were formulated based on numerous factors: the purpose of the question and, the target population in the target country -Saudi Arabia. Most of the questions were Closed-Ended questions in order to simplify answering, coding, and analysing. However, to avoid the restriction of the Closed-Ended questions there was an option as 'other' with an open space for aditional answers. The questionnaire was divided into six parts; personal information, sun exposure, health information, supplement and medications, FFQ, and anthropometric measurements (a copy of a blank questionnaire is shown in appendices 7-8)

The personal information section was designed to allow for the collection of general information. This section included questions on age, gender, place of birth, the current city of residence, education level, and socioeconomic status (Section 5.2.). The second section, on sun exposure, was designed to investigate the participants' sun exposure for the three months prior to this study. Questions were asked about the frequency and duration of exposure to direct sunlight, as well as the body part exposed to the sun and the usage of any sun protections (Section 4.3.4.). After that was the health information section, which covered questions on health status and lifestyle. The questions were about smoking, pregnancy and breastfeeding (for females only), and diseases that the participant had, if any (Section 5.2.). The health condition was categorised relative to the presence of heart disease, hypertension, diabetes (type I or type II), or no cardiovascular disease. The category of health condition was filled in by the participants and then reviewed from the participant's hospital files, where available. The hospital guidelines for determining the prevalence and incidence of any disease followed "The International Classification of Diseases" guidelines (Hamburg et al., 2008; Saudi Expert Panel, 2014). The next section was designed to cover detailed questions on the use of food supplements and medications (Sections 4.3.3. and 6.2.). The dietary intake of vitamin D section included the FFQ table of food items that contain vitamin D to determine the amount,

and frequency of consumption of vitamin D from the diet (Section 4.3.2.). The last part of the questionnaire was the anthropometric measurement (Section 5.2.7) and skin tone section (Section 4.5.3), which was filled in by the researcher. It included measurements of height, weight, waist and hip circumference, and a scale of skin tone.

A pilot study was undertaken to examine the validity of a questionnaire before it was used in the current study (Appendix 10). The pilot study included N= 12 participants (6 males and 6 females) aged 49.9 ± 6.7, who were otherwise not included in the current study. Piloting the questionnaire ensured that the questions were clear and understandable for the participants, as they were initially intended from their design.

### *4.2.4. Blood sample collection*

Fasting blood samples were taken from participants for biochemical assays, after the participants had read the information sheet and signed the consent form. Before taking the blood sample, the phlebotomist confirmed the participants' name, date of birth, and how long they had been fasting. Participants had not to have had any food or drinks except for clear water for at least 10 hours prior to the test. The samples were drawn and handled by a trained phlebotomist in an equipped room in the hospital laboratory. Ten ml of blood were taken from each participant, divided into three aliquots, and sent to three different laboratory departments. A vacutainer plain tube was used for the 25(OH)D test in the hormone department; the second sample tube was to test for CRP in the immunology and pathology department. Additionally, a lithium heparin tube was used to collect blood for the analysis of lipids and fasting glucose. To each tube, a sticker was attached which stated the patient's name, date of birth, ID, and request number. Each tube was kept in a plastic bag along with a copy of the request sheet and sent to the relevant department. The blood samples were either tested within two hours or stored at 2-8 °C and then tested within 7 days. Analysis methods were those routinely used by the hospital laboratories for all the blood tests. The results were reported on the hospital database system to which the

researcher was allowed access. An example of a blood test report is provided in Appendix 11.

### *4.2.5. Vascular structural and functional characteristics*

Vascular structural and functional characteristics were determined using two devices. Measurements of plus wave velocity (PWV) and central pulse pressure (CPP) were determined using a non-invasive Complior device (V1.9.4, Alam Medical, Vincennes, France). Carotid artery assessment was performed using a colour Doppler ultrasound instrument (MyLab 70 ultrasound, Esaote, Genoa, Italy) that included carotid intima media thickness (IMT), and carotid artery inter-adventitial diameter (IAD). The measurements were used to assess the participants' arterial stiffness to determine atherosclerosis status. Detailed methods are presented in chapter 7 of the current thesis.

### *4.2.6. Data coding, reduction, and analysis*

All of the data were coded in a numerical form first in Excel 2013, then transferred to SPSS (IBM SPSS statistics 24, Inc., Chicago, IL, USA) electronically to minimise human error. There were two types of data: categorical and scale. Categorical data were coded by giving a number from 1 to the number of values in each tested variable. For instance, for gender there were 2 values, "1" for "male" and "2" for "female". The second type of data was scale, thus ratio level data such as circulation 25(OH)D values, which were recorded by the actual value of the vitamin for each participant e.g. "21.36" or "14.00". Missing values were represented by a blank space.

To eliminate errors that might have occurred during data collection and coding, the data were reviewed and checked continuously (i.e. each data entry was reviewed twice: once after coding and again after completion of all the data entry). After that, data screening was commenced once the coding had been completed. Each of the variables was screened for any outliers (outlier labelling role for interquartile range (IQR) of 3), missing data, or inconsistent entries. In each case of an error being found,

the entry was fixed by going back to the data source such as the questionnaire or the program where the data were first recorded. Outliers were easier to identify in the box and whiskers plot. SPSS defines an IQR of three by a * shape. If the entry was correct, an individual discussion about the case was undertaken in the discussion section. For the sake of completeness, the data were also analysed after having removed any outliers and the effects of these are discussed. Confidence interval percentage (CI) were set at 95% for all of the data. Statistical significance was set at $P \leq 0.05$. Table 4-1 illustrates the strength of the relationship for the $R^2$ value.

*Table 4-1 $R^2$ coefficient of correlation strength of the relationship (edited from (Leech et al., 2011))*

| **Value of $R^2$** | **Strength of relationship** |
|---|---|
| -1.0 to -0.25 or 1.0 to 0.25 | Strong |
| -0.25 to -0.09 or 0.09 to 0.25 | Moderate |
| -0.09 to -0.1 or 0.1 to 0.09 | Weak |
| -0.01 to 0.01 | None or very weak |

Multiple linear regressions (forced entry method, run until stability was reached) were applied to create a model to predict serum 25(OH)D based on the significant predictors identified in each chapter. Using the final multiple linear regression stable model an equation, was derived to predict 25(OH)D concentrations. The equation will be as follow:

$$Y = (BX1) + (BX2) + (BX3) + (BX4) + \cdots . + C\ (\pm SEE)\ (\text{Leech et al.}, 2011)$$

Y is the value of dependent variable (the predicted value of 25(OH)D); B 1,2,… are the values of the slope for X (the value of the un-standardised coefficients); X 1,2,… are the values of the significant independent variables (value of the predictors); C is the intercept (constant); SEE is the standard error of the estimate. The predicted vitamin D concentrations will be determined by substituting the values of B, X, C, and SEE in the equation based on the values of the predictors in the study.

## 4.3. Chapter Methods

### *4.3.1. Measurement of serum 25(OH)D*

Determination of vitamin D concentrations was performed using the electrochemiluminescence binding assay on Elecsys 2010 (Cobas e E601 immunoassay analysers, Indianapolis, IN, USA) (Roche, 2015a). It is a competitive electro-chemiluminescent protein-binding assay (CPBA). The CPBA technique is a reliable technique for measuring 25(OH)D in serum or plasma (van den Ouweland et al., 2013). The assay uses vitamin D binding protein (VDBP) as a capture protein that binds 25(OH)D3 and 25(OH)D2. The assay can measure total 25(OH)D in serum or plasma.

The process of analysing 15µl of serum sample was completed using the following steps. First, the sample were incubated with pre-treatment reagents (4 mL of Dithiothreitol and 4mL of Sodium hydroxide) to release 25(OH) vitamin D from VDBP. Then, the pre-treated sample were incubated with ruthenium labelled VDBP that forms a complex between the 25(OH)D and the ruthenylated VDBP. After that, streptavidin-coated micro-particles and 25(OH)D labelled with biotin were both added to the mix. The free sites on the ruthenium labelled VDBP become occupied. A complex containing the ruthenium-labelled VDBP and the biotinylated 25(OH)D formed and bound to the solid phase via interaction of biotin and streptavidin. The reaction mixture was aspirated into the measuring cell and the micro-particles magnetically captured on the electrode surface. At this stage, the unbound substances were removed with ProCell. Then, a voltage was applied to the electrode, inducing chemiluminescent emission, which was measured with a photomultiplier. The results were obtained via a calibration curve specifically generated by 2-point calibration and a master curve provided via the reagent barcode. The total duration of the assay was about 27 minutes. Vitamin D serum concentration thresholds were defined as shown in table 4-2 (CV%= 11 to 13) (Lensmeyer et al., 2006 ; Reis et al., 2009 ; Abdel-Wareth et al., 2013 ; Al-Daghri et al., 2016).

*Table 4-2 Definition of serum 25(OH)D concentration thresholds (Reis et al., 2009 ; Al-Daghri et al., 2016)*

| 25(OH)D concentration | ng/ml | nmol/L |
|---|---|---|
| **Deficient** | < 20 | < 50 |
| **Insufficient** | 20 ≤ 30 | 50 ≤ 75 |
| **Adequate** | 30 < 100 | 75 < 250 |
| **Toxicity** | ≥ 100 | ≥ 250 |

### *4.3.2. Food frequency questionnaire (FFQ)*

The FFQ table listed food items that contain vitamin D to determine the dietary intake of the vitamin (Appendices 7 and 8). Vitamin D intake was assessed using a previously validated FFQ (Azhar, 2009; Shirazi et al., 2013; Hatfield et al., 2014; Bushnaq, 2016). The FFQ was designed to include 19 food items that naturally contain, or are fortified with, vitamin D in Saudi Arabia; in addition to foods that might be consumed by people from different ethnicities (Table 4-3) (Holick, 2007; Roe et al., 2015). The FFQ table was designed with an option to record the amount consumed from each item by cup, grams, can, slice, or spoon. The measurement of the estimated amount consumed was illustrated to the participants using samples of the food items (Figure 4-2). The frequency of consumption of each food item was recorded by choosing from nine responses ranging from never to three-times a day for the past three-months. There was an option for more than three times a day with an open answer but none of the participants chose it (Patterson et al., 1999).

*Table 4-3 Food Items used for the FFQ and the amount of vitamin D in 100 g of each food item.*

| **Food item** | **Amount of vitamin D µg / 100 g** | **Amount of vitamin D IU / 100 g** |
|---|---|---|
| Camel's liver* | 0.3 | 12 |
| Calf's liver | 0.3 | 12 |
| Lamb's liver | 0.5 | 20 |
| Chicken's liver | 0.2 | 8 |
| Wild salmon, fresh | 4.7 | 188 |
| Salmon, canned** | 13.6 | 544 |
| Sardines, fresh | 4.0 | 160 |
| Sardines, canned | 3.3 | 132 |
| Mackerel, canned | 8.5 | 340 |
| Tuna, fresh | 3.1 | 124 |
| Tuna, canned | 1.1 | 44 |
| Portobello mushrooms, fresh | 0.3 | 12 |
| Egg yolk | 12.8 | 512 |
| Fortified milk*** | 1 | 40 |
| Fortified yogurts*** | 1 | 40 |
| Fortified butter*** | 0.9 | 36 |
| Fortified margarine | 8.8 | 352 |
| Fortified cheese*** | 1 | 40 |
| Fortified breakfast cereals*** | 4.6 | 184 |

* The amount of vitamin D in camel liver was assumed similar to that in calf's liver.
** The amount of vitamin D in canned salmon includes skin and some small bones (Roe et al., 2015)
*** Amount of fortification was recorded based on the products in Saudi Arabia (Almarai, 2017).

The vitamin D intake for each of the food items was estimated using McCance and Widdowson's composition of foods integrated dataset (Roe et al., 2015) and Nutritics software (Nutritics, Dublin, Ireland). The amount of vitamin D in fortified products was recorded based on the products available in Saudi Arabia (Almarai, 2017). To determine the average daily vitamin D intake for each participant, the amount of vitamin D consumed from each food item in IU (Table 4-3) was multiplied by the frequency of consumption of the food item (Table 4-4) (Welch et al., 2005). The calculation was automated using a pre-designed Excel model with all the conversion factors for the amount of vitamin D in each food item and the daily intake frequency to minimise the chance of inputting errors. The total value of dietary intake of vitamin D (DI-VitD) in IU/day was recorded and analysed.

*Table 4-4 Conversion amount of frequent food consumption*

| Frequency of use in three months | Conversion to daily frequency |
|---|---|
| Never | 0/90 = 0 |
| Less than once a month | 1.5/90 = 0.0167 |
| 1-3 times a month | 6/90 = 0.0667 |
| Once a week | 13/90 = 0.1444 |
| 2-4 times a week | 39/90 = 0.4333 |
| 5-6 times a week | 71.5/90 = 0.7944 |
| Once a day | 90/90 = 1 |
| Twice a day | 180/90 = 2 |
| Three or more times a day | 270/90 = 3 |

### *4.3.3. Vitamin D supplements*

In order to collect data on the use of vitamin D supplements by participants, the questionnaire included a series of relevant questions. The questions required details of the commercial name of the brand, dose, and frequency of use. The frequency of use was given as daily, weekly, monthly, or less often. An additional question was on whether usage was preventative [without a prescription] or therapeutic [prescribed by their doctor]. During data coding, the dose and frequency of the supplement usage were calculated to give the daily consumption of vitamin D supplements in international units (IU), whether it was consumed separately or as part of multi-supplement regime. During coding, the total intake of vitamin D (TI-VitD) was calculated by adding the amount of DI-VitD to the daily dose of vitamin D supplements, if any, for each participant.

### *4.3.4. Sun exposure measurement*

There is no standard questionnaire for validated sun exposure measurement and analysis. However, studies have agreed that a self-reporting sun exposure questionnaire is the best indicator for estimating sun exposure (McCarty, 2008; Holick et al., 2011). Based on a review of the literature, a questionnaire was designed to determine vitamin D synthesis in the body from sunlight for the three months prior to this study (Azhar, 2009; Holick et al., 2011; Tuffaha et al., 2015) (Appendices 7 and 8). When calculating sun exposure data, studies have used different methods

depending upon the purpose of the study (Holick, 2009; Ardawi et al., 2011; Bushnaq, 2016; Aljefree et al., 2017b). A model has been designed for the current study based on the sun exposure questions to give a total value of sun exposure for each participant. The coding values for sunlight exposure were 1 (none/ limited), 2 (low), 3 (medium), and 4 (high). Table 4-5 illustrates the procedure for coding answers to these questions.

The first question was about the frequency of exposure to direct sunlight (not through glass, window, or parasol). The highest values were given to the most time spent exposed, starting from daily. For example, a value of "4" was given to daily exposure of sunlight and "1" for none or rarely. The next two questions asked about the times of the day when exposure to the sunlight took place during weekdays and at weekends. The highest values in the model were given to the noon times and the lowest to no exposure. Participants could choose more than one answer for this question and the coding in this case was for the highest value because the highest value will include a higher value of UVB radiation. For instance, if a participant chose exposure time between 7-9 am, which has a coding of "2", and 1-3 pm ,which has a coding of "4", the final coding for that question will be "4" as it will inclusive both amount of UVB radiation. Then there were two more questions about the length of sun exposure on weekdays and during the weekend. The highest value was also given to the most time. Furthermore, there was a question about the body parts exposed to sunlight, and participants were able to provide more than one answer. The coding model gave the highest value for the largest skin area exposed directly to the sunlight. The follow-up question asked about the use of sun protection (sunscreen or head cover). The highest value was given for no use of any sun protection. Skin pigmentation was part of the anthropometric measurement section in the questionnaire and was added to the sun exposure model. The category of the skin pigmentation was determined using the Fitzpatrick scale (Figure 4-4) by looking at the participant's inner arm colour while carrying out the examination during the meeting. Then, the observation was recorded. For the sun exposure model, the skin pigmentation was coded with the highest value given to lightest skin tone and the lowest value to the darkest skin tone (Table 4-5). Finally, all of the given values were

added up to provide a single value representing the total sun exposure (TSE) score for each participant.

To define the TSE score the range of minimum and maximum expected values were categorised as none/limited, low, medium and high sunlight exposure as well. The TSE values were divided equally to 4 groups as illustrated in table 4-6.

*Table 4-5 TSE coding model based on the questions from the questionnaire*

| Questions | None/ limited (1) | Low (2) | Medium (3) | High (4) |
|---|---|---|---|---|
| How often are you exposed directly to the sun (not through glass, window, or parasol)? | -None<br>-Rarely | - 1-2 times a month<br>- 3-4 times a month | - 1-2 times a week<br>- 3-4 times a week | - 5-6 times a week<br>-Daily |
| What time of the day are you exposed directly to the sun (not through glass, window, or parasol)? | -None | - 7-9 am<br>- 5-7 pm | - 9-11 am<br>- 3-5 pm | - 11 am -1 pm<br>- 1-3 pm |
| How much time do you usually spend outdoors in daylight? | -None | - < 10 min | - 10-20 min | - > 20 min |
| What areas of your body are often exposed to sunlight? | Non | -Only face<br>-Only hands | -Hands and feet<br>-Face, hand, and feet | -Face, hands, feet, and half arms.<br>-Face, hand, feet, half arms, and half legs. |
| What kind of sun protection do you usually use? | | -Sunscreen | -Shomak or Qutra | -None |
| Skin pigmentation | -Black<br>-Brown | - Olive | -Medium | -Fair<br>-very fair |


*Figure 4-4 The Fitzpatrick skin pigmentation classification (MDSkin, 2015)*

*Table 4-6 Classification of TSE model scores*

| TSE category | TSE values |
|---|---|
| **Non/limeted** | 8.0 – 14 |
| **Low** | 14 < 20 |
| **Medum** | 20 < 26 |
| **High** | 26 < 32 |

#### *4.3.3.1. Sun exposure factors*

For full determination of sun exposure, other factoers such as location and season were considered. Geographic location was determined by the city of residence. It was an open question and was categorised to one of 3 cities, during the coding phase, based on the participant's answers as provided in table 4-7. All of the participants' cities of residence were in the Makkah area. The cities of residence were used to indicate the local climate conditions. All of the participants were living in a similar climate and latitute in the Makkah area. Additionally, all of the data were obtained between the months of May and July in 2016 to ensure that there would be minimal seasonal variations between the participants.

*Table 4-7 Distribution of participants' place of residence and geographical location*

| City of residence | Frequency | Location |
|---|---|---|
| **Makkah** | n=93 | 21.4° N |
| **Jeddah** | n=2 | 21.5° N |
| **AL-Qunfudhah** | n=2 | 21.4° N |
| **TOTAL** | n=97 | |

## 4.4. Statistical analysis of the data

Statistical analysis was performed using SPSS v24 (Inc., Chicago, IL, USA). Data were tested for normal distribution using the Kolgomorov-Smirnov test. Similarly, the homogeneity of variance was assessed using Levene's test. In the current chapter, only data from TSE were normally distributed. Descriptive analysis is presented as mean ± standard deviation, median (interquartile range), and mean rank. One-way ANOVA, with Bonferroni corrected *post-hoc* pairwise mean comparisons, was carried out where there were three groups to compare (clinical groups). Independent t-test was used to compare the mean in two groups (i.e. gender). The rest of the chapter measurements were not-normally distributed, and such data was deemed suitable for non-parametric tests. Therefore, Kruskal-Wallis pairwise comparisons, with appropriate *post-hoc* Mann-Whitney tests were carried out where there were three groups, or simply Mann-Whitney tests for simple pairwise mean comparisons.

Additionally, where data were continuous rather than grouped, Spearman rho correlations were carried out. Multiple linear regressions (forced entry method, was run until stability was reached) were applied to create a model to predict serum 25(OH)D based on the significant predictors.

## 4.5 Results

In this section, a descriptive analysis and relationships are presented between serum 25(OH)D levels and the other markers of vitamin D including vitamin D supplements, vitamin D dietary intake, and sun exposure. The total study sample size was 97. Data was analysed as a pooled sample and in sub-groups to investigate any variation between the groups. First sub-group included, the clinical groups ((control group (CG), at risk group (ARG), and diagnosed with atherosclerosis group (DAG)). The second sub-group was gender (male, female). Table 4-8 gives a descriptive analysis of the chapter variables in the pooled sample and in clinical groups.

*Table 4-8 Descriptive statistics of the chapter variables by clinical groups and in the pooled sample. Data were presented as N (%) for categorical variables and mean ± standard deviation for numerical variables. * DI-VitD (dietary intake of vitamin D), TI-VitD (total intake of vitamin D), TSE (Total sun exposure).*

| **Variable** | **CG**<br>**N=33** | **ARG**<br>**N=30** | **DAG**<br>**N=34** | **Total**<br>**N=97** |
|---|---|---|---|---|
| **Male** | 5 (15.2%) | 3 (10%) | 7 (20.6%) | 15 (15.5%) |
| **Female** | 28 (84.8%) | 27 (90%) | 27 (79.4%) | 82(84.5%) |
| **Age** (years) | 41.3 ± 6.6 | 50.3 ± 6.6 | 53.1 ± 6.8 | 48.2 ± 8.4 |
| **25(OH)D** (ng/mL) | 18.5 ± 10.3 | 23.9 ± 14.9 | 18.3 ± 14.6 | 20.1 ± 13.4 |
| **DI-VitD** (IU/day) | 239.1 ± 155.6 | 264 ± 132.5 | 211.8 ± 101.7 | 237.3 ±131.9 |
| **TI-VitD** (IU/day) | 3330 ± 10193.6 | 3611.4 ± 10431 | 7396.5 ±16787.6 | 4842.3 ± 12959.6 |
| **TSE** Value | 21.2 ± 3.96 | 20.9 ± 4.47 | 21.24 ± 4.9 | 21.14 ± 4.4 |

### *4.5.1. Serum 25(OH)D results*

#### *4.5.1.1. Descriptive analysis of serum 25(OH)D in the pooled sample and between sub-groups*

Serum 25(OH)D was measured in 97 participants of whom 15 were males. The minimum 25(OH)D concentration was 3.00 ng/mL and the maximum was 58.94 ng/mL. Table 4-8 illustrates the mean values of 25(OH)D among the clinical groups (control group (CG), at risk group (ARG), and diagnosed with atherosclerosis group (DAG)) and figure 4-6 shows the distribution of 25(OH) concentrations among the clinical groups. Concentrations of 25(OH)D < 20 ng/mL are deficient, 20 ≤ 30 ng/mL are insufficient, 30 < 100 ng/mL are adequate, and above 100 ng/mL considered toxic. The majority of the participants (51.5%) had vitamin D deficiency (mean ± SD= 9.88 ± 4.2 ng/mL), 28.9% had insufficient concentrations (23.37 ± 2.57 ng/mL), and only 19.6% had adequate concentrations (42.13 ± 9.28 ng/mL) of serum 25(OH)D (figure 4-5). There were no outliers in 25(OH)D data at an IQR of 3. Among the participants, 30.9 % were taking vitamin D supplements while the rest (69.1%) were not. Descriptive statistic of 25(OH)D concentrations among the pooled sample, clinical groups, and gender (male and female) with consideration of the consumption of vitamin D supplements are all illustrated in table 4-9.

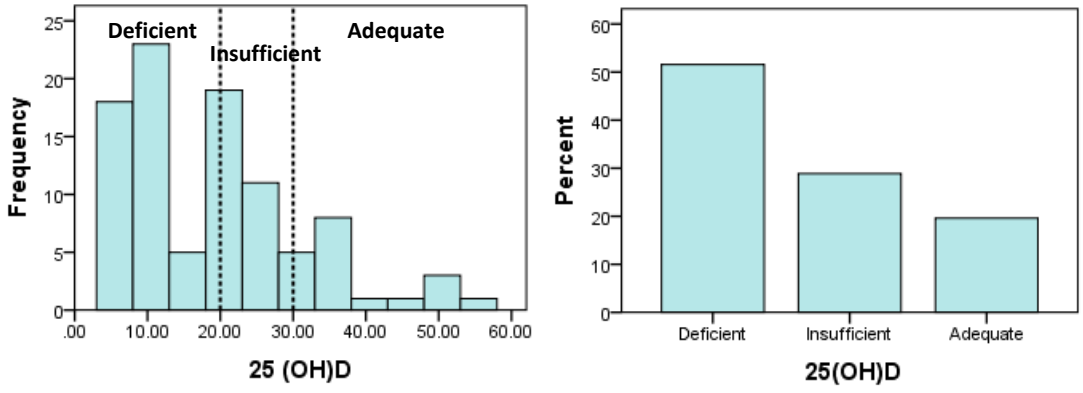


*Figure 4-5 Distribution of 25(OH)D concentrations (ng/mL) among the participants. 25(OH)D < 20 ng/mL are deficient, 20 ≤ 30 ng/mL are insufficient, 30 < 100 ng/mL are adequate, and above 100 ng/mL toxic.* Cut off values are shown between the dotted lines.

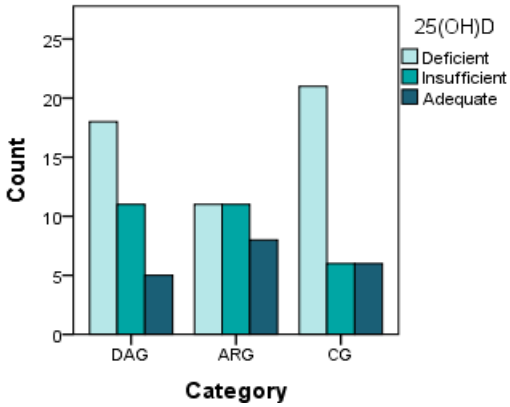


*Figure 4-6 Distribution of 25(OH) concentrations (ng/mL) among the clinical groups. 25(OH)D < 20 ng/mL are deficient, 20 ≤ 30 ng/mL are insufficient, 30 < 100 ng/mL are adequate.*

*Table 4-9 Descriptive statistic of 25(OH)D concentrations (ng/mL) among the pooled sample and clinical groups, gender when consuming vitamin D supplements or not. Data are presented as mean ± standard deviation, median (interquartile range), and mean rank.*

| | Take Vitamin D supplements | N | Mean ± SD | Median (IQR) | Mean Rank |
|---|---|---|---|---|---|
| **CG** | Yes | 6 | 40.27 ± 16.16 | 41.92 (25.01) | 20.17 |
| | No | 27 | 13.37 ± 8.63 | 11.54 (14.29) | 30.57 |
| | Total | **33** | 18.5 ± 10.31 | 18.64 (16.09) | 43.30 |
| **ARG** | Yes | 12 | 32.51 ± 17.99 | 30.37 (33.16) | 15.67 |
| | No | 18 | 18.17 ± 8.94 | 20.07 (15.54) | 41.64 |
| | Total | 30 | 23.91 ± 14.85 | 21.87 (19.54) | 56.68 |
| **DAG** | Yes | 12 | 27.5 ± 9.4 | 24.46 (14.87) | 13 |
| | No | 22 | 13.56 ± 6.96 | 10.64 (11.20) | 31.57 |
| | Total | 34 | 18.26 ± 14.57 | 12.7 (15.18) | 43.30 |
| **Male** | Yes | 0 | 0 | 0 | 0 |
| | No | 15 | 15.75 ± 8 | 15.47(13.29) | 36.87 |
| | Total | 15 | 15.75 ± 8 | 15.47 (13.29) | 42.07 |
| **Female** | Yes | 30 | 32.08 ± 14.96 | 33.41 (20.84) | 15.50 |
| | No | 52 | 14.43 ± 8.50 | 11.78 (13.27) | 33.17 |
| | Total | 82 | 20.888 ± 14.10 | 20.04 (17.73) | 50.27 |
| **Vitamin D supplements** | Yes | 30 | 32.08 ± 14.96 | 33.4 (20.84) | 72.0 |
| | No | 67 | 14.72 ± 8.35 | 12.05 (13.17) | 38.70 |
| **Total sample** | | **97** | **20.09 ± 13.44** | **18.77 (16.64)** | |

*4.5.1.2. 25(OH)D concentration and the difference between sub-groups*

To investigate the results further, a series of non-parametric tests were performed. Kruskal-Wallis test indicated no significant differences in 25(OH)D concentrations between the clinical groups (P=0.161) as shown in the box and whiskers plot (figure 4-7). In addition, Mann-Whitney test indicated no significant diffrences between male and female in terms of their 25(OH)D status (P=0.299). Box and whiskers plots in figures 4-7 illustrated no differences between the sexes. A Mann-Whitney test performed using the consumption of vitamin D supplements as a grouping variable indicated, as expected, a significant difference (U=315, P<0.001) (table 4-9). Box and whiskers plots of 25(OH)D and consumption of supplements is illustrated in figure 4-7 and shows the significant difference.

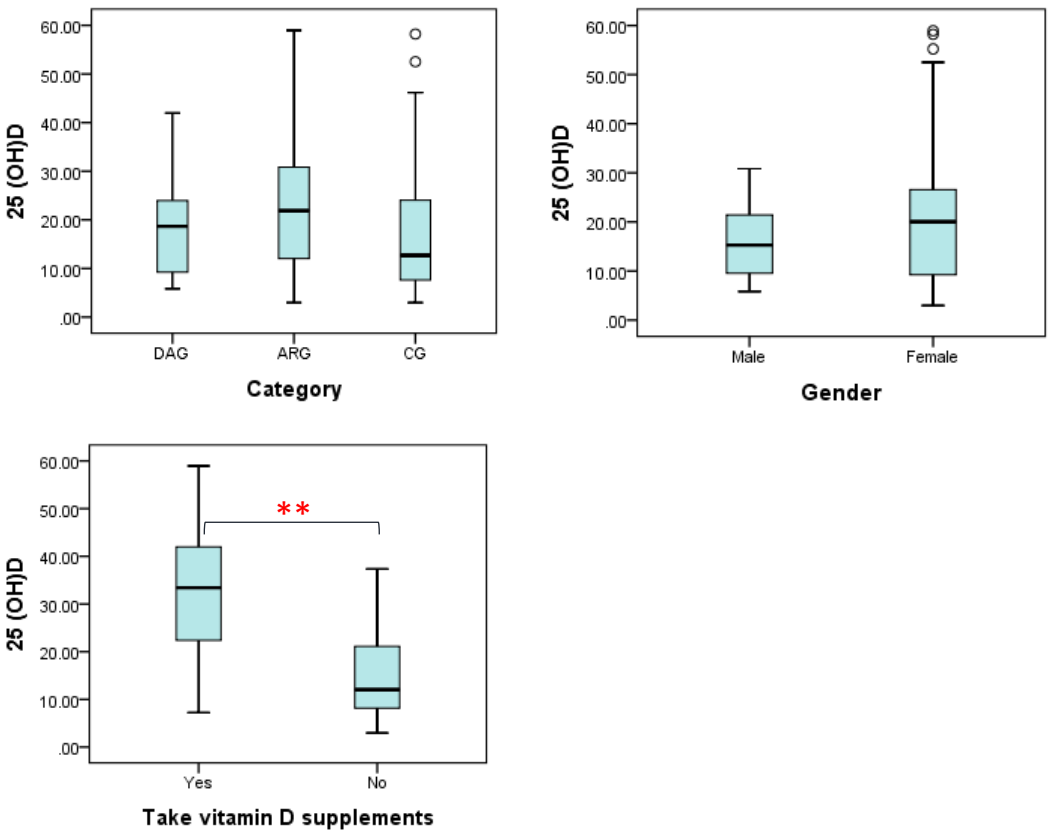


*Figure 4-7 Box and whiskers plots for 25(OH)D (ng/mL) by clinical, gender, and vitamin D supplementation grouping. A figure include the whole sample and B without the outliers. Data shows medians, interquartile range, whiskers and outliers. ** Indicates significant between group differences (P<0.01).*

For additional investigation, data were analysed based on the vitamin D supplement consumption. Results using a Kruskal-Wallis test indicated no significant differences in 25(OH)D concentrations between the clinical groups when taking the supplements (P=0.265), nor when not taking supplements (P=0.146). None of the male participants were taking vitamin D supplements thus, there was no comparison between male and female who were taking supplements. A Mann-Whitney test was performed to investigate any gender bias when vitamin D supplements were not used, the comparison revealed no significant difference (P=0.518).

On the other hand, when comparing the data from the clinical groups the results were different. A Mann-Whitney test revealed a significant difference between the participants who use vitamin D supplements and those who do not within each of the clinical groups CG (U=11, P<0.001), ARG (U=56, P= 0.028), and DAG (U=31, P<0.0001) (figure 4-8).

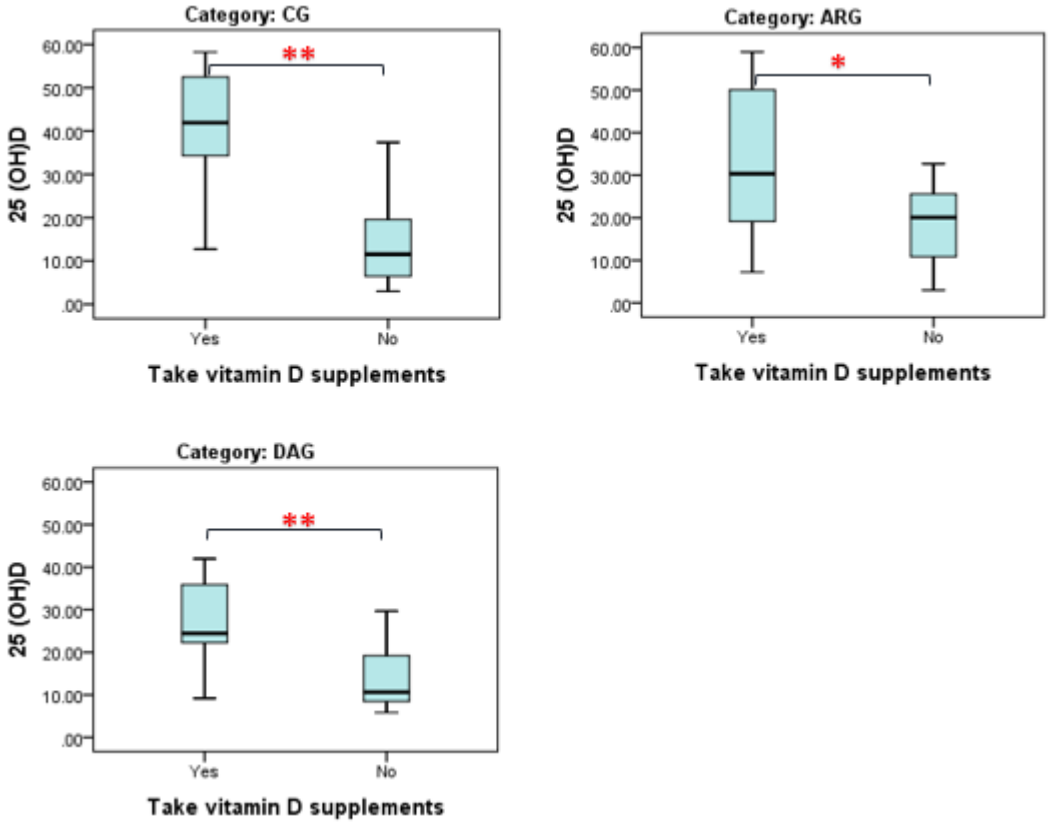


*Figure 4-8 Box and whiskers plots for 25(OH)D (ng/mL) in the clinical groups whether consume vitamin D supplements or not. Data shows medians, interquartile range, whiskers and outliers. Significant differences between groups indicated by (* = P<0.05) and (** = P<0.01).*

### *4.5.2. Dietary intake of vitamin D (DI-VitD) and total intake of vitamin D from diet and supplements (TI-VitD).*

#### *4.5.2.1. Descriptive analysis of DI-VitD and TI-VitD in the pooled sample and between sub-groups.*

The FFQ was filled out by all of 97 participants and analysed for total dietary vitamin D intake (section 4.3.2.) and the dose of supplements consumed (section 4.3.3.). The average DI-VitD for the pooled sample was 237.27 ± 131.9 IU/day with minimum and maximum values of 26.04 and 589 IU/day. Figure 4-9 illustrate the data distribution without outliers. Table 4-10 shows descriptive results of dietary intake of vitamin D among the pooled sample and in the sub-groups.

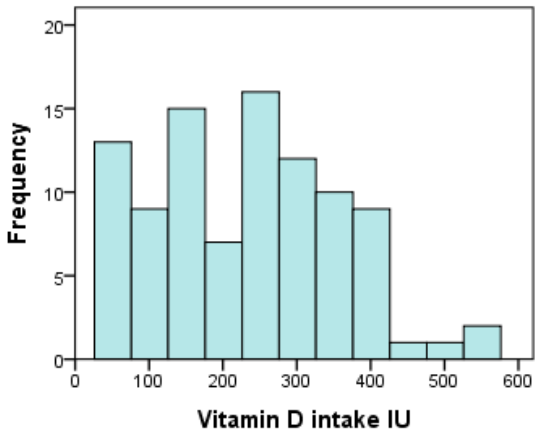


*Figure 4-9 Distribution of daily dietary intake of vitamin D IU in the pooled sample*

*Table 4-10 Descriptive analysis of dietary intake of VitD (IU/day) in the pooled sample and in the sub-groups.*

| | N | Mean ± SD | Median (IQR) | Mean Rank |
|---|---|---|---|---|
| **CG** | 33 | 239.14 ± 155.67 | 238.80 (225) | 47.77 |
| **ARG** | 30 | 264.55 ± 132.543 | 273.00 (201) | 55.2 |
| **DAG** | 34 | 211.84 ± 101.785 | 214.10 (164) | 44.74 |
| **Male** | 15 | 226.76 ± 139.619 | 172.92 (147) | 688.5 |
| **Female** | 82 | 239.19 ± 131.261 | 238.64 (197) | 4064.5 |
| **Total** | 97 | 237.27 ± 131.911 | 237.32 (192) | |

Conversely, the TI-VitD values from diet and supplements for the pooled sample was 4842.29 ± 12959.6 IU/day with minimum and maximum values 26 and 50363 IU/day (table 4-11). The data were not normally distributed (figure 4-10). In the pooled sample 23 values above 750 IU/day were pointed as outliers at an IQR of 3 (figure 4-11 (A)). However, these high values were the result of supplement consumption. Therefore, figure 4-11 (B) indicates no outliers when data were divided by the consumption of supplements. This indicates that these outliers are part of the TI-VitD data and should not be removed.

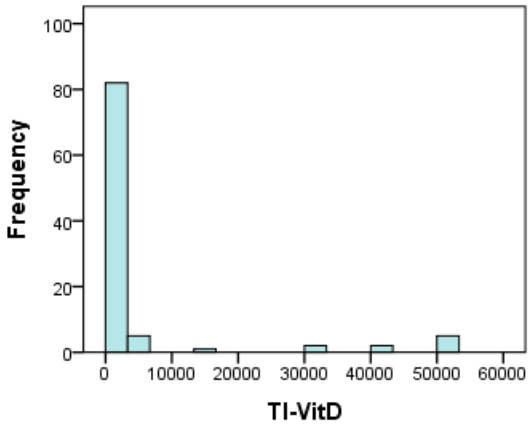


*Figure 4-10 Distribution of the total intake of vitamin D from diet and supplements (IU/day) in the pooled sample.*

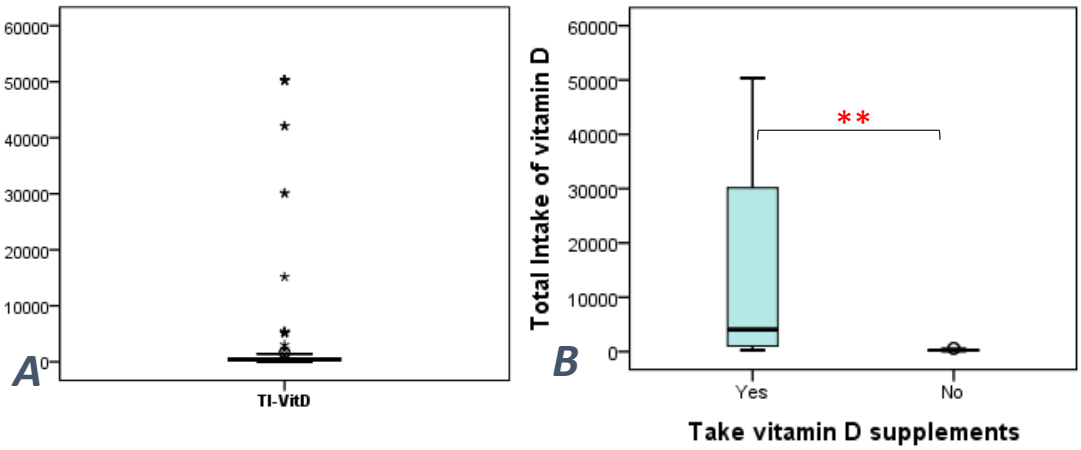


*Figure 4-11 A box and whiskers plot of the total intake of vitamin D from diet and supplements (IU/day) (A) in the pooled sample and (B) when vitamin D supplements were taken or not. ** Indicates significant between group differences (P<0.01).*

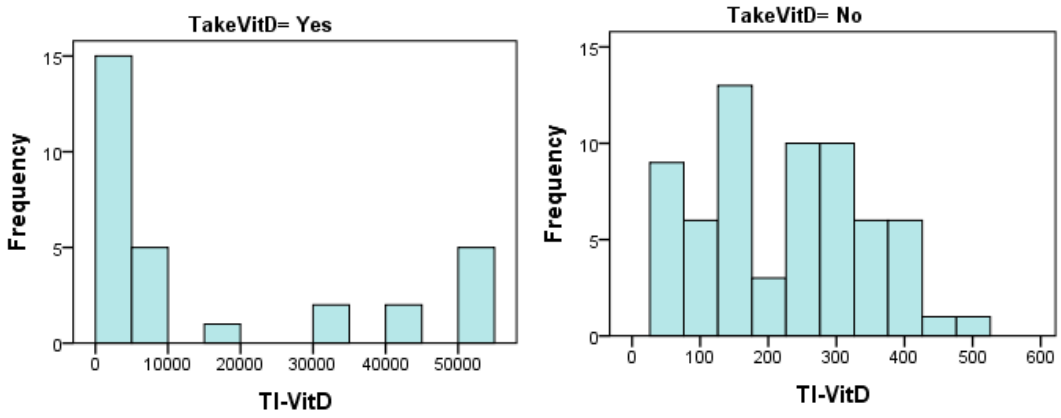


*Figure 4-12 Histograms of the TI-VitD levels (IU/day) when vitamin D supplements were taken of not*

*Table 4-11 Descriptive analysis of TI-VitD (IU/day) in the pooled sample and in the sub-groups.*

| | N | Mean ± SD | Median (IQR) | Mean Rank |
|---|---|---|---|---|
| **CG** | 33 | 3330.1 ± 10193.65 | 290 (391) | 46.11 |
| **ARG** | 30 | 3611.4 ± 10431.07 | 332 (1229) | 51.38 |
| **DAG** | 34 | 7396.2 ± 16787.6 | 297 (1278) | 49.71 |
| **Male** | 15 | 226.80 ± 139.67 | 173 (148) | 497 |
| **Female** | 82 | 5686.6 ± 13942.2 | 323 (1161) | 4256 |
| **Total** | **97** | **4842.29 ± 12959.6** | **298 (529)** | |

### *4.5.2.2. DI-VitD and TI-VitD values and the difference between sub-groups*

To investigate the difference between the sub-groups, non-parametric tests were performed. There were no significant differences between the clinical groups (P=0.318) nor between male and female (P=0.643) in their DI-VitD values as shown in the box and whiskers plots in figure 4-13. Similarly, there was no significant difference in TI-VitD values between the clinical groups (P=0.74). In contrast, there was a significant difference between male and female in TI-VitD values (U=377, P=0.018) as shown in the box and whiskers plot (figure 4-14).

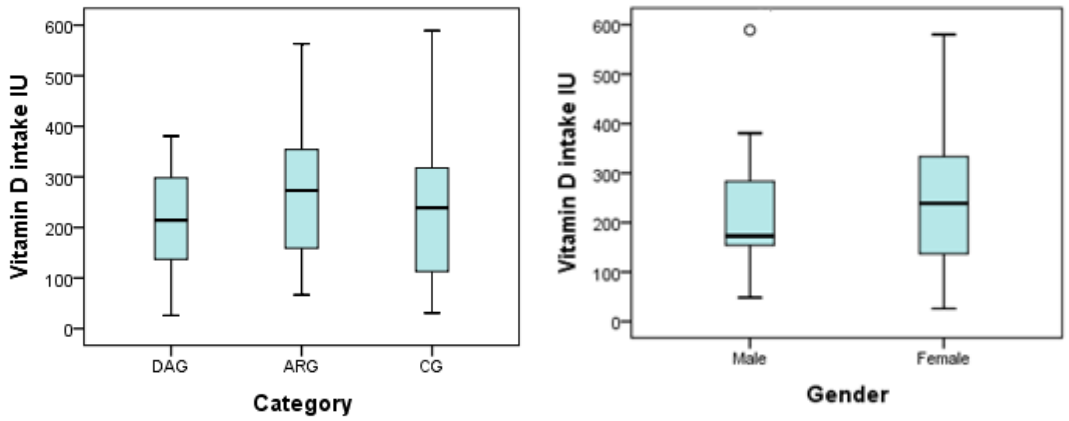


*Figure 4-13 Box and whiskers plots of DI-VitD and TI-VitD (IU/day) by clinical groups, and gender. Data shows medians, interquartile range, whiskers and outliers.*

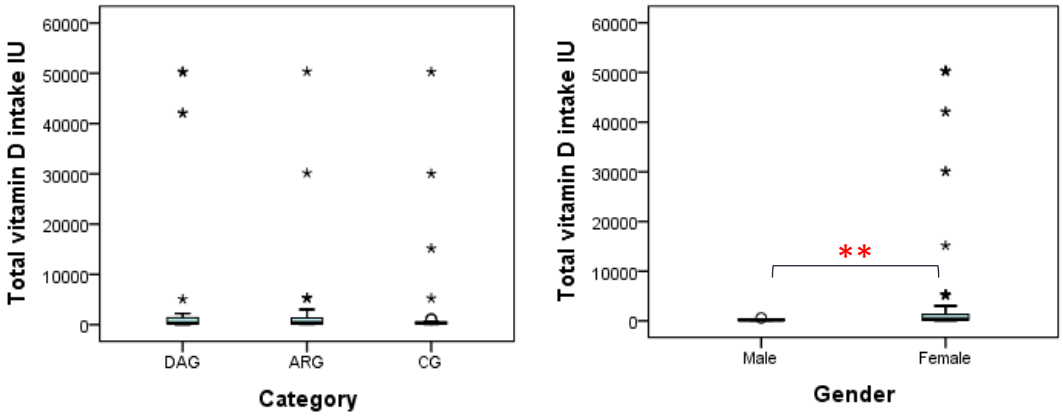


*Figure 4-14 Box and whiskers plots of DI-VitD and TI-VitD (IU/day) by clinical groups, and gender. Data shows medians, interquartile range, whiskers and outliers. Outliers were not removed as they represents the consumption of vitamin D supplements. ** Indicates significant between group differences (P<0.01).*

### *4.5.3.3. Correlations between DI-VitD, TI-VitD values and Serum 25(OH)D in the pooled sample*

To examine the relationship between dietary intake of VitD and the 25(OH)D concentration, correlation and regression tests were performed. There were statically significant negative Spearman correlation between DI-VitD and the 25(OH)D (rho= -0.195, P=0.028, $R^2$= 0.04). Contrariwise, a positive significant Spearman correlation was observed between TI-VitD and the 25(OH)D (rho=0.338, P<0.0001, $R^2$= 0.11) (Table 4-12). Figures 4-15 and 4-16 shows a scatter plots

between DI-VitD and, TI-VitD with 25(OH)D concentrations to illustrate the relationships.

*Table 4-12 Spearman correlations between 25(OH)D (ng/mL) and dietary intake of vitamin D (IU/day), total intake of vitamin D (IU/day)*

| | | | 25 (OH)D | DI-VitD | TI-VitD |
|---|---|---|---|---|---|
| Spearman's rho | 25 (OH)D | Correlation Coefficient | 1.000 | -0.195* | 0.338** |
| | | Sig. (1-tailed) | . | 0.028 | < 0.000 |
| | | N | 97 | 97 | 97 |
| | DI-VitD | Correlation Coefficient | | 1.000 | 0.532** |
| | | Sig. (1-tailed) | | | < 0.000 |
| | | N | | | 97 |
| | TI-VitD | Correlation Coefficient | | | 1.000 |
| | | Sig. (1-tailed) | | | . |
| | | N | | | 97 |
| *. Correlation is significant at the 0.05 level (1-tailed). | | | | | |
| **. Correlation is significant at the 0.01 level (1-tailed). | | | | | |

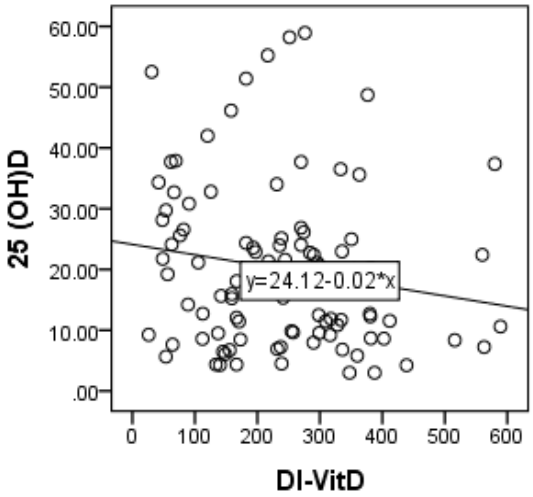


*Figure 4-15 X-Y Scatter graph of 25(OH)D ng/mL and dietary intake of vitamin D IU/day.*

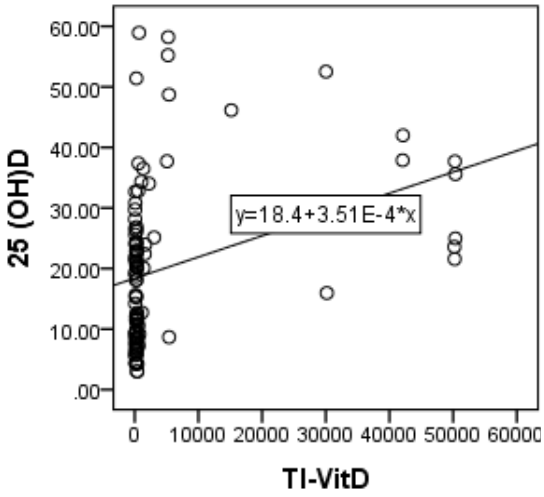


*Figure 4-16 X-Y Scatter graph of 25(OH)D ng/mL and total intake of vitamin D IU/day.*

### *4.5.3. Sun Exposure results*

#### *4.5.3.1. Descriptive analysis of Total sun exposure (TSE) in the pooled sample and between sub-groups*

Data on TSE were scale data designed by a sun exposure model (section 4.3.4). The maximum value was 30 TSE and the minimum was 12 TSE with a mean ± SD of (21.14 ± 4.44) (Table 4-13). Figure 5-17 shows the TSE distribution of the participants. Among the participants, 11.34% had limited TSE, 36.1% had Low TSE, 41.2% had medium TSE and only 11.34% had high TSE. Table 4-13 illustrates descriptive results of TSE based on the clinical groups and on gender. There were no outliers in the TSE data.

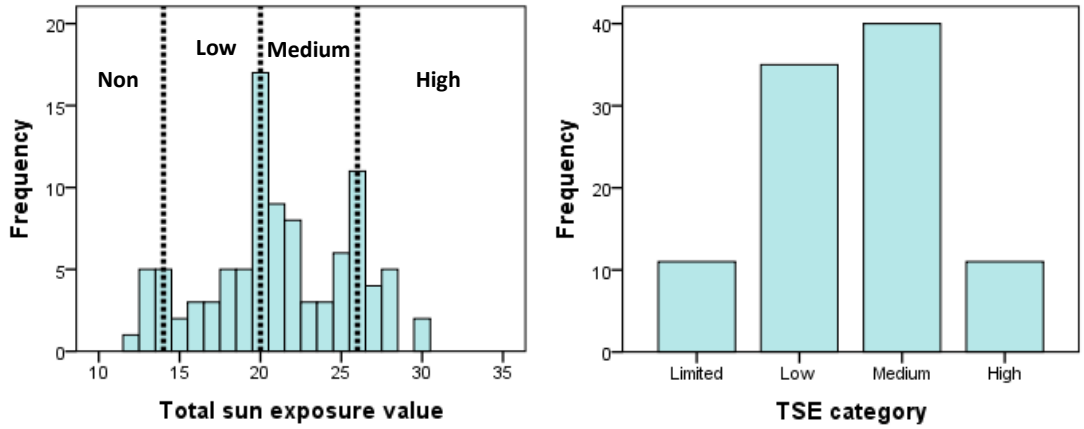


*Figure 4-17 Distribution of TSE values among the participants in pooled sample*. Cut off values are shown between the dotted lines.

*Table 4-13 Descriptive statistic of TSE among the pooled sample and in the study sub-groups*

| | N | Mean ± SD | Median (IQR) |
|---|---|---|---|
| **CG** | 33 | 21.24 ± 3.96 | 20.00 (8) |
| **ARG** | 30 | 30.93 ± 4.48 | 20.50 (7) |
| **DAG** | 34 | 21.24 ± 4.94 | 22.00 (7) |
| **Male** | 15 | 24.53 ± 4.19 | 25.00 (6) |
| **Female** | 82 | 20.52 ± 4.22 | 20.00 (6) |
| **Total** | 97 | 21.14 ± 4.44 | 21.00 (7) |

### *4.5.3.2. TSE values and the difference between sub-groups*

To investigate the difference in sun exposure between the sub-groups, a series of parametric statistical inference tests were performed. One-way ANOVA revealed no significant differences in sun exposure habits between the clinical groups (P=0.953). However, there was a significant difference in the sun exposure habits between male and female when an independent t-test was performed (T=3.38, P=0.001). Figure 4-18 are box and whiskers plots of the difference in TSE within the sub-groups.

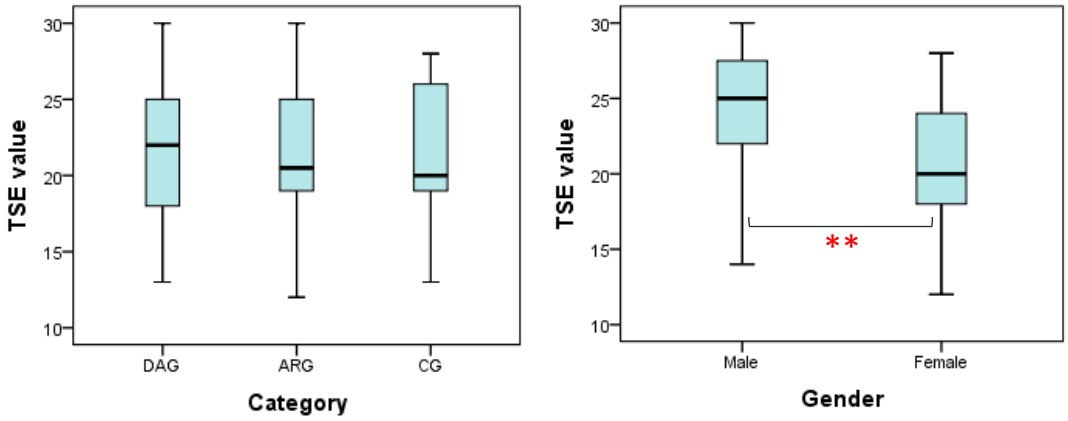


*Figure 4-18 Box and whiskers plots of TSE value among the clinical groups, and gender. Data shows medians, interquartile range, whiskers and outliers. . ** Indicates significant between group differences (P<0.01).*

### *4.5.3.3. Correlations and Regressions between TSE and Serum 25(OH)D in the pooled sample*

To examine the relationship between TSE and 25(OH)D a Spearman correlation test was performed and indicate a statically significant correlation (rho= -0.22, P=0.015, $R^2$=0.05) (Table 4-14). Figure 4-19 illustrate Scatter diagram between TSE and 25(OH)D concentration.

*Table 4-14 Spearman correlations between 25(OH)D concentrations (ng/mL) and total sun exposure values*

| | | | 25 (OH)D | TSE value |
|---|---|---|---|---|
| Spearman's rho | 25 (OH)D | Correlation Coefficient | 1.000 | -0.220* |
| | | Sig. (1-tailed) | . | 0.015 |
| | | N | 97 | 97 |
| *. Correlation is significant at the 0.05 level (1-tailed). | | | | |

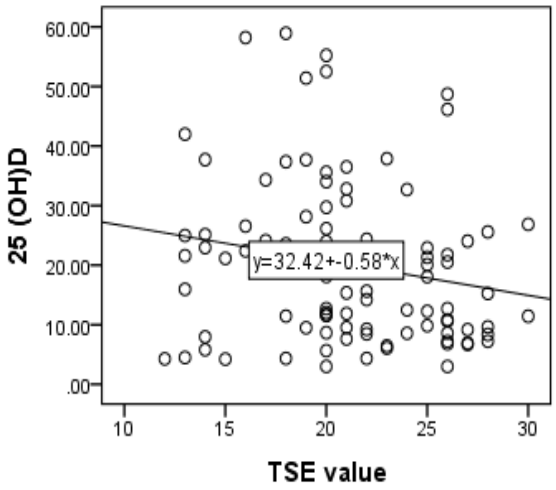


*Figure 4-19 X-Y Scatter graph of 25(OH)D concentrations ng/mL and total sun exposure values.*

## *4.5.4 Comparison of the size of the correlation*

Serum 25(OH)D concentration was significantly correlated to all of chapter modulators (see table 4-15).The strength of correlation was moderate between 25(OH)D and total intake with vitamin D supplements (TI-VitD) ($R^2 = 0.11$) and the correlations were weak between 25(OH)D and dietary intake of vitamin D (DI-VitD) ($R^2 = 0.04$) and total sun exposure ($R^2 = 0.05$).

*Table 4-15 Spearman's rho correlations between 25(OH)D and the chapter variables*

| | | | 25 (OH)D | DI-VitD | TI-VitD | TSE |
|---|---|---|---|---|---|---|
| Spearman's rho | 25 (OH)D | Correlation Coefficient | 1.000 | -0.195* | 0.338** | -0.220* |
| | | Sig. (1-tailed) | . | 0.028 | < 0.000 | 0.015 |
| | | N | 97 | 97 | 97 | 97 |
| | DI-VitD | Correlation Coefficient | | 1.000 | 0.532** | 0.115 |
| | | Sig. (1-tailed) | | . | < 0.000 | 0.132 |
| | | N | | 97 | 97 | 97 |
| | TI-VitD | Correlation Coefficient | | | 1.000 | -0.210* |
| | | Sig. (1-tailed) | | | . | 0.020 |
| | | N | | | 97 | 97 |
| | TSE | Correlation Coefficient | | | | 1.000 |
| | | Sig. (1-tailed) | | | | . |
| | | N | | | | 97 |

*. Correlation is significant at the 0.05 level (1-tailed).
**. Correlation is significant at the 0.01 level (1-tailed).

### *4.5.5. Regression model*

A multiple linear regression model was performed to create a composite model predictor of serum 25(OH)D using all of the significant modulators identified above. These significant modulators included consumption of vitamin D supplements, total sun exposure (TSE), dietary intake of vitamin D (DI-VitD), and total intake of vitamin D (TI-VitD). The model was statistically significant (P< 0.0001) and explains 39.7% of the variance of 25(OH)D concentration. The model is statistically powerful due to the low shrinkage of R (Adjusted $R^2$= 0.370). However, in further analysis the model was unstable because of the present of non-significant contributors (TI-VitD P=0.917, and TSE P=0.83) (see table 4-16). Therefore, the model was re-run to attain a stable model that incorporated the consumption of vitamin D supplements and DI-VitD to predict 25(OH)D. The stable model explains 39.6% of the variance in 25(OH)D and is statistically significant and has lower shrinkage of R (adjusted $R^2$= 0.383) than the first model. The predictors of this model are all significant, consumption of vitamin D supplements (P=0.0001) and DI-VD (P=0.019) (Table 4-17).

*Table 4-16 Unstable model predictor of serum 25(OH)D*

| Coefficients [a] | | | | | | |
|---|---|---|---|---|---|---|
| Model | | Unstandardized Coefficients | | Standardized Coefficients | T | Sig. |
| | | B | Std. Error | Beta | | |
| 1 | (Constant) | 53.269 | 7.198 | | 7.401 | <0.000 |
| | Consumption of vitamin D supplements | -17.582 | 2.830 | -0.608 | -6.213 | <0.000 |
| | Dietary intake of vitamin D | -0.020 | 0.008 | -0.193 | -2.350 | 0.021 |
| | Total intake of vitamin D | 1.061E-5 | 0.000 | 0.010 | 0.105 | 0.917 |
| | Total sun exposure | 0.055 | 0.264 | 0.018 | 0.207 | 0.836 |
| a. Dependent variable: 25(OH)D | | | | | | |

*Table 4-17 Stable model predictor of serum 25(OH)D*

| Coefficients [a] | | | | | | |
|---|---|---|---|---|---|---|
| Model | | Unstandardized Coefficients | | Standardized Coefficients | T | Sig. |
| | | B | Std. Error | Beta | | |
| 1 | (Constant) | 54.433 | 4.574 | | 11.901 | <0.000 |
| | Consumption of vitamin D supplements | -17.579 | 2.320 | -0.608 | -7.576 | <0.000 |
| | Dietary intake of vitamin D | -0.019 | 0.008 | -0.191 | -2.380 | 0.019 |
| a. Dependent variable: 25(OH)D | | | | | | |

## 4.6. Discussion

### *4.6.1. Vitamin D status discussion*

The current study has found a relatively high prevalence of vitamin D deficiency among adults who live in Saudi Arabia, mainly Makkah city. 51.5% of the participants were vitamin D deficient (25(OH)D < 20 ng/mL) with a 25(OH)D mean value of 9.88 ± 4.2 ng/mL, and 28.9% had an insufficient concentration of vitamin D in their blood plasma (25(OH)D 20 ≤ 30 ng/ml) (23.37 ± 2.57 ng/mL). Only 19.6% of the participants had adequate concentrations of vitamin D (25(OH)D > 30 ng/ml) (42.13 ± 9.28 ng/mL). These results confirmed the issue of vitamin D deficiency in Saudi Arabia reported in previous research (Al Faraj and Al Mutairi, 2003; Azhar, 2009; Ardawi et al., 2012; Tuffaha et al., 2015; Abulkhair et al., 2016; Al-Daghri, 2016; Aljefree et al., 2016; Qadhi, 2016; Kaddam et al., 2017). These results were also similar to results from other studies, especially those performed on a similar age group population in the same region (Western region) of Saudi Arabia (Ardawi et al., 2012; Aljefree et al., 2016). For instance, Ardawi et al., (2012), indicated that 51.5% of males aged 20-60 years were deficient and 38.8% had insufficient concentrations of 25(OH)D. Another study by Aljefree et al., (2016) studied adult participants (control and patients with coronary heart disease) and found that 57.5% of these had 25(OH)D concentrations < 20 ng/mL were deficient and the rest exhibited values > 20 ng/mL which they considered adequate. Both Ardawi et al., (2012) and Aljefree et al., (2016) had comparable samples and results to the present study, which support the findings of the present study.

The consumption of vitamin D supplements has a significant impact on 25(OH)D status (Holick et al., 2011; Aljefree et al., 2017a). In the current study, almost a third of the participants (i.e. 30.9%) were taking vitamin D supplements and had a mean ± SD 32.08 ± 14.96 ng/mL of 25(OH)D which is within the adequate range for vitamin D status. The difference in vitamin D status between the group who consume vitamin D supplements and those who did not, was significant ($U=315$, $P<0.001$), which indicates the positive impact of vitamin D supplements on vitamin D status.

The significant differences in 25(OH)D concentrations when vitamin D supplements were consumed or not, was also observed within the clinical groups; control group (CG), at risk group (ARG), and the diagnosed with atherosclerosis group (DAG) (U=11, P<001) (U=56, P=0.028) (U=31, P<0.001) respectively. Although these results are all significant, it was observed that the CG had the biggest difference between the groups who took or did not take vitamin D supplements. There are many explanations for these results. The CG was a healthy group whom are free from any systemic disease, which can impair the body's ability to absorb vitamin D (Holick, 2007). Since the CG were free from those diseases, the effect of supplementation was clearly significant. In addition, the CG are younger in age (41.3 ± 6.6 years) compared to the other 2 groups (ARG 50.3 ± 6.6 years and DAG 53.3 ± 6.8 years) but not significantly, however, aging is one of the factors that affects vitamin D absorption and bioavailability in the body (Holick et al., 2011).

Additionally, the participants who were taking vitamin D supplements represented 18.1% of the CG, 40% of the ARG, and 35.2% of the DAG. The explanation for the low number of participants taking vitamin D supplements in the CG compared to the other 2 groups is that, the participants in the CG were taking vitamin D supplements for prevention or treatment because of a low vitamin D status. On the other hand, participants in the ARG and DAG were mainly taking the supplements prescribed by their physician, in line with the new recommendation in Saudi Arabia, whereby vitamin D deficiency in diabetic patients and patients with cardiovascular disease should be treated with vitamin D supplements (section 2.1.7.) (Lee et al., 2008; Al-Daghri et al., 2016).

### *4.6.2. Discussion of vitamin D intake from diet (DI-VitD) and diet with supplements (TI-VitD)*

Dietary vitamin D is the second source of vitamin D after sun exposure, however, very few foods naturally contain vitamin D as demonstrated in the literature (Holick et al., 2011; Dalan et al., 2014) Additionally, in Saudi Arabia, food that naturally contains the highest amount of vitamin D i.e. Salmon, Mackerel, and Sardines are not commonly consumed. The highest amount of dietary vitamin D comes from the consumption of eggs and fortified dairy products such as milk and yogurt, as discussed in the literature and observed from the current study (Aljefree et al., 2017b). The mean intake of vitamin D from the diet (DI-VitD) in the current study was 237.27 ± 131.9 IU/day which represents only 39.5% of the recommended dietary intake of vitamin D (600 IU/day) (Ministry of Health, 2017). Many studies concur with this issue of low intake of vitamin D rich food in Saudi Arabia (Yousef et al., 2013; Kearney et al., 2015). Yousef et al. have observed similar results in 120 breast cancer patients and 120 control volunteers in Saudi Arabia. They found that <34% of cancer patients and <39% of the control group had a low consumption of oral vitamin D from the diet. The low intake of vitamin D from the diet could be one for the reasons of vitamin D deficiency in Saudi dwellers. However, there was a negative significant correlation between DI-VitD and 25(OH)D concentrations ($P<0.05$). The strength of the correlations was ($R^2= 0.04$) which indicates a weak relationship and it explains only 4% of the variance in 25(OH)D concentrations. Additionally, there were no gender or clinical variations in DI-VitD in the current study.

Conversely, when supplement consumption was added to the dietary intake of vitamin D to make the total intake of vitamin D (TI-VitD), there was a moderate positive correlation with serum 25(OH)D concentrations ($P<0.0001$) ($R^2= 0.11$) which explains 11% of the variance in 25(OH)D concentrations. The higher the values of TI-VitD the greater concentrations of 25(OH)D, which enhanced the supplement effect on vitamin D status. The mean value of TI-VD was 4842.29 ± 12959.6 IU/day. There was a statistical variation between males and females in TI-VD as none of the male participants were taking vitamin D supplements ($U=377$, $P=0.018$). As demonstrated

in the literature review, the new recommendations suggest consumption of vitamin D supplements for people at risk of vitamin D deficiency and for treatment of vitamin D deficiency, which is supported by the results of the current study (Al-Daghri et al., 2016; Holick, 2017).

In the TI-VitD data, there were 23 apparent outliers above 750 IU/day out of the total number of the participants n=97. In fact, they were not removed from the data because they represented 76.6% of the participants who were taking vitamin D supplements n=30. The values were reviewed to confirm they were correct entries. It was estimated that any DI-VitD analysis without the outliers would skew that data towards a greater proportion of one population than was truly representative of our sample; as such, this outlier removal was not performed.

### *4.6.3. Discussion of sun exposure*

While exposure to sunlight is the main source of vitamin D, especially in a country with sunny climate throughout the year like Saudi Arabia, the current study indicated that 50.1% of the participants had vitamin D deficiency. Using the sun exposure analysis model designed for this study, total sun exposure (TSE) was determined from the questionnaire. Among the participants, TSE was limited in 11.3% of participants, low in 36.1%, medium in 41.2% and only 11.3% had a high TSE value. As discussed in the literature review, many factors limit sun exposure behaviour in Saudi Arabia. The excessive high temperature reduces outdoor activities during daytime (Tuffaha et al., 2015; Aljefree et al., 2017b). The data collection period was between May and July, during which time the temperature rose up to 48°C in Makkah and Al-Qunfudhah cities and to 40°C in Jeddah city (AccuWeather, 2016). Additionally, 51.5% of the participants were unemployed housewives, which meant they did not have to go out during the daytime. These housewives were female participants and so that also explained the gender-based significant variation in TSE ($P< 0.001$). Overall, it was observed from the current study and other studies that the majority of the Saudi dwellers were not fully aware of the importance of sun exposure and the right way to obtain it (Al-Agha et al., 2016; Aljefree et al., 2017b).

The outcome of the TSE value was un-expectedly negatively correlated to 25(OH)D concentration (rho=-0.22, P<0.01) the relationship was weak and TSE represented only 5% of 25 (OH)D variance ($R^2$=0.05). However, similar results were also observed from other studies (Binkley et al., 2007; Bushnaq, 2016). Binkley et al. (2007) measured serum 25(OH)D in 93 adults in Hawaii (latitude 21° N). A self-reporting sun exposure questionnaire was used. They found no correlation between 25(OH)D concentration and sun exposure. Similarly, Bushnaq (2016) also found that the average of exposed body surface area in populations from the United Kingdom (latitude 53.46° N) and Saudi Arabia (latitude 21.41° N) n=79 has a negative effect on 25(OH)D concentrations. The negative, or no, association between sun exposure and 25(OH)D could be due to the low concentration of vitamin D as Binkley et al. suggested (2007). Additionally, the low exposure to sunlight could be the reason for the unpredicted association with 25(OH)D concentration. Moreover, the lack of awareness of the right way to expose oneself to the sun to maximise the potential for synthesising vitamin D could be the reason for this negative relationship (Aljefree et al., 2017a).

### *4.6.4. Multiple linear regression model*

The multiple linear regression model suggested that together, consumption of vitamin D supplements and dietary intake of vitamin D, create a stable model to predict 25(OH)D serum concentration, more so that any model including other factors such as TI-VD, and TSE. The model explains 39.6% of the variance in 25(OH)D concentrations.

## 4.7. Summary and conclusion

The current study confirmed the huge problem of vitamin D deficiency in Saudi Arabia. Additionally, the study confirmed the positive impact of vitamin D supplements on circulating 25(OH)D levels in the pooled sample and in between the clinical groups (CG, ARG, DAG). The current study proved the significant association between 25(OH)D and the participants' vitamin D status when vitamin D supplements were consumed. The current study designed dietary intake and the sun exposer models for the people who live in Saudi Arabia.

The current study has confirmed that Saudi dwellers are not consuming sufficient vitamin D rich food. Additionally, they are not exposing themselves (uncovered skin) enough to sunlight in order to synthesise vitamin D. Out of the sources of vitamin D, diet and the consumption of vitamin D supplements were good indicators of circulating 25(OH)D concentration, but sun exposure was not. In all, this data would suggest that for Saudis, at least, this simplifies the formulation of government recommendations for lifestyle to maximise the potential for a healthy vitamin D status.

# Chapter 5 - Determination of the effect of life-style factors on circulating 25(OH)D levels in a sample of Saudi Arabia dwellers

## 5.1. Introduction

Certain traits and life style factors, such as the socioeconomic status and education level, have an impact on the health of individuals (Shirazi et al., 2013). Many factors can adversely affect vitamin D status and confer a risk of vitamin D deficiency. Some studies have demonstrated the impacts of these factors (Shirazi et al., 2013; Holick and Chen, 2008). The current chapter presents the association between serum 25(OH)D status and gender, age, education level, occupation, socioeconomic status, smoking, BMI body mass index (BMI) and waist-to-hip ratio (WHR).

### *5.1.1. Chapter aim*

- To establish whether there are any relationships between circulating 25(OH)D concentrations and lifestyle factors in a sample of Saudi Arabia dwellers.

### *5.1.2. Chapter objectives*

1- To assess lifestyle factors and determine any differences between the sub-groups.
2- To determine any association between the lifestyle factors and circulating 25(OH)D concentrations.

## 5.2. Methods

### *5.2.1. Gender*

Participants were asked to state their sex in the general information section of the questionnaire. The answer had two options, male and female. The tendency was to have a similar number of participants from each sex in each of the study groups (control group (CG), at risk group (ARG), and diagnosed with atherosclerosis group (DAG)). However, females were more cooperative and hence a greater number participated in the study.

### *5.2.2. Age*

In the general information section of the questionnaire, there was a question about the date of birth. The inclusion criteria for age was 35 to 60 years old, a range which covered middle aged adults and was found to include participants whose statuses were within the 3 categories, control, at-risk and confirmed disease. The majority of the participants provided their date of birth in a Hijiri date as it is the standard calendar used in Saudi Arabia. The date was converted to a Gregorian date using a website converter (IslamiCity, 1995-2018). The same converter was used to determine all of the participants' ages to ensure accuracy of the data.

### *5.2.3. Education level*

The participants were asked to choose their level of achievement in education. The question was part of the general information section of the questionnaire. There were six levels of education that the participants could choose from. The seventh option was "other", with an open space for an answer, but none of the participants chose it. Table 5-1 describes each level of education and the ideal age of the person at each level.

*Table 5-1 Description of education levels in Saudi Arabia*

| Education level | Description of the level | Ideal age during level |
|---|---|---|
| **Illiterate** | Did not go to school | ----- |
| **Primary** | 6 levels from year 1 to 6 | 6 to 11 years |
| **Secondary** | 3 levels from year 7 to 9 | 12 to 14 years |
| **High school** | 3 levels from year 10 to 12 | 15 to 17 years |
| **Diploma** | 1 or 2 years | at any age after high school |
| **Bachelor** | 4 to 6 levels | at any age after high school |
| **Postgraduate** | Masters or doctoral degree | at any age after bachelor |

### *5.2.4. Occupation*

In the general information section of the questionnaire, participants were asked about their job. The question was targeting the nature of the job rather than the job itself. The question had seven answers to choose from: education, healthcare, public sector, private sector, housewife, retired and other. Education, if they worked in school, university, or any sector related to education. Healthcare, if they worked in a hospital, clinic, or any related place. Public sector included jobs that are run by the government but not in education or healthcare. Private sector represented jobs that are run by a private institution or company that were also not under education or healthcare. Housewife represented people who did not have a job. Finally, the option for "other" was for any other answer with an open space for the answer, but none of the participants chose it.

### *5.2.5. Socioeconomic status*

Socioeconomic status assessment consisted of two parts: monthly income and number of family members benefiting from said income. The questions were included in the general information part of the questionnaire.

The income question was asked according to the monthly family income in Saudi Riyals (SR), the currency that is used in Saudi Arabia. Rather than provide a specific

value, the question provided five income ranges (Table 5-2) from which a choice could be made. Details of the mean value of the ranges of income and the amounts in Great British Pounds (GBP) are also shown in table 5-2.

The number of members in the family, who were benefiting from the income, was an open question. Participants were asked to state how many members of the family were using the income value which was selected in the previous question. The number should also have included the participant.

*Table 5-2 Monthly income in SR and GBP*

| Range in SR | Equivalent in GBP | Mean value SR | Mean value GBP |
|---|---|---|---|
| Less than 5000 | Less than 1050 | 2500 | 520 |
| 5000 to 10000 | 1040 to 2080 | 7500 | 1560 |
| 10000 to 15000 | 2080 to 3120 | 12500 | 2600 |
| 15000 to 20000 | 3120 to 4160 | 17500 | 3640 |
| 20000 and above | 4160 and above | 22500 | 4680 |

### *5.2.6. Smoking status*

Smoking status was determined using four questions in the health information section of the questionnaire. The first question asked about the current smoking status. The answer to this question was either yes or no. The second question was about the history of smoking if the participants were not current smokers. The third question was about the participants' smoking habits, for participants who were smokers or used to be smokers. That question had four possible answers: Heavy smoker, moderate smoker, light smoker and rare smoker. The last question was also for both smokers and those who used to be smokers, regarding the type of smoking products used. The participants could choose from four different types of smoking products that were cigarettes, electronic cigarettes, hookah and shisha. There was an option for "other" with a blank space to write any additional type but none of the participants wrote anything on it.

### *5.2.7. BMI and WHR*

Anthropometric measurements were performed to establish the participant's body mass index (BMI) and waist-to-hip ratio (WHR). For the BMI the measurements, height and mass were taken. Participants' height (cm) and mass (kg) were measured using an electronic scale (Doran Scales, Charles, IL, USA) (Appendix 12). The participants stood on the scale without their shoes, purse, wallet, or any additional items. The weight was taken for most of the participants when they arrived in a 10-hour fasted state for the purpose of the blood collection sample. Some of participants first arrived not fasted and their anthropometric measurements were taken when they had come back for the blood test while they were fasting. For the height measurement, the participants were asked to stand straight and position their head in the Frankfort plane (Hirani, 2010). The head plate was gently lowered on to the top of the head and the height was then measured. Height and weight were used to identify the body mass index (BMI). The BMI is calculated as the ratio of body mass in kilograms to the height in meters, squared (body mass/height$^2$). BMI is defined in table 5-3.

*Table 5-3 Illustration of BMI (kg/m$^2$) categories for adult (Hirani, 2010)*

| **Category** | **BMI kg/m$^2$** |
|---|---|
| Underweight | < 18.5 |
| Healthy weight | 18.5 - 24.9 |
| Overweight | 25 - 29.9 |
| Obese | > 30 |

The waist and hip circumference measurements were taken to provide an indication of abdominal fat. A standard tape measure in centimetres (cm) was used without any pressure to the body surface over light clothing. The participants were asked to stand straight with feet close together, arms down at the sides, and to relax the abdominal muscles. The waist was defined as the midpoint between the hip and the last rib cage bone. The hip was defined as the widest point over the buttocks. Waist-to-hip ratio (WHR) was calculated by dividing the circumferences of waist over hip. The category of WHR ranges are displayed in Table 5-4.

*Table 5-4 Illustration of WHR (cm) categories for males and females (Dalton et al., 2003; Consultation, 2008)*

| Category | WHR for males | WHR for females |
|---|---|---|
| Normal adipose | ≤0.90 | ≤ 0.80 |
| High adipose | 0.90 - 0.99 | 0.80 - 0.84 |
| Morbidly adipose | ≥ 1.00 | ≥ 0.85 |

### *5.2.8. Circulation of 25(OH) D*

Concentrations of 25(OH)D were determined using an electrochemiluminescence binding assay that uses Elecsys 2010 (Cobas e E601 immunoassay analysers, Indianapolis, IN, USA) (Roche, 2015a). Details of the methods used in blood sample collection and laboratory analysis of 25(OH)D are presented in chapter 4.

## 5.3. Statistical analysis of the data

Statistical analysis were performed using SPSS v24 (Inc., Chicago, IL, USA). Descriptive and summary statistics are reported as mean ± standard deviation, median (interquartile range), and mean rank for continuous variables and count (N) and percent for categorical variables. In the current chapter, none of the outcome measures were normally distributed, as such data was deemed suitable for non-parametric statistical tests. Therefore, the Kruskal-Wallis test, with appropriate *post-hoc* Mann-Whitney tests were carried out where there were more than three groups, or Mann-Whitney tests alone for simple mean pairwise comparisons. Where data was continuous rather than grouped, Spearman Rho correlations were carried out. Statistical significance was set at $p \leq 0.05$. Multiple linear regressions model was performed for further investigation to study an effect of variables on 25(OH)D if there were any.

## 5.4. Results

In this section determination of the separate, and where appropriate, combined impact of life-style factors including gender, age, education level, socioeconomic status, smoking status, skin tone, BMI and WHR on 25(OH)D plasma level is presented. The overall study sample size was 97. General data descriptive of the participants' life-style factors in each of the study groups CG, ARG, and DAG are illustrated in table 5-5.

*Table 5-5 Distributions of life-style factors among the participants in total and in each of the study groups*

| Variable | CG N=33 | ARG N=30 | DAG N=34 | Total N=97 |
|---|---|---|---|---|
| ***Gender*** | | | | |
| *Male (% total)* | 5 (15.2%) | 3 (10%) | 7 (20.6%) | 15 (15.5%) |
| *Female (% total)* | 28 (84.8%) | 27 (90%) | 27 (79.4%) | 82(84.5%) |
| **Age (years)** | 41.3 ± 6.6* | 50.3 ± 6.6* | 53.1 ± 6.8* | 48.2 ± 8.4* |
| ***Education level*** | | | | |
| *Illiterate* | 1 (3 %) | 4 (13.3 %) | 3 (8.8 %) | 8 (8.2 %) |
| *Primary* | 3 (9.1 %) | 5 (16.7 %) | 10 (29.4 %) | 18 (18.6 %) |
| *Secondary* | 1 (3 %) | 10 (33.3 %) | 5 (14.7 %) | 16 (16.5 %) |
| *High school* | 5 (15.2 %) | 4 (13.3 %) | 2 (5.9 %) | 11 (11.3 %) |
| *Diploma* | 2 (6.1 %) | 1 (3.3 %) | 5 (14.7 %) | 8 (8.2 %) |
| *Bachelor* | 16 (48.5 %) | 5 (16.7 %) | 6 (17.6%) | 27 (27.8 %) |
| *Postgraduate* | 5 (15.2 %) | 1 (3.3 %) | 3 (8.8 %) | 9 (9.3 %) |
| ***Occupation*** | | | | |
| *Education* | 7 (21.2 %) | 5 (16.7 %) | 8 (23.5 %) | 20 (20.6 %) |
| *Health care* | 12 (36.4 %) | 1 (3.3 %) | 2 (5.9 %) | 15 (15.5 %) |
| *Public sector* | 3 (9.1 %) | 0 (0 %) | 0 (0 %) | 3 (3.1 %) |
| *Private sector* | 0 (0 %) | 2 (6.7 %) | 3 (8.8 %) | 5 (5.2 %) |
| *Housewife* | 10 (30.3 %) | 22 (73.3 %) | 18 (52.9) | 50 (51.5 %) |
| *Retired* | 1 (3 %) | 0 (0 %) | 3 (8.8 %) | 4 (4.1 %) |
| ***Income by SR*** | | | | |
| *Less than 5000* | 5 (15.2 %) | 17 (56.7 %) | 16 (47.1 %) | 38 (39.2 %) |
| *5000-10000* | 9 (27.3 %) | 3 (10 %) | 6 (17.6 %) | 18 (18.6 %) |
| *10000-15000* | 6 (18.2 %) | 5 (16.7 %) | 2 (5.9 %) | 13 (13.4 %) |
| *15000-20000* | 7 (21.2 %) | 4 (13.3 %) | 3 (8.8 %) | 14 (14.4 %) |
| *>20000* | 6 (18.2 %) | 1 (3.3%) | 7 (20.6 %) | 14 (14.4 %) |
| ***Number of family members*** | 4.7 ± 2.2 * | 4.6 ± 2.5 * | 4 ± 2.9 * | 4.4 ± 2.6 * |
| ***Smoking status*** | | | | |
| *Smokers* | 12 (36.4 %) | 1 (3.3 %) | 3 (8.8 %) | 16 (16.5 %) |
| *Used to smoke* | 0 (0 %) | 0 (0 %) | 4 (11.8 %) | 4 (4.1 %) |
| *Non-smokers* | 21 (63.6 %) | 29 (96.7) | 72 (79.4 %) | 77 (79.4 %) |
| ***BMI* (kg/m$^2$)** | 30.6 ± 6.9* | 32.1 ± 4.6* | 33 ± 5.87* | 31.9 ± 5.9* |
| ***WHR (cm)*** | 0.85 ± 0.08* | 0.95 ± 0.08* | 0.93 ± 0.15* | 0.91 ± 0.12* |

Categorical data are calculated as count (% total)

* = Scale data are calculated as mean ± standard deviation (SD)

### *5.4.1. Association between gender and vitamin D level*

Data on gender included male n=15 and female n=82 of a total sample n=97 as illustrated in table 5-5 and figure 5-1. To examine the difference between male and female in 25(OH)D concentrations a Mann-Whitney test was performed and revealed no significant gender effect (P=0.299) as discussed in section 4.5.1.2. and shown in table 5-6. A box and whiskers plot of gender and 25(OH)D concentrations was illustrated in section 4.5.1.2. figure 4-7.

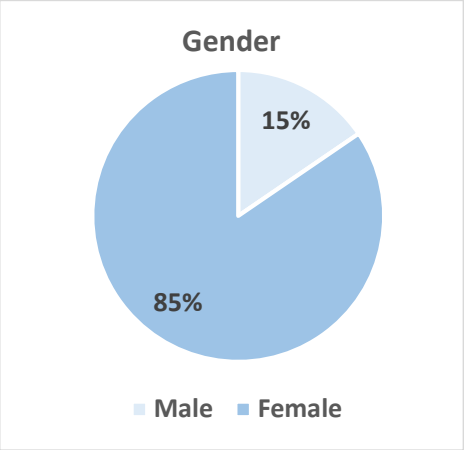


*Figure 5-1 Distribution of gender among the participants. Data shown in relative percentage (to the whole sample).*

*Table 5-6 Mean rank of 25(OH)D concentrations ng/mL among sexes.*

| | Gender | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| 25 (OH)D | Male | 15 | 42.07 | 631.00 |
| | Female | 82 | 50.27 | 4122.00 |
| | Total | 97 | | |

### *5.4.2. Association between age and 25(OH)D concentrations*

Data on age were scale data. The participants (n=97) ranged in age between 35 to 60 years with mean 48.22 ± 8.367 years (Table 5-5). Figure 5-2 shows the age distribution of the participants. Table 5-5 illustrate the mean of age among the pooled sample and the clinical groups. Running a Kruskal-Wallis test, age was significantly different between the study groups ($\chi^2$ (2) =35.48, P<0.0001) (table 5-7). Running a post-hoc Mann-Whitney, the difference was found to be significant

between the CG vs DAG (U=135, P<0.0001) and between CG vs ARG (U=163, P<0.0001). Figure 5-3 shows a box and whiskers plot of age in the study groups. Additionally, there was a significant differences in age between participants who take vitamin D supplements and who do not (U=618, P=0.002) as it is shown in the box and whiskers plot (figure 5-3).

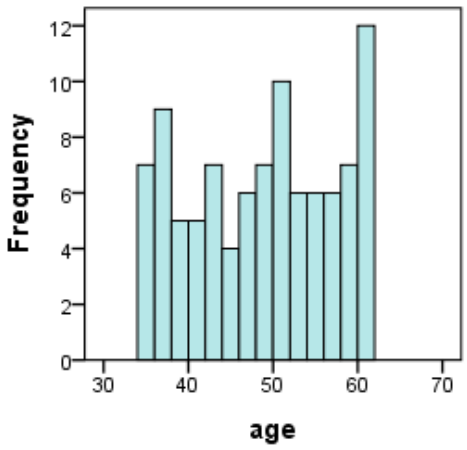


*Figure 5-2Distribution of age among the participants in the pooled sample*

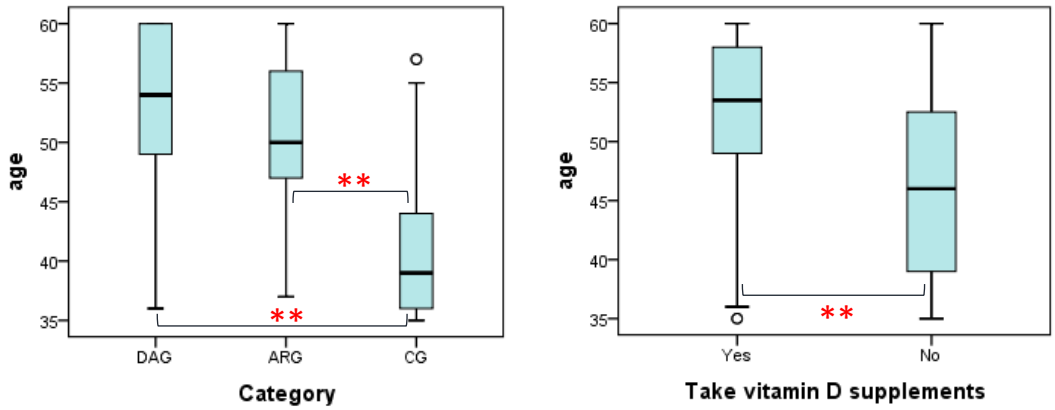


*Figure 5-3 A box and whiskers plot of age among the study groups and the consumption of vitamin D supplements. Data shows medians, interquartile range, whiskers and outliers. ** Indicates significant between group differences (P<0.01).*

*Table 5-7 Mean ranks of age among the study groups*

| | Category | N | Mean Rank |
|---|---|---|---|
| age | DAG | 34 | 65.56 |
| | ARG | 30 | 55.50 |
| | CG | 33 | 26.03 |
| | Total | 97 | |

Whilst age was statistically correlated to 25(OH)D (rho=0.29, P=0.0001, $R^2$=0.08) as illustrated in table 5-8, the strength of this relationship is considered weak (table 4-1). That analysis indicates that as the participants aged they were more likely to take vitamin D supplements and they had better 25(OH)D status. A scatter plot of the relationship between 25(OH)D and age is illustrated in figure 5-4.

*Table 5-8 Spearman's rho correlation between 25(OH)D (ng/mL) and age (years)*

| | | | age |
|---|---|---|---|
| Spearman's rho | 25 (OH)D | Correlation Coefficient | 0.291** |
| | | Sig. (1-tailed) | 0.002 |
| | | N | 97 |
| **. Correlation is significant at the 0.01 level (1-tailed). | | | |

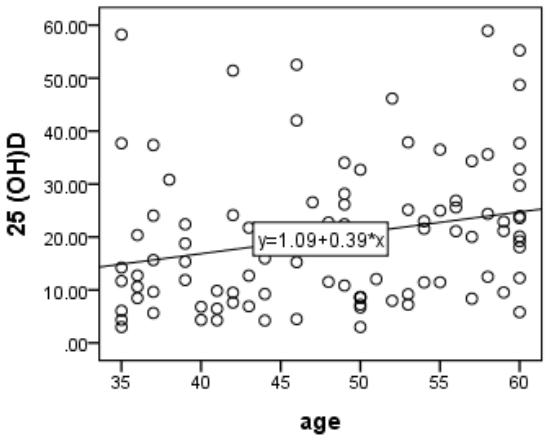


*Figure 5-4 X-Y Scatter graph of 25(OH)D (ng/mL) and age (years)*

## *5.4.3. Impact of education level on vitamin D level*

There were seven levels of education in the current study, ranging from illiterate to postgraduate (figure 5-5). A Kruskal-Wallis test revealed no main effect of education on 25(OH)D levels (P= 0.165). Table 5-9 shows the mean rank of vitamin D on the seven levels of education. Figure 5-6 illustrate the box and whiskers plot for education level and 25(OH)D.

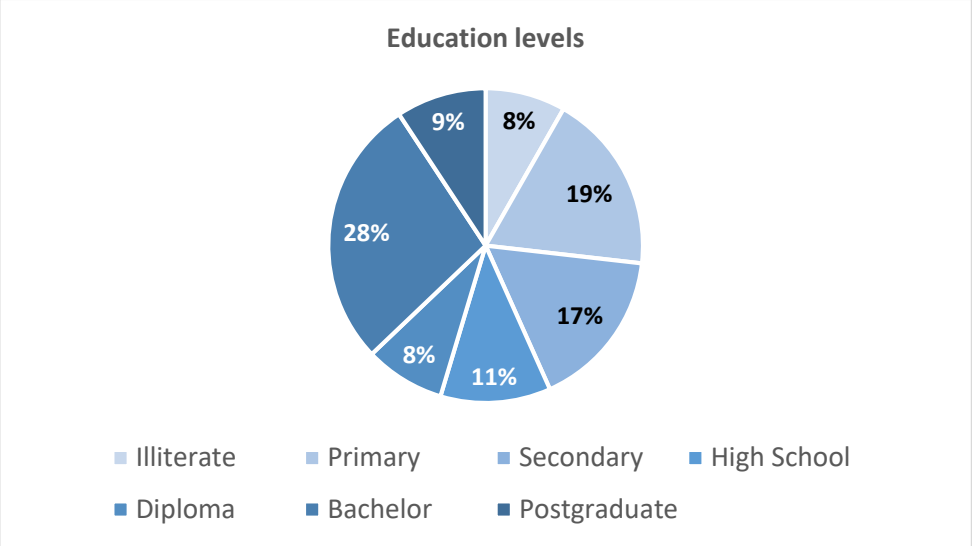


*Figure 5-5 Illustration of the percentile per education level. Distribution was non-normal.*

*Table 5-9 Mean Rank of vitamin D among education level*

| | education level | N | Mean Rank |
|---|---|---|---|
| 25 (OH)D | Illiterate | 8 | 71.75 |
| | Primary | 18 | 37.69 |
| | Secondary | 16 | 52.03 |
| | High School | 11 | 42.86 |
| | Diploma | 8 | 53.75 |
| | Bachelor | 27 | 49.83 |
| | Postgraduate | 9 | 46.78 |
| | Total | 97 | |

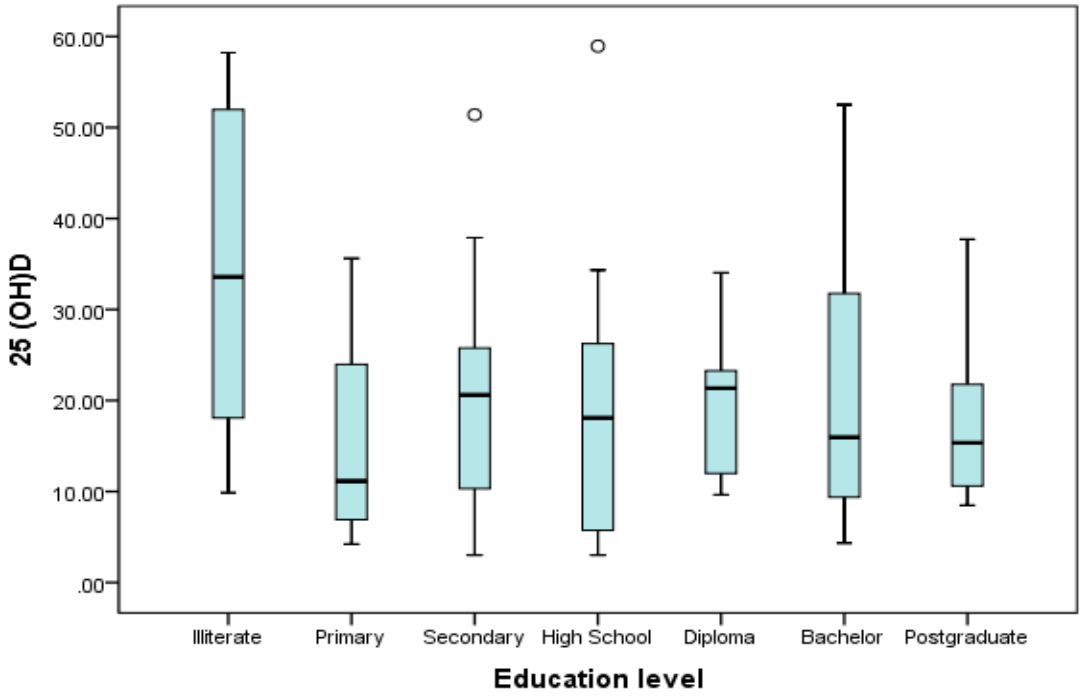


*Figure 5-6 A box and whiskers plot of education level and 25(OH)D concentrations (ng/mL). Data shows medians, interquartile range, whiskers and outliers.*

### *5.4.4. Occupation*

Data on occupation included six different jobs as distributed in figure 5-7. The majority of the participants were housewives. Running a Kruskal-Wallis test revealed no difference in 25(OH)D status by occupation (P=0.488), mean ranks are illustrated in table 5-10. Figure 5-8 illustrate the box and whiskers plot for education level and 25(OH)D.

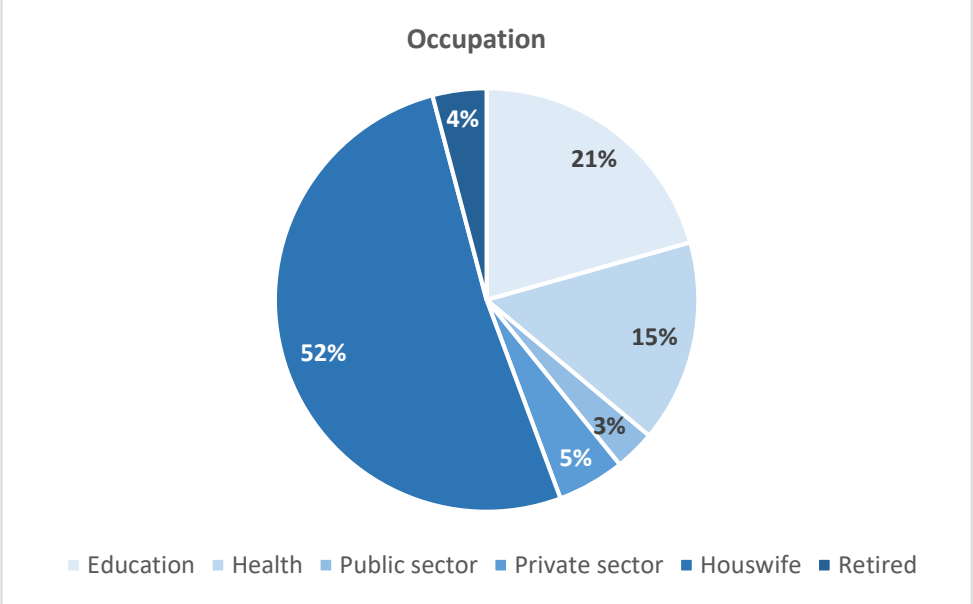


*Figure 5-7 Distribution of participant's jobs. Distribution is not normal*

*Table 5-10 Mean rank of 25(OH)D concentrations (ng/mL) by occupation*

| | job | N | Mean Rank |
|---|---|---|---|
| 25 (OH)D | Education | 20 | 58.95 |
| | Health | 15 | 45.10 |
| | Public sector | 3 | 30.00 |
| | Private sector | 5 | 49.60 |
| | Housewife | 50 | 47.75 |
| | Retired | 4 | 43.00 |
| | Total | 97 | |

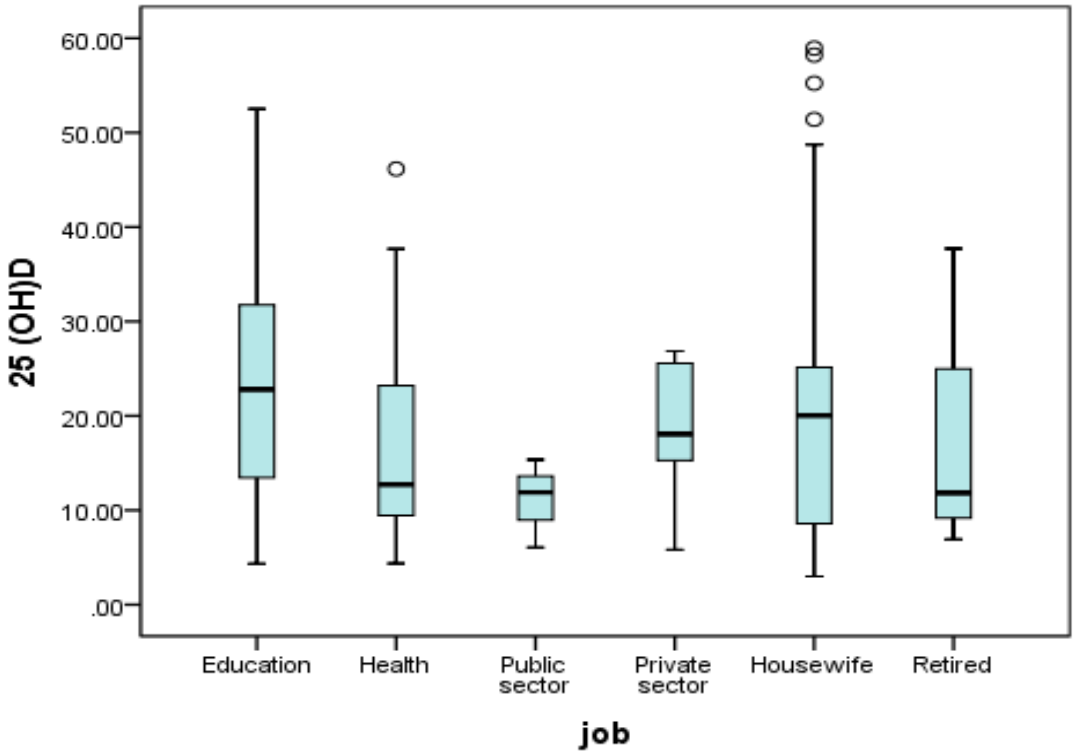


*Figure 5-8 A box and whiskers plot for 25(OH)D concentrations (ng/mL) and occupations. Data shows medians, interquartile range, whiskers and outliers.*

### *5.4.5. Impact of socioeconomic status on 25(OH)D level*

Data on socioeconomic status includes five levels of income and the number of family members who are benefiting from the income. Figure 5-9 illustrates the distribution of family income among the participants. The number of family members benefiting from the income ranged 1 to 13 (minimum to maximum). Also notable, that income normalised for the number of family members ranged 3.913 to 4.962. It is interesting that there was a significant positive correlation between the number of family members and income (rho=0.24, P=0.009) (Table 5-11).

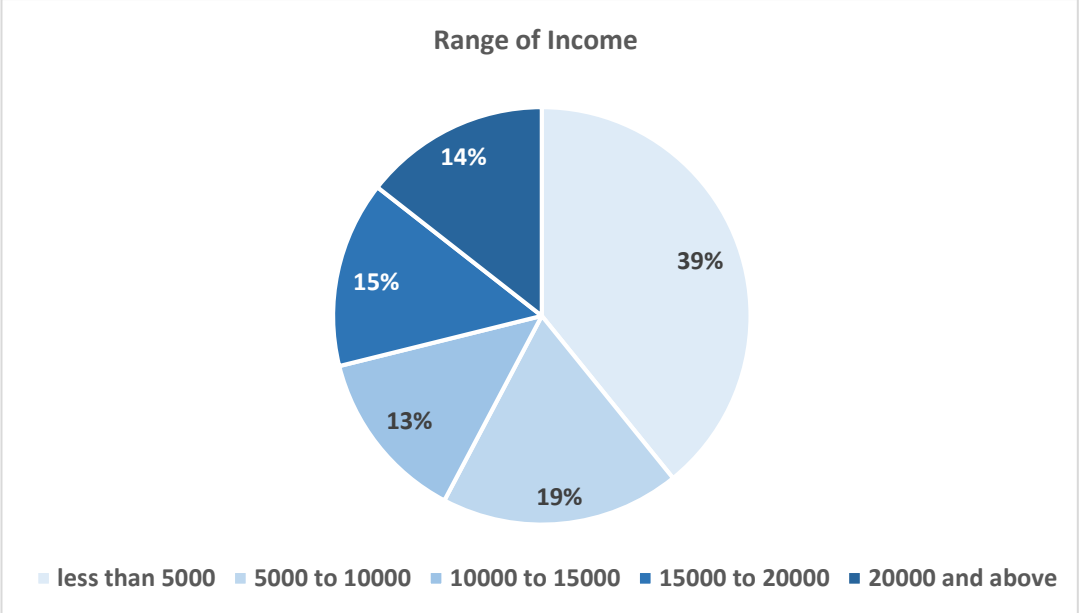


*Figure 5-9 Distribution of income among the participants. Data shown in relative percentage (to the whole sample)*

*Table 5-11 Spearman's correlations between 25(OH)D and family mean income, number of family members, and normalised income by family size.*

| | | | 25 (OH)D | Mean income | N of family members | income / family size |
|---|---|---|---|---|---|---|
| Spearman's rho | 25 (OH)D | Correlation Coefficient | 1.000 | 0.016 | -0.146 | 0.062 |
| | | Sig. (1-tailed) | . | 0.439 | 0.076 | 0.275 |
| | | N | 97 | 97 | 97 | 97 |
| | Mean income | Correlation Coefficient | | 1.000 | 0.240** | 0.814** |
| | | Sig. (1-tailed) | | . | 0.009 | 0.000 |
| | | N | | 97 | 97 | 97 |
| | number of family members | Correlation Coefficient | | | 1.000 | -0.321** |
| | | Sig. (1-tailed) | | | . | 0.001 |
| | | N | | | 97 | 097 |
| | Income / family size | Correlation Coefficient | | | | 1.000 |
| | | Sig. (1-tailed) | | | | . |
| | | N | | | | 97 |

**. Correlation is significant at the 0.01 level (1-tailed).

The mean ranks of 25 (OH) D by socio-economic status are presented in table 5-12 below. A Kruskal-Wallis test revealed no significant difference between overall income and 25(OH)D level (P=0.77). Similarly, a Spearman's rho showed no significant correlation between income normalised for number of family members and 25(OH)D level (P=0.275) (Table 5-11). In other words, there is no statistical difference or correlation in 25(OH)D level by socioeconomic status, whether accounted for as a total amount or normalised for number of dependant in the household. Figure 5-10 and figure 5-11 illustrate the box and whiskers plot for income and number of family members and 25(OH)D.

*Table 5-12 Mean rank of 25(OH)D by income (SR)*

| | income | N | Mean Rank |
|---|---|---|---|
| 25 (OH)D | less than 5000 | 38 | 46.88 |
| | 5000 to 10000 | 18 | 53.36 |
| | 10000 to 15000 | 13 | 49.23 |
| | 15000 to 20000 | 14 | 54.61 |
| | 20000 and above | 14 | 43.32 |
| | Total | 97 | |

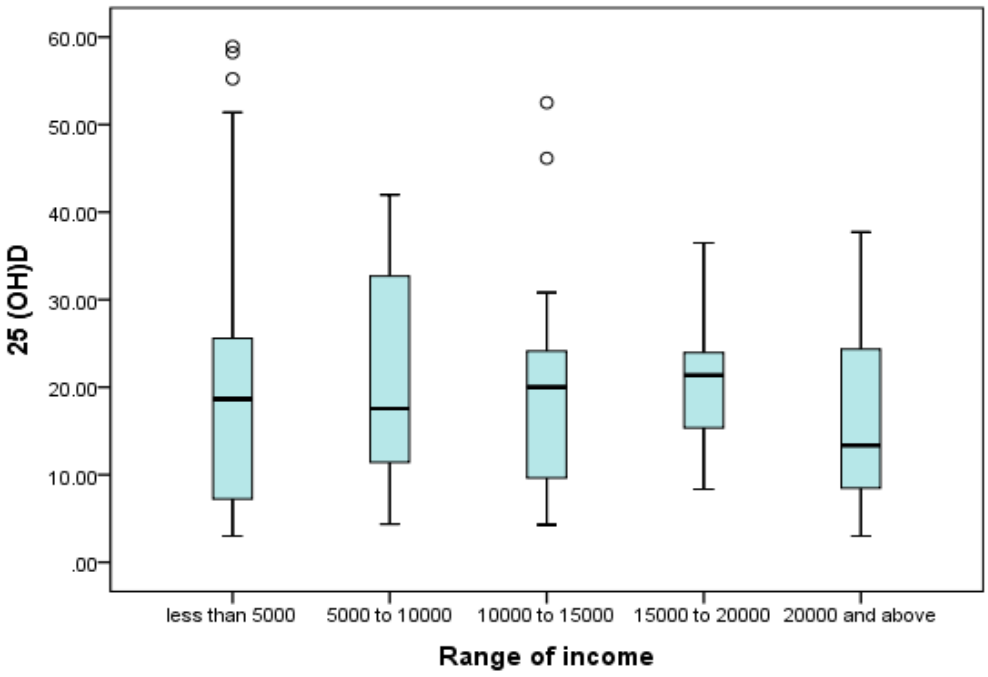


*Figure 5-10 Box and whiskers plot for income (SR) and 25(OH)D concentrations (ng/mL). Data shows medians, interquartile range, whiskers and outliers.*

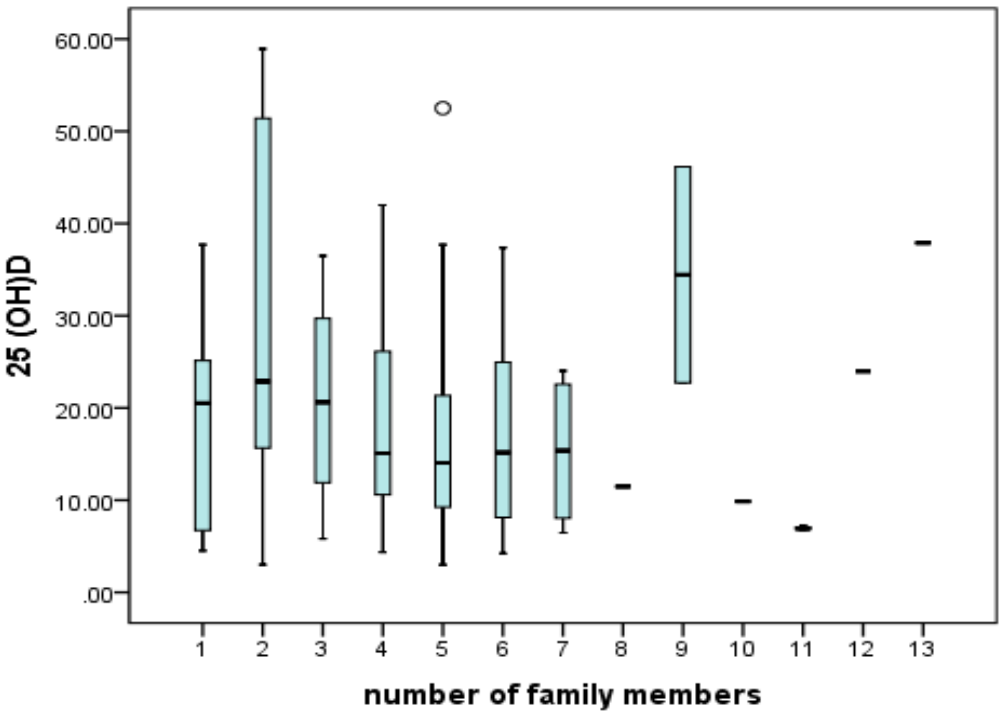


*Figure 5-11 Box and whiskers plot for number of family members and 25(OH)D concentrations (ng/mL). Data shows medians, interquartile range, whiskers and outliers.*

### *5.4.6. Impact of Smoking on 25(OH)D level*

There were three questions related to smoking status. The questions enquired about current smoking status, previous history of smoking, amount of smoking (i.e. as they define the amount of daily smoking events), and type of smoking implement.

In terms of the current smoking status, there were only 15 smokers, 4 used to be smokers and the majority of the participants 78 were non-smokers (Figure 5-12). In order to explore the impact of smoking status on 25(OH)D concentrations, a Kruskal Wallis was run with three levels (current smokers' vs previous smokers, who have stopped, vs no-smoking history participants). Consequently, analysis showed no main effect of smoking status on 25(OH)D level (P=0.721). Mean ranks were presented in table 5-13. Figure 5-13 shows a box and whiskers plot of smoking status and 25(OH)D.

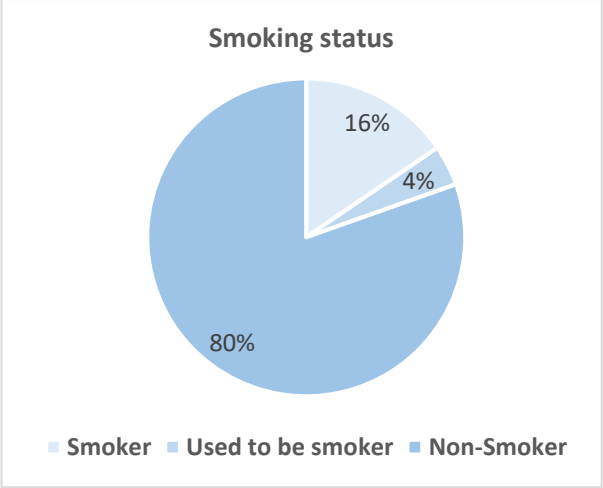


*Figure 5-12 Smoking status among the participant. Distribution is not normal.*

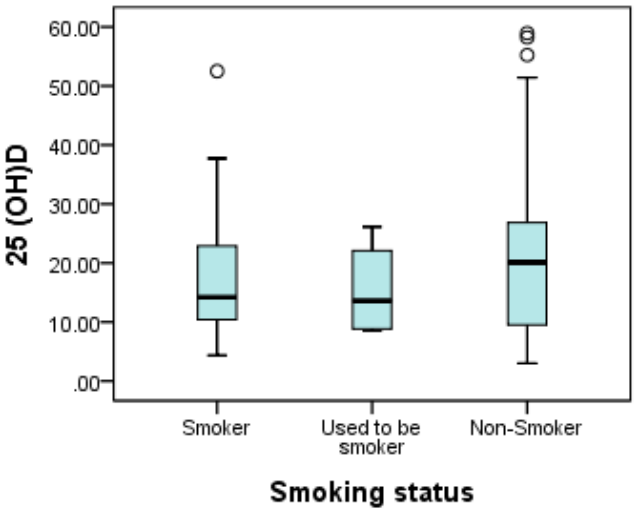


*Figure 5-13 A box and whiskers plot for smoking status and 25(OH)D (ng/mL). Data shows medians, interquartile range, whiskers and outliers.*

*Table 5-13 Mean rank of 25(OH)D among participants smoking status*

| | Smoking status | N | Mean Rank |
|---|---|---|---|
| 25 (OH)D | Smoker | 15 | 45.47 |
| | Used to be smoker | 4 | 41.25 |
| | Non-Smoker | 78 | 50.08 |
| | Total | 97 | |

The second question in this category was aimed at both current smokers and participants with a history of smoking, asking them to define their smoking quantity from four levels. Only 20 participants fitted this category. The mean rank of vitamin

D level are illustrated below (Table 5-14). However, any apparent difference was found to not be statistically significant through a Kruskal-Wallis test (P= 0.06). A box and whiskers plot of smoking habits and 25(OH)D is shown in figure 5-16.

*Table 5-14 Mean rank of 25(OH)D among smoking habits*

| | smoke habits | N | Mean Rank |
|---|---|---|---|
| 25 (OH)D | Heavy smoker | 5 | 9.40 |
| | Moderate smoker | 6 | 13.00 |
| | light smoker | 5 | 5.20 |
| | Rarely smoke | 4 | 14.75 |
| | Total | 20 | |

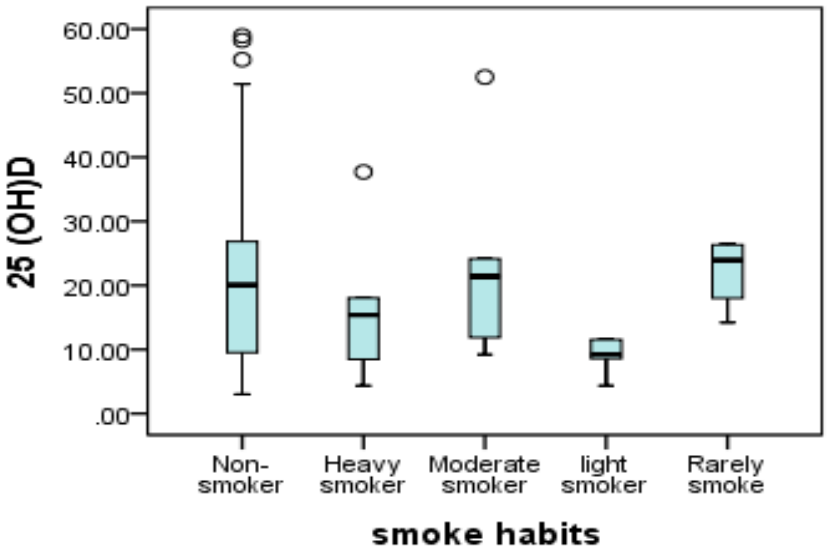


*Figure 5-14 A box and whiskers plot of smoking habits and 25(OH)D levels (ng/mL). Data shows medians, interquartile range, whiskers and outliers.*

The last question in the smoking section related to the type of smoking apparatus participants had used, with four possible levels. The mean rank of vitamin D level was 11.33 for Cigarettes smokers, 10.31 for Hookah smokers, 10.50 for Shisha smokers (none of the participants was using/had used electronic cigarettes) (Table 5-15). A box and whiskers plot of smoking types and 25(OH)D is illustrated in figure 5-15. A Kruskal-Wallis test showed no main effect of type of smoking implement on 25(OH)D level (P=0.964). There was no significant association between smoking and vitamin D level in the current study.

*Table 5-15 Mean rank of 25(OH)D among types of smoking*

| | Type smoke | N | Mean Rank |
|---|---|---|---|
| 25 (OH)D | Cigarettes | 3 | 11.33 |
| | Hookah | 13 | 10.31 |
| | Shisha | 4 | 10.50 |
| | Total | 20 | |

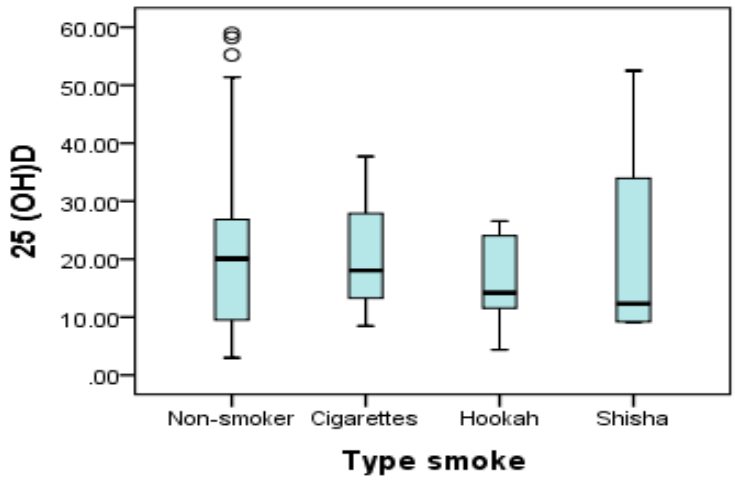


*Figure 5-15 A box and whiskers plot of type of smoking and 25(OH)D. Data shows medians, interquartile range, whiskers and outliers.*

## *5.4.7. Impact of Anthropometric measurements (BMI & WHR) on 25(OH)D*

Data on anthropometric measurements n=97 included two categories BMI and WHR. Calculation of BMI from height and weight is described in detail in section 5.2.7. For BMI values < 18.5 are underweight, 18.5-24.9 are healthy weight, 25-29.9 are overweight, and >30 kg/m$^2$ are obese (Table 5-3). The BMI of the entire group (97) was 31.93 ± 5.96kg/m$^2$ which falls within the obese range however, segregation of the participants according to gender shows that the female participants bias the data because their BMI is 32.38 ± 5.86kg/m$^2$ (table 5-5) which indicates obesity whilst the male participants, BMI 29.4 ± 6.01kg/m$^2$ are generally classed as overweight (Table 5-16). The overall minimum BMI value was 18.00 and the maximum was 49.1 kg/m$^2$.

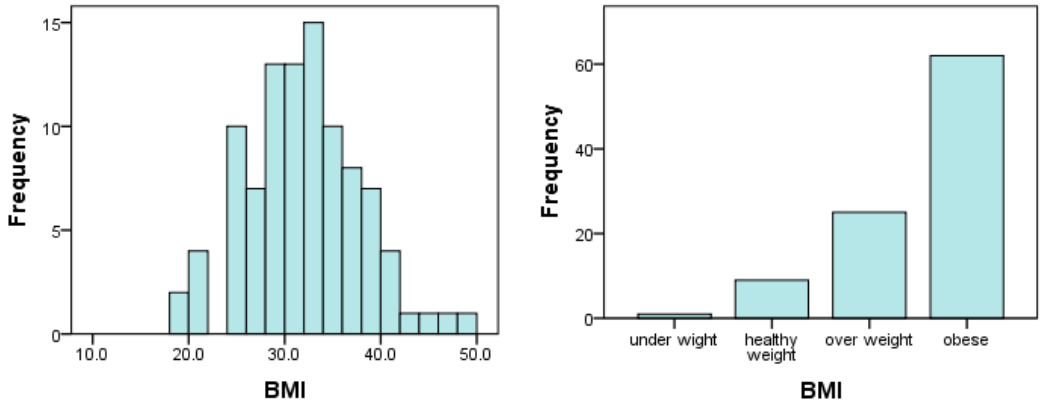


*Figure 5-16 Distribution* of BMI (kg/m$^2$) values and frequency under BMI categories among *the participants*

*Table 5-16 Descriptive analysis of BMI (kg/m$^2$) in the pooled sample and in sub-group*

| | N | Mean ± SD | Median (IQR) | Mean Rank |
|---|---|---|---|---|
| **CG** | 33 | 30.61 ± 6.96 | 30.63 (11.4) | 44.42 |
| **ARG** | 30 | 32.13 ± 4.61 | 31.57 (6.4) | 49.63 |
| **DAG** | 34 | 33.02 ± 5.87 | 32.02 (7.3) | 52.88 |
| **Male** | 15 | 29.42 ± 6.01 | 29.04 (4.9) | 34.67 |
| **Female** | 82 | 32.38 ± 5.86 | 32.84 (7.8) | 51.62 |
| **Total** | 97 | 31.92 ± 5.95 | 31.84 (7.6) | |

There was no difference in BMI between the study groups CG, ARG, and DAG when running a Kruskal-Wallis test (P=0.46) (Table 5-16). However, there was a significant difference between male and female in BMI when running a Mann-Whitney test (U=400, P=0.03) (Table 5-16). Box and whiskers plots of BMI among the clinical groups and gender are illustrated in figure 5-17 and 5-18. There was no spearman's rho correlation between BMI and 25(OH)D (P=0.47) (Table 5-18). Figure 5-19 represents the scatter diagrams of the lack of any relationship between 25(OH)D and BMI.

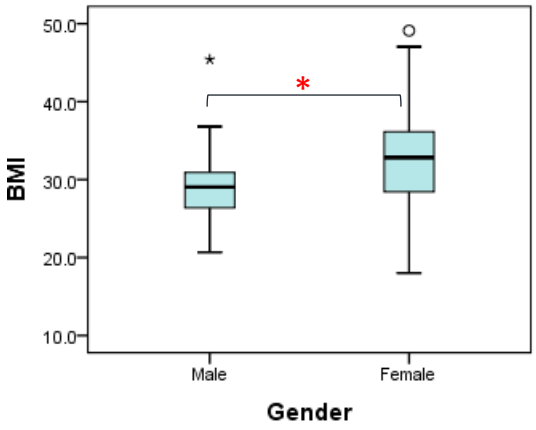


*Figure 5-17 A box and whiskers plot for BMI (kg/m$^2$) and Gender. Data shows medians, interquartile range, whiskers and outliers. * Indicates significant difference between groups (P<0.05).*

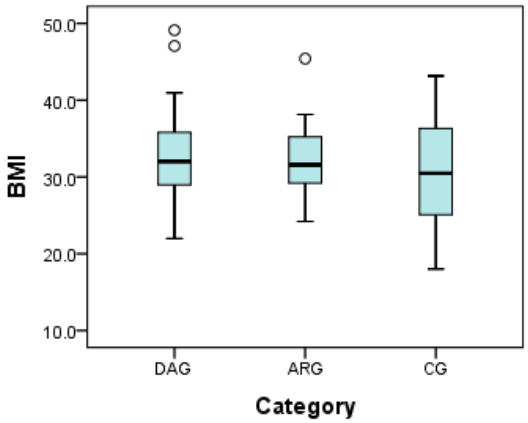


*Figure 5-18 A box and whiskers plot for BMI (kg/m$^2$) and study groups. Data shows medians, interquartile range, whiskers and outliers.*

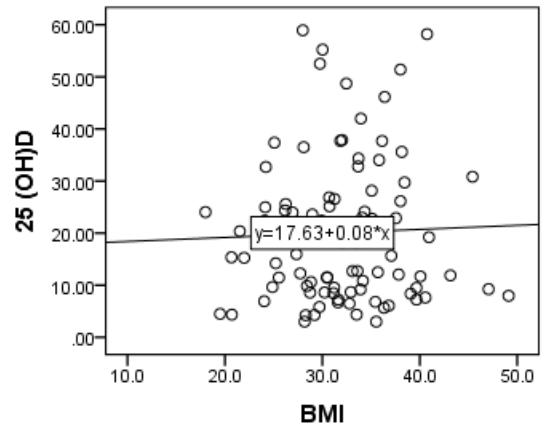


*Figure 5-19 X-Y Scatter graph of 25(OH)D (ng/mL) against BMI (kg/m$^2$).*

The second category of anthropometric measurements was WHR. Results were calculated from the measurement of waist and hip circumference as was explained in section 5.2.7. Results were considered to show normal body adipose if the WHR ≤0.90 for males and ≤ 0.80 for females, high adipose between 0.90-0.99 for males and 0.80-0.84 for females, morbidly adipose ≥1.00 for males and ≥0.85 for females (Table 5-4 and Figure 5-20). The mean WHR for males was 0.947 ± 0.069 cm, which indicates high body adipose levels. The female participants' mean WHR was 0.903 ± 0.112 cm, which is considered morbidly adipose (table 5-17). Mann-Whitney test revealed a significant difference between male and female in WHR (U=412, P=0.043) as shown in the box and whiskers plot in (Figure 5-21). Moreover, the Kruskal-Wallis

test indicates a significant difference in WHR between the clinical groups ($\chi^2$ (2) =19.38, P<0.0001). *Post-hoc* Mann-Whitney tests showed that the significant difference were between CG vs ARG (U=169, P<0.0001) and CG vs DAG (U=344, P=0.006) as shown in the box and whiskers plot in figure 5-22.

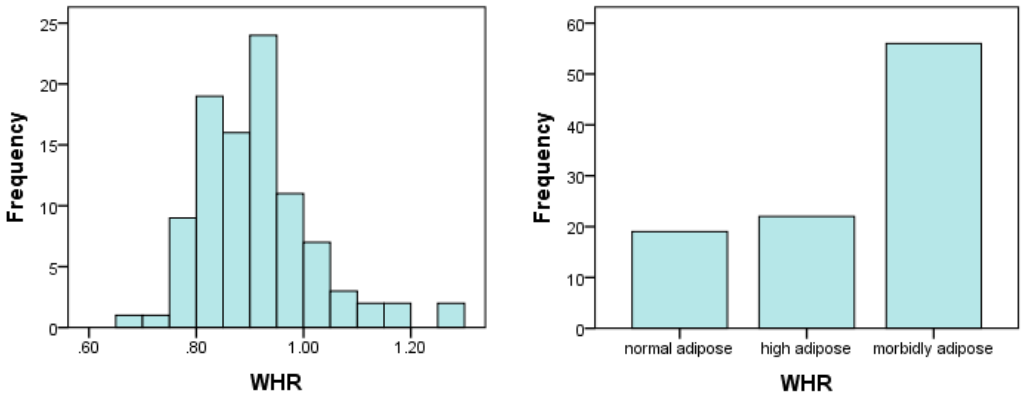


*Figure 5-20 Distribution of WHR values (cm) and frequency under WHR categories among the participants*

*Table 5-17 Descriptive analysis of WHR (cm) in the pooled sample and in sub-group*

| | N | Mean ± SD | Median (IQR) | Mean Rank |
|---|---|---|---|---|
| **CG** | 33 | 0.852 ± 0.085 | 0.849 (0.12) | 32.55 |
| **ARG** | 30 | 0.951 ± 0.083 | 0.937 (0.08) | 63.15 |
| **DAG** | 34 | 0.939 ± 0.150 | 0.922 (0.15) | 52.49 |
| **Male** | 15 | 0.947 ± 0.069 | 0.925 (0.11) | 62.53 |
| **Female** | 82 | 0.907 ± 0.126 | 0.892 (0.13) | 46.52 |
| **Total** | 97 | 0.913 ± 0.119 | 0.906 (0.12) | |

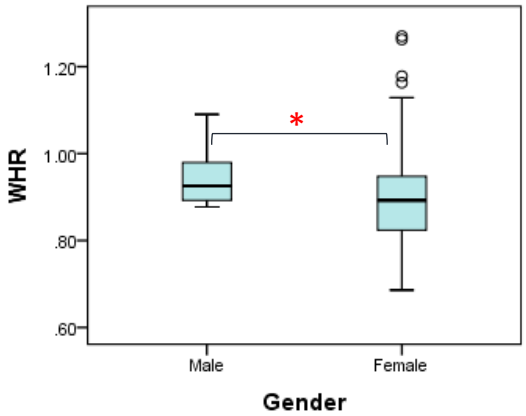


*Figure 5-21 A box and whiskers plot for WHR and Gender. Data shows medians, interquartile range, whiskers and outliers. * Indicates significant between group differences (P<0.05).*

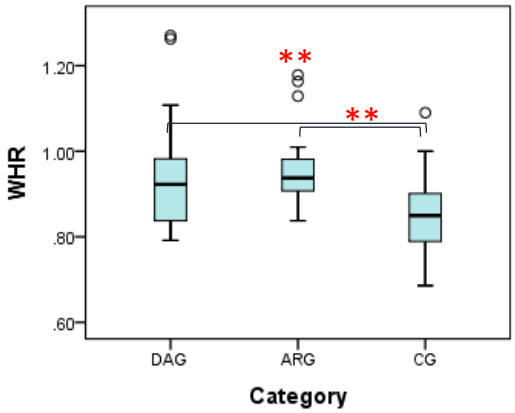


*Figure 5-22 A box and whiskers plot for WHR and study groups. Data shows medians, interquartile range, whiskers and outliers. ** Indicates significant between group differences (P<0.01).*

A spearman's rho correlation indicate no significant relation between WHR and 25(OH)D. A simple scatter plot between WHR and 25(OH)D illustrates the lack of relationship between the two variables in the pooled sample (Figure 5-23) For additional investigation, the correlation between WHR and age was tested and found to be significant (rho=0.29, P=0.002) table 5-18.

*Table 5-18 Spearman's correlations between 25(OH)D (ng/mL), BMI (kg/m$^2$), and WHR (cm)*

| | | | 25 (OH)D | BMI | WHR |
|---|---|---|---|---|---|
| Spearman 's rho | 25 (OH)D | Correlation Coefficient | 1.000 | 0.017 | 0.134 |
| | | Sig. (1-tailed) | . | 0.434 | 0.095 |
| | | N | 97 | 97 | 97 |
| | BMI | Correlation Coefficient | | 1.000 | -0.026 |
| | | Sig. (1-tailed) | | . | 0.402 |
| | | N | | 97 | 97 |
| | WHR | Correlation Coefficient | | | 1.000 |
| | | Sig. (1-tailed) | | | . |
| | | N | | | 97 |

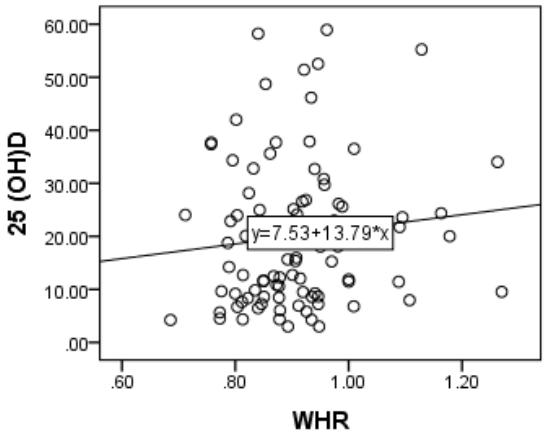


*Figure 5-23 X-Y Scatter graph of 25(OH)D (ng/mL) and WHR (mm).*

## *5.4.8 The chapter correlation and regression model*

Serum 25(OH)D concentration was only significantly correlated with age out of all of the potential modulating life style factors (table 5-20). The strength of the correlation, $R^2$, was 0.08, which is considered weak. Table 5-20 represent all of the chapter non-parametric correlations between all the chapter variables.

To create a composite model predictor of serum 25(OH)D levels, a multiple linear regression model was performed. The stable model in chapter 4 of the current study incorporated the use of vitamin D supplements and dietary intake of vitamin D (DI-VitD). Since age was the only modulator that was significantly correlated to 25(OH)D in this chapter, a model was designed which included those three modulators (Table 5-19). The model was statistically significant ($P<0.0001$) and explains 40.1% of the variance of 25(OH)D concentrations. The model was statistically powerful due to the low shrinkage of R (Adjusted $R^2= 0.382$). However, in further analysis the model was unstable because age was a non-significant contributor (age $P=0.389$). Age will therefore not be added to the final regression model in chapter 8 of the study.

*Table 5-19 Unstable model predictor of serum 25(OH)D*

| Coefficients[a] | | | | | | |
|---|---|---|---|---|---|---|
| Model | | Unstandardized Coefficients | | Standardized Coefficients | t | Sig. |
| | | B | Std. Error | Beta | | |
| 1 | (Constant) | 47.732 | 8.999 | | 5.304 | 0.000 |
| | Take vitamin D supplements | -16.928 | 2.443 | -0.585 | -6.930 | 0.000 |
| | Dietary vitamin D intake IU | -0.020 | 0.008 | -0.193 | -2.406 | 0.018 |
| | age | 0.117 | 0.136 | 0.073 | 0.865 | 0.389 |
| a. Dependent Variable: 25 (OH)D | | | | | | |

*Table 5-20 Spearman's correlation and analysis of the chapter measurements*

| | | | 25 (OH)D | Gender | age | education level | Mean Income | family members | Income / family size | BMI | WHR |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Spearman's rho | 25 (OH)D | Correlation Coefficient | 1.000 | 0.106 | 0.291** | -0.031 | 0.016 | -0.146 | 0.062 | 0.017 | 0.134 |
| | | Sig. (1-tailed) | . | 0.151 | 0.002 | 0.382 | 0.439 | 0.076 | 0.275 | 0.434 | 0.095 |
| | | N | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 97 |
| | Gender | Correlation Coefficient | | 1.000 | 0.008 | -0.101 | -0.014 | -0.144 | 0.050 | 0.219* | -0.207* |
| | | Sig. (1-tailed) | | . | 0.470 | 0.162 | 0.447 | 0.080 | 0.315 | 0.016 | 0.021 |
| | | N | | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 97 |
| | age | Correlation Coefficient | | | 1.000 | -0.296** | -0.175* | -0.209* | -0.092 | -0.010 | 0.295** |
| | | Sig. (1-tailed) | | | . | 0.002 | 0.044 | 0.020 | 0.184 | 0.462 | 0.002 |
| | | N | | | 97 | 97 | 97 | 97 | 97 | 97 | 97 |
| | education level | Correlation Coefficient | | | | 1.000 | 0.622** | 0.205* | 0.474** | -0.106 | -0.124 |
| | | Sig. (1-tailed) | | | | . | 0.000 | 0.022 | 0.000 | 0.150 | 0.114 |
| | | N | | | | 97 | 97 | 97 | 97 | 97 | 97 |
| | Mean Income | Correlation Coefficient | | | | | 1.000 | 0.240** | 0.814** | -0.110 | -0.160 |
| | | Sig. (1-tailed) | | | | | . | 0.009 | 0.000 | 0.141 | 0.059 |
| | | N | | | | | 97 | 97 | 97 | 97 | 97 |
| | number of family | Correlation Coefficient | | | | | | 1.000 | -0.321** | -0.215* | -0.074 |
| | | Sig. (1-tailed) | | | | | | . | 0.001 | 0.017 | 0.236 |
| | | N | | | | | | 97 | 97 | 97 | 97 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Normalised income | Correlation Coefficient | | | | | | | 1.000 | -0.013 | -0.074 |
| | | Sig. (1-tailed) | | | | | | | . | 0.451 | 0.235 |
| | | N | | | | | | | 97 | 97 | 97 |
| | BMI | Correlation Coefficient | | | | | | | | 1.000 | -0.026 |
| | | Sig. (1-tailed) | | | | | | | | . | 0.402 |
| | | N | | | | | | | | 97 | 97 |
| | WHR | Correlation Coefficient | | | | | | | | | 1.000 |
| | | Sig. (1-tailed) | | | | | | | | | . |
| | | N | | | | | | | | | 97 |

*. Correlation is significant at the 0.05 level (1-tailed).
**. Correlation is significant at the 0.01 level (1-tailed).

## 5.5. Discussion

This chapter is a general descriptive chapter of the lifestyle factors that could have an effect on vitamin D and atherosclerosis status. The factors proposed initially included gender, age, education level, occupation, socioeconomic status, smoking, BMI, and WHR.

The majority of the participants in the current study were female n=82 compared to 15 males. The initial target was to have the same number of participants of each sex however, males were not as cooperative in participating in the study as females. It was observed that men were not comfortable to participate in the study because the researcher conducting the data collection was female. Additionally, the majority of the male participants were invited through the first recruitment approach, which was during their routine visit to the cardiovascular clinic at the hospital. Few were approached as they were local hospital staff and/or relatives who had attended the hospital with patients as companions. None of the male participants who participated in the study were recruited through the third recruitment approach, which was a text message sent to hospital patients, asking them to participate if they fitted the criteria. There was no difference between the sexes in 25(OH)D concentration. Studies have suggested that there is no physical difference between sexes in synthesising vitamin D (Giovannucci, 2009; Holick et al., 2011).

One of the main factors that is commonly associated with vitamin D status is age (Holick et al., 2011). In the current study, there was a positive correlation between age and 25(OH)D concentrations (rho=0.291, P=0.002), which conflicts with what has been proposed in the literature (Arain et al., 2015). This correlation was no longer apparent when only the participants who were not taking vitamin D supplements were considered (P=0.055). Participants who were taking vitamin D supplements (52.07 ± 7.67 years) tended to be significantly older than those who were not taking them (46.49 ± 8.14 years) (U=618, P<0.01). Additionally, those participant were mainly from the ARG and DAG who were mostly recommended to take vitamin D supplements by their physician, which make them tend to have higher values of 25(OH)D. The reverse association between age and 25(OH)D levels when vitamin D

supplements were consumed was also observed in previous studies which lends to further support to the current study's findings (Lertratanakul et al., 2014; Degerud, 2016).

The participants were from all educational backgrounds, economic status, and doing different jobs. All of these potential covariates showed no effect on circulating 25(OH)D concentrations in the current study. Additionally, smoking habits were examined as a status, frequency of smoking, and the method of smoking used. None of these showed a relationship with 25(OH)D concentrations ether. Previous studies have had similar observations whereby there was no association between these lifestyle factors and vitamin D status (Ardawi et al., 2011; Shirazi et al., 2013), opposed to other studies that did observe such associations (Azhar, 2009; Kilkkinen et al., 2009). In the study by Kilkkinen et al. (2009) high concentrations of 25(OH)D were associated with older, and highly educated participants, whereas heavy smoking was associated with low concentrations of 25(OH)D. The associations were obvious with vitamin D status probably because of the large number of participants (n=6219) which gave greater accuracy to their results. Moreover, the study by Azhar observed associations only between type of job and smoking status with 25(OH)D levels. However, the study also had slightly larger number of participants (n=118) than the current study and they were all healthy participants. The health condition of participants definitely had an impact on the results, which could be one of the explanations for the absence of an association between these factors and 25(OH)D concentration in the current study.

Anthropometric measurements indicated that the majority of the participants (63.9%) were obese (25.8%) were overweight (8.1%) had healthy weight and the rest were under weight. According to the BMI measurements. Similarly, the WHR indicated that (57.7%) of the participants were morbidly adipose and (22.7%) were high adipose and the rest were normal adipose. These data highlight the huge issue of obesity among the Saudi dwellers as addressed in the literature review (Ardawi et al., 2012; Aljefree et al., 2016). In the current study, significant correlations between anthropometric measurements and 25(OH)D concentration were not observed. Also,

worryingly, obesity and vitamin D deficiency were both apparent in the study. Vitamin D tends to be deposited in the adipose tissues in the body which decreases the bioavailability of the vitamin (Holick and Chen, 2008; Ardawi et al., 2011).

Anthropometric measurements showed significant differences between sexes and clinical groups. Female participants had significantly higher BMI than males ($P<0.05$). Conversely, females had a significantly lower WHR than males ($P<0.05$) which indicates less abdominal obesity in the female than in the males. On the other hand, the WHR variation was obvious between the clinical groups ($P<0.0001$). The control group (CG) had the lowest WHR, then the diagnosed with atherosclerosis group (DAG) ($U=344$, $P<0.01$), and then the at-risk group (ARG) ($U=169$, $P<0.0001$). This indicates an association between abdominal obesity and the incidence and development of atherosclerosis.

### *5.5.1. Multiple linear regression model*

The multiple linear regression model suggested that age is a non-significant contributor ($P=0.389$) when added to the stable model from chapter 4. Therefore, the stable model from chapter 4 that includes the usage of vitamin D supplements and dietary intake of vitamin D, is the stable model, so far, for predicting 25(OH)D serum concentration.

## 5.6. Summary and conclusion

None of the lifestyle factors examined in the current study was associated with circulating 25(OH)D concentration. Only age was, in fact, positively associated with 25(OH)D concentration. However, this effect was a result of increased vitamin D supplement use with greater age. Obesity was prevalent in the current study sample of Saudi dwellers and was associated with the participants' clinical conditions. In conclusion, the current study suggests that gender, education level, occupation, socioeconomic status, smoking, BMI, and WHR are not indicators of vitamin D status. In addition, age also was not a good indicator of vitamin D status after accounting for vitamin D supplement usage.

# Chapter 6 - Association between circulating 25(OH)D levels and other blood markers of health

## 6.1. Introduction

A review of the literature indicates a potential relationship between vitamin D status, atherosclerosis risk factors, such as having hyperglycaemia and/or hyperlipidaemia, and inflammatory markers such as high concentrations of C-reactive protein (CRP) (Menezes et al., 2014; Faridi et al., 2017). Within this chapter, an investigation of these associations is presented. Experimental work includes the measurement and analysis of markers such as C-reactive protein (CRP), fasting blood glucose (FBG), total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL), and triglycerides (TG) as indicators of the markers discussed above. The relationships between these markers were investigated in the pooled study sample, and in each of the clinical groups (control group (CG), at-risk group (ARG), and participants diagnosed with atherosclerosis group (DAG)). The consumption of vitamin D supplements was considered when investigating the relationships. Other factors such as gender, consumption of related medications, and diseases were also taken into account in the analysis.

### *6.1.1. Chapter aim*

The chapter aims to investigate the association between vitamin D status and the biochemical parameters.

### *6.1.2. Chapter objectives:*

1- To assess each of the biochemical parameters (CRP, FBG, and lipids) in the pooled sample and in each of the study sub-groups.
2- To investigate any association between the parameters monitored and the concentration of 25(OH)D in the pooled sample and in each of the study sub-groups, considering general factors influencing the parameters.
3- To elucidate any observable relationship between the monitored parameters.

## 6.2. Methods

### *6.2.1. Measurement of high sensitivity C-reactive protein (CRP)*

CRP is a protein produced by the liver. It is present in human serum, and increases after most forms of tissue injury, bacterial and viral infections, inflammation and malignant neoplasia (van Wissen et al., 2002). In the current study, measurement of CRP in serum was carried out using an *in vitro* diagnostic reagent (Siemens BN II / BN ProSpect® system, Marburg, Germany) (Siemens, 2014). Polystyrene particles coated with monoclonal antibodies (goat IgG) specific to human CRP are aggregated when mixed with samples containing CRP. These aggregates scatter a beam of light passed through the sample. The intensity of the scattered light is proportional to the concentration of the relevant protein in the sample. The result is evaluated by comparison with a standard of known concentration (CV%= 3.1 to 5.8) (van Wissen et al., 2002; Siemens, 2014). Normal concentrations of CRP for adults are presented in table 6-1 (Ministry of Health Portal. 2016).

*Table 6-1 Normal ranges of blood parameters concentrations (Ministry of Health Portal. 2016)*

| **Test** | **Normal Range** | **unit** |
|---|---|---|
| High-sensitivity C-Reactive Protein (CRP) | 0.0001 - 0.3 | mg/dL |
| Glucose (FBG) | 70 -115 | mg/dL |
| **Lipid Profile** | | |
| Cholesterol, Total (TC) | 50 -200 | mg/dL |
| Triglycerides (TG) | 30 -200 | mg/dL |
| HDL Cholesterol (HDL) | 35 - 55 | mg/dL |
| LDL Cholesterol (LDL) | 0.01< -150 | mg/dL |

### *6.2.2. Measurement of fasting blood glucose (FBG)*

Fasting blood glucose concentration was determined using the hexokinase enzymatic reference method using a Roche Cobas c 501 analyser (Indianapolis, IN, USA) (Roche, 2014b). Using 2µl of the participants' serum sample, the hexokinase catalyses the

phosphorylation of glucose to glucose-6-phosphate by ATP. Then, glucose-6-phosphate dehydrogenase oxidises glucose-6-phosphate in the presence of NADP to gluconate-6-phosphate. No other carbohydrate is oxidised. The rate of NADPH formation during the reaction is directly proportional to the glucose concentration and is measured by an automatic fluorescence analyser (CV%= 0.7 to 1.2) (Roche, 2014b). The normal FBG concentrations for adults are shown in table 6-1(Ministry of Health Portal. 2016).

## *6.2.3. Lipid profile*

Enzymatic methods were used to measure lipids including high-density lipoprotein (HDL-C), low-density lipoprotein (LDL-C), triglycerides (TG), and total cholesterol (TC). Optimal concentrations of lipids in adults are shown in table 6-1 (Ministry of Health Portal. 2016).

### *6.2.3.1. Measurement of HDL and LDL cholesterol*

HDL and LDL cholesterol were measured using the Roche enzymatic colorimetric assay system (Roche c 501, Indianapolis, IN, USA). The concentrations of HDL-C and LDL-C were determined enzymatically using cholesterol esterase and cholesterol oxidase. Cholesterol esters are hydrolysed into free cholesterol and fatty acids by cholesterol esterase. In the presence of oxygen, cholesterol is oxidised by cholesterol oxidase to 4-cholestenone and hydrogen peroxide. In the presence of peroxidase, the generated hydrogen peroxide reacts with 4-amino-antipyrine and HSDA to form a purple-blue product. The colour intensity is directly proportional to the cholesterol concentration and was measured photometrically using the Cobas analyser e501. A volume of 2.5µl of serum was used to measure HDL-C and 2µl of serum for LDL-C. The control intervals for HDL-C were set as 23.7-32.5 mg/dL for the lower limit and 53.2-73.6 mg/dL for the upper limit using PreciControl ClinChem Multi 1 and 2 (CV%= 0.4 to 1.0). The control intervals for LDL-C were set as 44.6-61.4 mg/dL for the lower limit and 80.3-110.7 mg/dL for the upper limit, using PreciControl Multi 1 and 2 respectively CV%= 0.9 to 2.1) (Roche, 2013a; Roche, 2014a)

#### *6.2.3.2. Measurement of total cholesterol (TC)*

A Roche assay was used to measure TC (Roche c 501, Indianapolis, IN, USA). Cholesterol esters are cleaved by the action of cholesterol esterase to yield free cholesterol and fatty acids following the same method that was used when measuring HDL and LDL cholesterol. The colour intensity is directly proportional to the TC concentration. TC was determined by measuring the increase in absorbance using the Cobas e501 analyser. A volume of 2µl of serum was used and the control intervals set as 74.1-90.5 mg/dL for the lower limit and 155-191 mg/dL for the upper limit using PreciControl ClinChem Multi 1 and 2 (CV%= 0.8 to 1.9) (Roche, 2013b).

#### *6.2.3.3. Measurement of triglycerides (TG)*

TG were measured using a Roche assay (Roche c 501, Indianapolis, IN, USA) that uses a lipoprotein lipase for the rapid and complete hydrolysis of triglycerides to glycerol followed by oxidation to dihydroxyacetone phosphate and hydrogen peroxide. The hydrogen peroxide produced then reacts with 4-aminophenazone and 4-chlorophenol under the catalytic action of peroxidase to form a red compound. The colour intensity of the red dye formed is directly proportional to the triglyceride concentration and can be measured automatically by the analyser. A sample size of 2 µl was used and the control intervals set as 93-113 mg/dL for the lower limit and 184-224 mg/dL for the upper limit using PreciControl ClinChem Multi 1 and 2 (CV%= 1.6 to 1.9) (Roche, 2015b).

### *6.2.4. Measurement of serum 25 (OH) D*

Measurement of 25(OH)D was accomplished using an electrochemiluminescence binding assay. Details of the measurements are provided in section 4.3.1. of the current study.

## 6.3. Statistical analysis of the data

Statistical analysis was performed using SPSS v24 (Inc., Chicago, IL, USA). Data were tested for normal distribution using the Kolgomorov-Smirnov test. In addition, homogeneity of variance was assessed using the Levene's test. Descriptive analysis are presented as mean ± standard deviation, median (interquartile range), and mean rank. In the current chapter, none of the outcome measures was normally distributed. As such, the data was deemed suitable for analysis using non-parametric tests. Therefore, categorical data were analysed using a Kruskal-Wallis test and/or a Mann-Whitney U test (as appropriate for the number of comparisons) to examine the mean differences between the groups. Thus, the Kruskal-Wallis test with appropriate post-hoc Mann-Whitney tests, were carried out to test the data for mean differences between more than two groups. The Mann-Whitney test was performed for simple pairwise comparisons. Box and whiskers plots were used to illustrate medians, interquartile range, whiskers, outliers, and indicate significant between group differences.

On the other hand, where data was continuous rather than grouped, a Spearman rho correlation analysis was carried out to examine associations. Furthermore, multinomial logistic regression was applied to further describe a comprehensive model predictor of 25(OH)D using the specified blood markers when significant association was observed. Throughout, statistical significance was set at $p \leq 0.05$.

## 6.4. Results

In this section blood markers of health including:- C-reactive protein (CRP) , fasting blood glucose (FBG), total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL), and triglycerides (TG) are explored. The overall study sample size was 97. As in the previous chapters, data were analysed as a pooled sample and as sub-groups. The first sub-group was, the clinical groups (control group (CG), at risk group (ARG), and diagnosed with atherosclerosis group (DAG)). The second sub-group was gender (male, female). The third sub-group was the consumption of vitamin D supplements groups (consume vitamin D supplements, do not consume vitamin D supplements). Some blood parameters can be directly affected by other factors, such as the use of hypoglycaemic agents or statins. Data is presented based on the usage of those factors . Table 6-2 shows statistical mean and standard deviation of the study blood markers within each of the clinical groups and the pooled study sample.

*Table 6-2 Descriptive statistic of the chapter parameters in each of the clinical groups and in the pooled sample. Data were presented as mean ± standard deviation. Parameters are 25-hydroxi vitamin D (24(OH)D), C-reactive protein (CRP) , fasting blood glucose (FBG), total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL), and triglycerides (TG)*

| Biochemical parameters | CG N=33 | ARG N=30 | DAG N=34 | Total N=97 |
|---|---|---|---|---|
| **25(OH)D (ng/mL)** | 18.50 ± 10.31 | 23.91 ± 14.85 | 18.26 ± 14.57 | 20.09 ± 13.44 |
| **CRP (mg/dL)** | 0.479 ± 0.804 | 0.837 ± 0.804 | 0.626 ± 1.520 | 0.641 ± 1.052 |
| **FBG (mg/dL)** | 89.73 ± 9.139 | 177.60 ± 68.93 | 133.71 ± 51.73 | 132.11 ± 60.25 |
| **TC (mg/dL)** | 196.76 ± 31.66 | 183.27 ± 42.60 | 182.88 ± 44.01 | 187.72 ± 39.88 |
| **HDL (mg/dL)** | 48.00 ± 12.80 | 44.23 ± 11.83 | 48.35 ± 16.87 | 46.71 ±13.06 |
| **LDL (mg/dL)** | 129.48 ± 27.85 | 109.70 ±38.44 | 107.35 ± 35.52 | 115.60 ± 35.21 |
| **TG (mg/dL)** | 108.70 ± 62.83 | 182.37 ± 114.02 | 161.03 ± 88.81 | 149.82 ± 94.40 |

## *6.4.1. Analysis of C-reactive protein (CRP)*

### *6.4.1.1. Descriptive analysis of CRP in the pooled sample and between sub-groups*

Measurement of CRP provided continuous data for the 97 participants. The minimum value was 0.0001 mg/dL and the maximum value was 8.55 mg/dL. 41.2% of the participants had normal concentrations of CRP (mean ± SD) (0.007 ± 0.047 mg/dL), whereas the rest 58.8% had high concentrations (1.086 ± 1.186 mg/dL). Normal values of CRP are ≥0.0001 and ≤0.3 mg/dL (table 6-1). Table 6-3 illustrates descriptive analysis of CRP based on the clinical groups and on gender. There were three outlier values above 2.00 mg/dL (i.e. 2.87, 4.00, 8.55 mg/dl). Table 6-4 shows CRP values without outliers n= 94. Figure 6-1 shows the distribution of the sample without CRP outliers. The results were reviewed for validation. None of the participants who had outliers' values in CRP had outliers in the rest of the blood markers. This appear to indicate that these outliers are simply related to the CRP data and have no common feature.

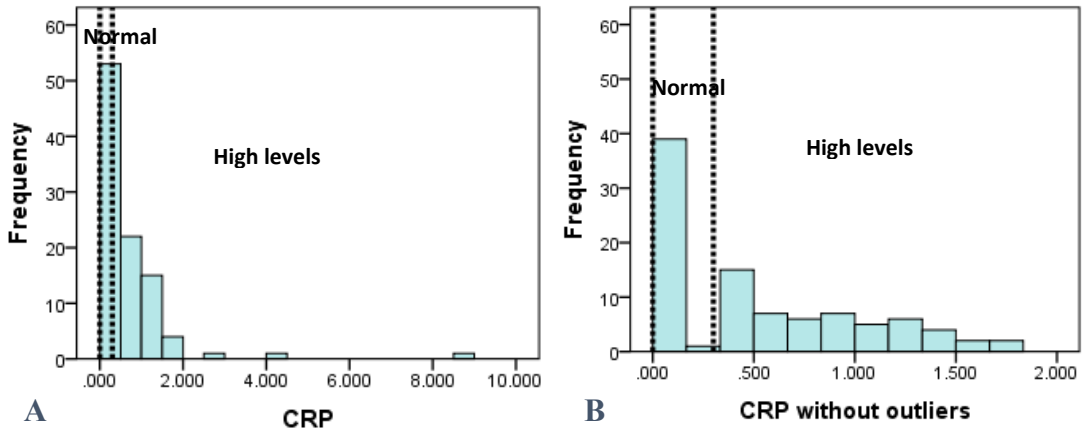


*Figure 6-1* Histograms of CRP (mg/dL) frequent among the whole sample and in clinical groups. A figure include the whole sample and B without the outliers. Normal range is shown between the dotted lines.

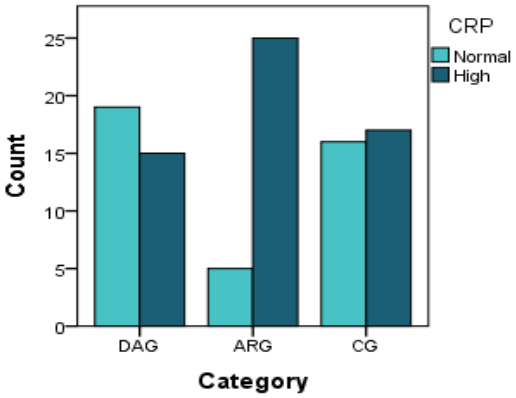


*Figure 6-2 Distribution of CRP concentration among the study clinical groups*

*Table 6-3 Descriptive statistic for CRP (mg/dL) among the pooled sample, clinical groups and gender*

| | N | Mean ± SD | Median (IQR) | Mean Rank |
|---|---|---|---|---|
| **CG** | 33 | 0.479 ± 0.533 | 0.389 (0.99) | 41.75 |
| **ARG** | 30 | 0.837 ± 0.804 | 0.542 (0.97) | 60.27 |
| **DAG** | 34 | 0.626 ± 1.52 | 0.000 (0.73) | 41.75 |
| **Male** | 15 | 0.444 ± 0.518 | 0.356 (0.96) | 44.73 |
| **Female** | 82 | 0.677 ± 1.121 | 0.423 (1.003) | 49.78 |
| **Total** | 97 | 0.641 ± 1.052 | 0.418 (0.97) | |

*Table 6-4 Descriptive statistic for CRP without outliers (mg/dl) among the pooled sample, clinical groups and gender*

| | N | Mean ± SD | Median (IQR) | Mean Rank |
|---|---|---|---|---|
| **CG** | 33 | 0.47 ± 0.53 | 0.38 (0.99) | 46.23 |
| **ARG** | 29 | 0.73 ± 0.52 | 0.51 (0.89) | 59.03 |
| **DAG** | 32 | 0.31 ± 0.41 | 0.00 (0.69) | 38.36 |
| **Male** | 15 | 0.44 ± 0.52 | 0.356 (0.96) | 44.73 |
| **Female** | 79 | 0.51 ± 0.53 | 0.41 (0.94) | 48.03 |
| **Total** | 94 | 0.49 ± 0.52 | 0.41 (0.94) | |

### *6.4.1.2. CRP concentration and the differences between sub-groups*

To investigate the differences between the sub-groups in their CRP concentrations a series of statistical tests were performed. A Kruskal-Wallis test indicates a significant difference in CRP among the clinical groups ($\chi^2$ (2) =7.89, P=0.019)). A post Hoc analysis revealed this main effect to be due to the differences between CG vs ARG (U=346, P=0.037) and, ARG vs DAG (U=321, P=0.009). Even after removing the three apparent outliers ($\chi^2$ (2) =9.53, P=0.009) the significant difference remained between CG vs ARG (U=346, P=0.057), and ARG vs DAG (U=262, P=0.003). Figures 6-3 A and B shows the box and whiskers plot of CRP among the clinical groups, which revealed significant difference in the total sample and without the outliers. Additionally, to determine if there was a difference between the sexes in CRP status a Mann-Whitney test was performed and revealed no significant difference (P=0.509) even when after removing the outliers (P=0.657).

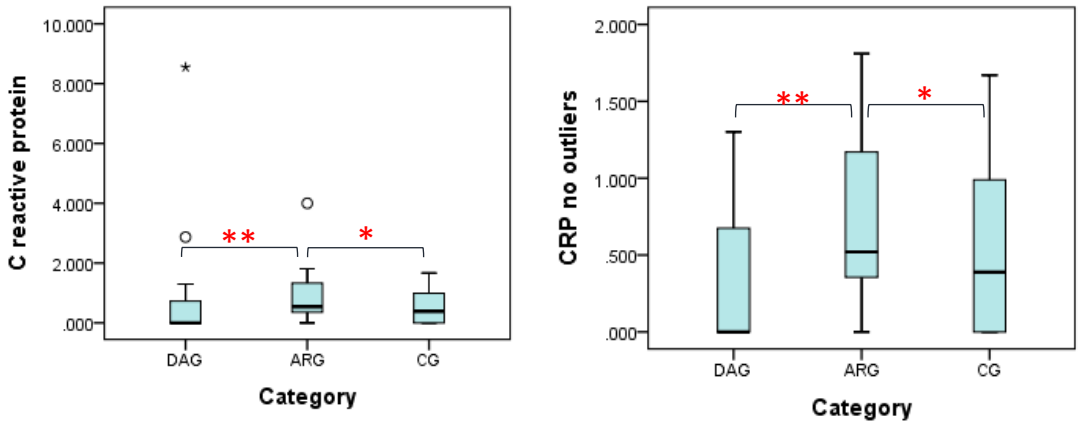


*Figure 6-3 A box and whiskers plot of CRP (mg/dL) among the clinical groups. Data shows medians, interquartile range, whiskers and outliers. A figure include the whole sample and B without the outliers Significant differences between groups indicated by (* = P<0.05) and (** = P<0.01).*

### *6.4.1.3. Correlations between CRP and 25(OH)D in the pooled sample and in sub-groups*

To examine the continuous relationship between CRP and the blood markers concentrations Spearman's correlation tests were performed. There was no significant correlation between CRP and 25(OH)D (P=0.388) nor when outliers were removed (P= 0.458) (table 6-5). Scatter plots illustrate these relationships (or lack thereof) in figure 6-4 A and B, in the total sample and without the outliers. However, there was a statistically significant correlation between 25(OH)D and CRP in the ARG

in the vitamin D supplements users only (rho=-0.666, P=0.009) (table 6-20). The remaining blood markers exhibited, there were statistically significant Spearman correlations between CRP and FBG (rho=0.046, P=0.046), and between CRP and TG concentration (rho=0.026, P=0.026) (table 6-19).

*Table 6-5 Spearman's correlation between 25(OH)D ng/mL and CRP mg/dl with and without outliers*

| | | | 25 (OH)D |
|---|---|---|---|
| Spearman's rho | 25 (OH)D | Correlation Coefficient | 1.000 |
| | | Sig. (1-tailed) | . |
| | | N | 97 |
| | C reactive protein | Correlation Coefficient | 0.029 |
| | | Sig. (1-tailed) | 0.388 |
| | | N | 97 |
| | CRP without outliers | Correlation Coefficient | 0.011 |
| | | Sig. (1-tailed) | 0.458 |
| | | N | 94 |

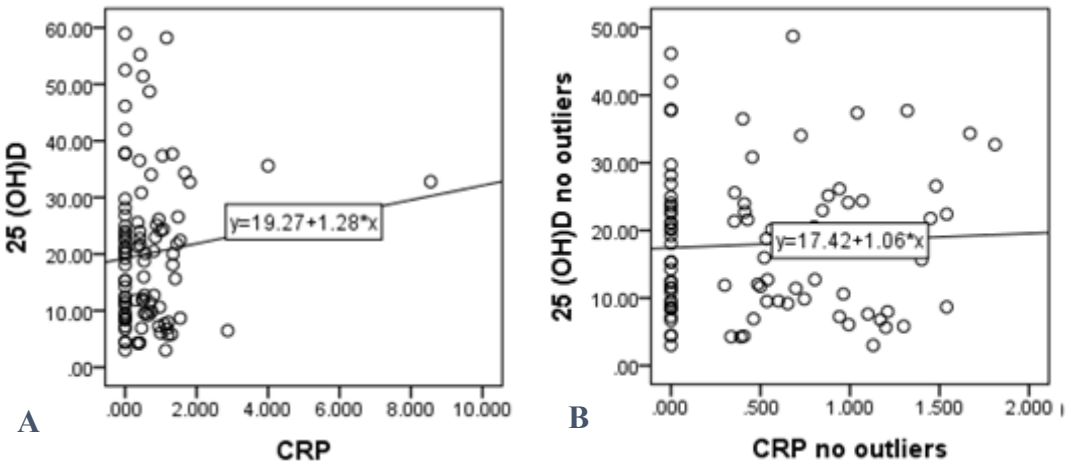


*Figure 6-4 X-Y Scatter graph of 25(OH)D (ng/mL) and CRP (mg/dL). A represent the total sample and B without outliers.*

## *6.4.2. Analysis of fasting blood glucose (FBG)*

### *6.4.2.1. Descriptive analysis of FBG in the pooled sample and between sub-groups*

Fasting blood glucose was measured in all 97 participants. The minimum concentration was 60 mg/dL and the maximum was 350 mg/dL. Normal

concentrations of FBG are between 70-115 mg/dL (table 6-1). The majority of the participants (55.7%) had normal FBG levels (mean ± SD) (93.70 ± 8.5), 43.3% high FBG levels (184.5 ± 63.5), and only 1% had low FBG levels (60 ± 0). Figure 6-5 illustrates the FBG concentrations among the clinical groups. Descriptive statistic of FBG concentrations in sub-groups among the participants under treatments (hypoglycaemic agent) are shown in table 6-6. In addition, tables 6-7 and 6-8 shows descriptive statistics and FBG concentration among the study clinical group and diabetic participants.

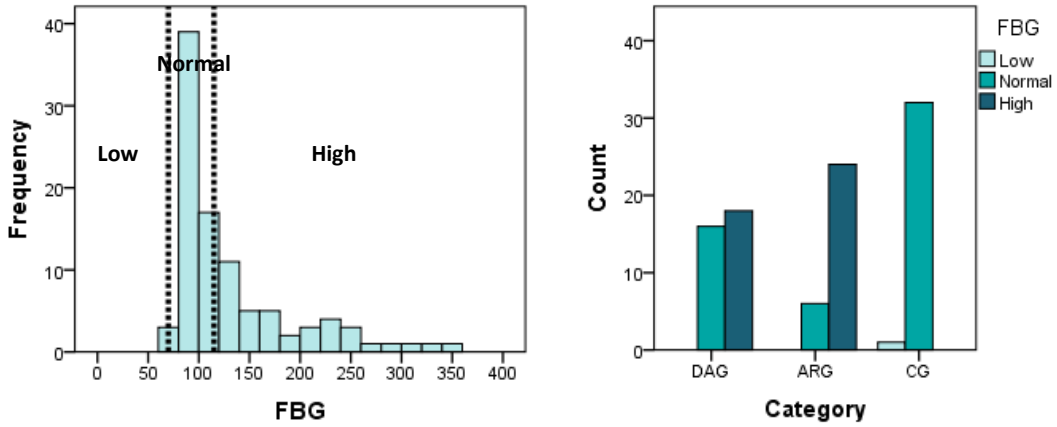


*Figure 6-5 Histograms of FBG concentrations (mg/dL) in the whole sample and among the clinical groups.* The dotted line shows the cut off values for FBG.

*Table 6-6 Descriptive statistic and mean ranks of FBG levels (mg/dL) among the clinical groups and sex considering the participants under medication*

| | Take hypoglycaemic agent | N | Mean ± SD | Median (IQR) | Mean Rank |
|---|---|---|---|---|---|
| **CG** | Yes | 0 | 0 | 0 | 0 |
| | No | 33 | 89.73 ± 9.139 | 90 (12) | 21.14 |
| | Total | **33** | **89.73 ± 9.139** | **90 (12)** | 22.29 |
| **ARG** | Yes | 26 | 189.88 ± 64.83 | 172.50 (115) | 22.69 |
| | No | 4 | 92.75 ± 2.87 | 91.50 (5) | 24.63 |
| | Total | 30 | 176.93 ± 68.93 | 156.0 (119) | 70.43 |
| **DAG** | Yes | 14 | 157.79 ± 63.15 | 136 (87) | 16.43 |
| | No | 20 | 116.85 ± 34.54 | 103 (30) | 42.85 |
| | Total | 34 | 133.71 ± 51.73 | 118.50 (49) | 22.29 |
| **Male** | Yes | 4 | 180 ± 90.35 | 166.50 (170) | 18.25 |
| | No | 11 | 110.27 ± 41.99 | 97 (15) | 36.68 |
| | Total | 15 | 128.93 ± 63.52 | 101.00 (35) | 47.03 |
| **Female** | Yes | 36 | 179.97 ± 67.12 | 158 (96) | 20.75 |
| | No | 46 | 96.87 ± 18.55 | 93 (12) | 27.64 |
| | Total | 82 | 132.70 ± 60.01 | 105.50 (59) | 49.36 |
| **Total** | | 97 | 132.67 ± 62.029 | 104.00 (58) | |

*Table 6-7 Descriptive statistic of the FBG concentration (mg/dL) among the clinical groups*

| FBG levels | CG | | ARG | | DAG | |
|---|---|---|---|---|---|---|
| | N | Mean ± SD | N | Mean ± SD | N | Mean ± SD |
| **Low** | 1(3%) | 60 ± 0 | 0 | 0 | 0 | 0 |
| **Normal** | 32 (97%) | 90 ± 7.53 | 6 (20%) | 98.17 ± 8.77 | 16 (47.1%) | 98.13 ± 8.18 |
| **High** | 0 | 0 | 24 (80%) | 197.5 ± 64.5 | 18 (52.9%) | 167.2 ± 59.6 |
| FG concentration are Low <70 mg/dL, Normal 70≤115 mg/dL, high >115 mg/dL | | | | | | |

*Table 6-8 Descriptive statistic of FBG concentrations (mg/dL) among diabetic participants*

| Diabetic status | CG | | ARG | | DAG | |
|---|---|---|---|---|---|---|
| | N | Mean ± SD | N | Mean ± SD | N | Mean ± SD |
| Diabetic | 0 | 0 | 26 (86.7%) | 190.6 ± 66.3 | 14 (41.2%) | 160.2 ± 69.9 |
| Non-diabetic | 33 (100%) | 89.7 ± 9.1 | 4 (13.3%) | 92.7 ± 2.9 | 20 (58.8%) | 116.8 ± 34.5 |

### *6.4.2.2. FBG concentration and the difference between sub-groups*

The difference in FBG concentrations within the sub-groups has been tested. Analysis showed significant differences in FBG between the clinical groups CG, ARG, and DAG using a Kruskal-Wallis test ($\chi^2$ (2) = 49.25, P<0.001), mean ranks are illustrated in table 6-6. The significant difference were between CG, ARG (U=53, P<0.0001), CG, DAG (U=121.5, P<0.0001) and ARG, DAG (U=309, P=0.007). However, a Mann-Whitney test on FBG by sex revealed no significant difference (P=0.768). On the other hand, a Mann-Whitney test revealed significant differences in FBG between the participants who are taking a hypoglycaemic agent and who do not (U=176.5, P<0.0001). Box and whiskers plots are illustrated in figure 6-6 and show the significant differences in FBG levels between the clinical groups and between the participants who take hypoglycaemic agent and who do not, In addition to the gender bias box and whiskers plot.

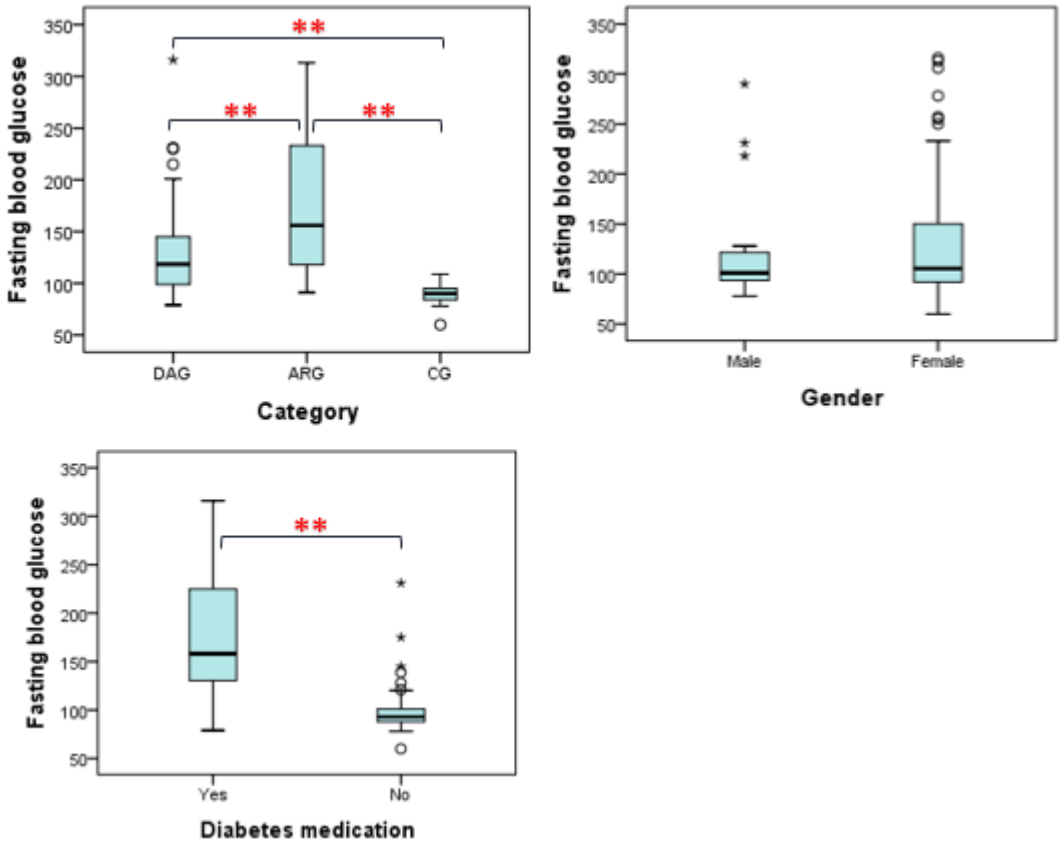


*Figure 6-6 A box and whiskers plot of FBG (mg/dL) among the clinical groups, gender, and consumption of hypoglycaemic agent. Data shows medians, interquartile range, whiskers and outliers. ** Indicates significant between group differences (P<0.01)*

For further analysis, data were sorted by consumption of hypoglycaemic medication. Obviously, none of the participants in the control group was under diabetic treatments nor had high FBG levels as it is shown in table 6-6. On the other hand, the other two groups had diabetic patients who were using hypoglycaemic medication. A Kruskal-Wallis test determined non-significant difference between the clinical groups taking hypoglycaemic medication (P=0.106) as it is shown in the box and whiskers plots in figure 6-7. There was however a significant difference within the clinical groups not taking diabetic medication ($\chi^2$ (2) =21.64, P<0.001) between CG vs DAG (U=84.50 P<0.0001), and ARG vs DAG (U=8.50, P=0.015).

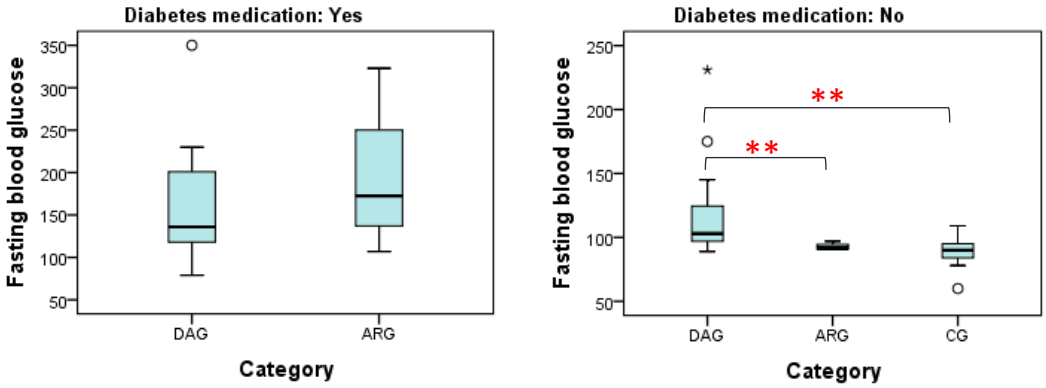


*Figure 6-7 Box and whiskers plots for FBG among clinical groups who consume diabetic medications and who do not. Data shows medians, interquartile range, whiskers and outliers. ** Indicates significant between group differences (P<0.01)*

### *6.4.2.3. Correlations between FBG and 25(OH)D in addition to the rest of the blood markers*

Correlation between FBG and 25(OH)D was performed to investigate any relationship. Spearman correlations revealed no significant correlation between FBG and 25(OH)D (table 6-9). For additional information on the relationship between 25(OH)D and FBG, a scatter diagram is presented for 25(OH)D against FBG (figure 6-8). However, FBG levels were significantly correlated with CRP (rho=0.172, P=.046), HDL (rho=0.221, P=.015), and TG (rho=0.541 P<0.0001) (table 6-19).

*Table 6-9 Spearman's correlation between 25(OH)D (ng/mL) and FBG (mg/dl) concentrations*

| | | | 25 (OH)D | FBG |
|---|---|---|---|---|
| Spearman's rho | 25 (OH)D | Correlation Coefficient | 1.000 | .027 |
| | | Sig. (1-tailed) | . | .398 |
| | | N | 97 | 97 |

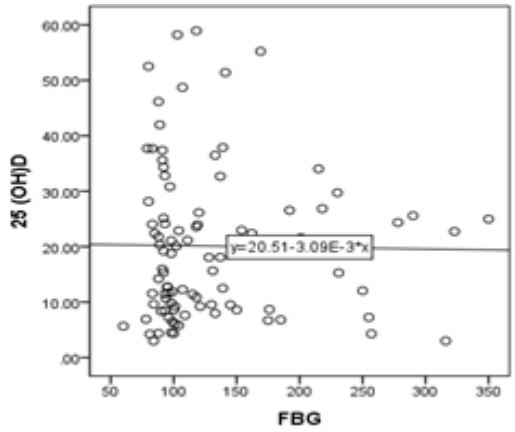


*Figure 6-8 X-Y Scatter graph of 25(OH)D against FBG*

## *Analysis of lipid profile*

## *6.4.3. Analysis of total cholesterol (TC)*

### *6.4.3.1. Descriptive analysis of TC in the pooled sample and between sub-groups*

Total cholesterol was measured in all 97 participants. The normal concentrations of TC are between 50 to 200 mg/dL (table 6-1). The majority of the participants 60.8% had normal levels of TC (162.1 ± 24.5), and 39.2% had high TC concentrations (227.5 ± 22.8). The minimum value was 105 mg/dL and the maximum was 295 mg/dL. Distribution of TC results among the participants in the clinical groups and in both genders is shown in table 6-10. In addition, all of the descriptive results are shown when dividing the sample according to usage of Statins.

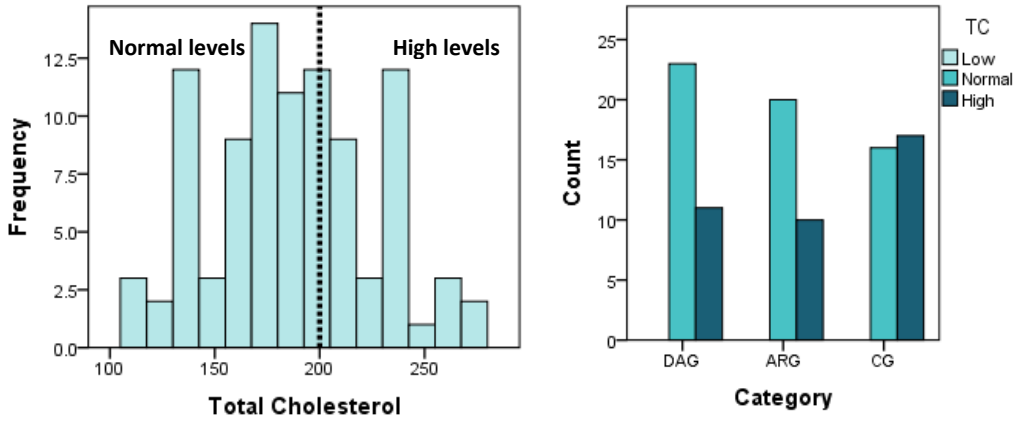


*Figure 6-9 Illustration of HDL (mg/dL) status among the whole participants and a histogram of TC levels among the clinical groups. Cut off values are shown between the dotted lines.*

*Table 6-10 Distribution of TC (mg/dL) among the clinical groups and gender when consume Statin medication or not*

| | Taking Statins | N | Mean | Median | Mean rank |
|---|---|---|---|---|---|
| **CG** | Yes | 0 | 0 | 0 | 0 |
| | No | 33 | 196.76 ± 31.66 | 202 (47) | 42.52 |
| | Total | 33 | 196.76 ± 31.66 | 202 (47) | 56.35 |
| **ARG** | Yes | 10 | 163.50 ± 37.56 | 156.50 (63) | 9.60 |
| | No | 20 | 193.15 ± 42.350 | 185.50 (48) | 38.63 |
| | Total | 30 | 183.27 ± 42.6 | 177.50 (48) | 45.25 |
| **DAG** | Yes | 10 | 180.50 ± 50.403 | 172.50 (99) | 11.40 |
| | No | 24 | 183.88 ± 42.205 | 183.50 (78) | 34.48 |
| | Total | 34 | 182.88 ± 44.01 | 178 (83) | 45.18 |
| **Male** | Yes | 4 | 152.50 ± 21.01 | 146.00 (38) | 8.88 |
| | No | 11 | 197.09 ± 58.82 | 185 (116) | 10.91 |
| | Total | 15 | 185.20 ± 54.61 | 172 (97) | 43.80 |
| **Female** | Yes | 16 | 176.88 ± 47.45 | 172.50 (90) | 38.95 |
| | No | 66 | 190.92 ± 33.86 | 191.50 (46) | 39.01 |
| | Total | 82 | 188.18 ± 36.99 | 188.50 (51) | 49.95 |
| **Total** | | 97 | 187.72 ± 39.88 | 185 (54) | |

### *6.4.3.2. TC concentration and the difference between sub-groups*

To investigate the difference between the sub-groups, a series of non-parametric tests were performed. A Kruskal-Wallis test was performed and revealed no statistical significant difference in TC between the three clinical groups (P=0.182). Consequently, a Mann-Whitney test indicated no significant difference in TC concentrations between genders (P=0.436). Mean ranks are shown in table 6-6. However, a Mann-Whitney test indicated a significant difference in the whole sample between participants who take Statin and those who do not (U=534, P=0.035). Box and whiskers plots of the difference within the clinical groups, gender, and the usage of Statin medication in TC concentrations are presented in figure 6-10.

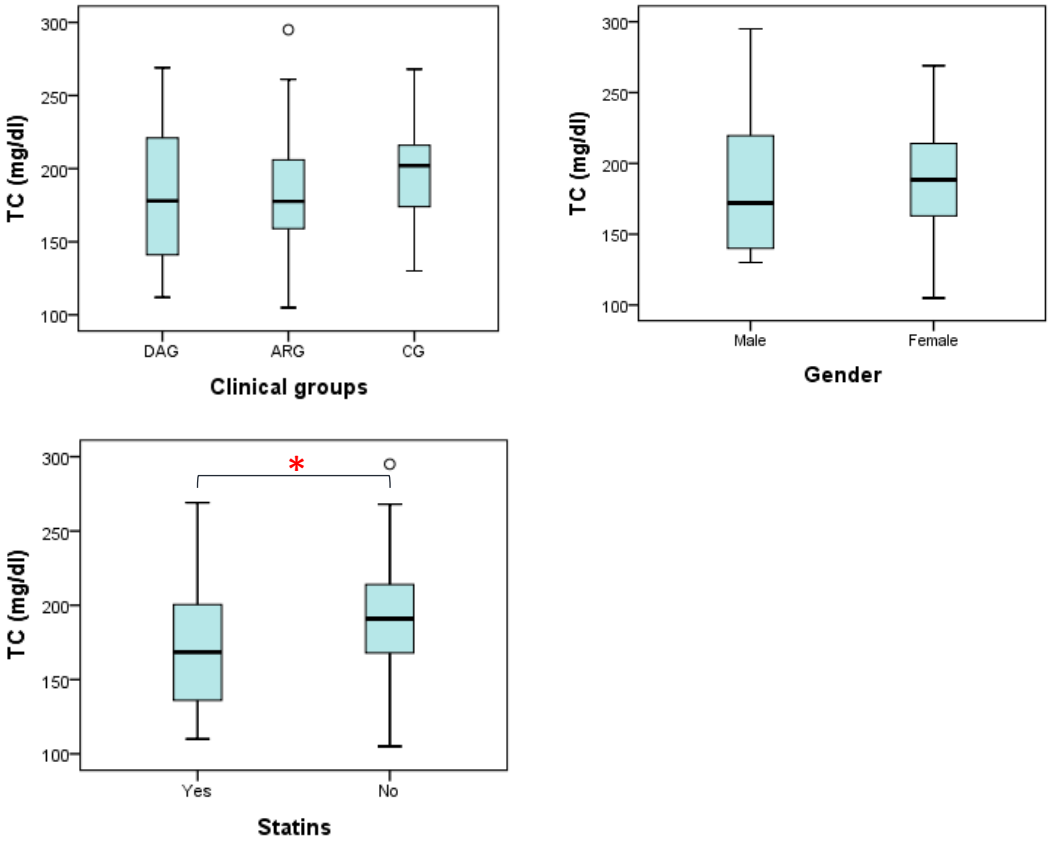


*Figure 6-10 Box and whiskers plots for TC levels (mg/dL) within clinical groups, gender, and usage of Statin medication. Data shows medians, interquartile range, whiskers and outliers.* Indicates significant between group differences (P<0.05)*

### *6.4.3.3. Correlation between TC and 25(OH)D concentrations in addition to the other blood parameters*

Further analysis of the association between TC and 25(OH)D was performed. Spearman rho correlation revealed no significant correlation between 25(OH)D status and TC status (P=0.392) (table 6-11). A scatter plot is presenting the association between 25(OH)D and TC levels in the total sample figure 6-11. However, there were significant correlations between TC and HDL (rho=0.215, P=0.017), LDL (rho=0.893, P<0.0001), and TG (rho=0.211, P=0.019) (table 6-19).

*Table 6-11 Spearman's correlation between 25(OH)D (ng/mL) and TC (mg/dl) concentrations*

| | | | 25 (OH)D | Total Cholesterol |
|---|---|---|---|---|
| Spearman's rho | 25 (OH)D | Correlation Coefficient | 1.000 | 0.028 |
| | | Sig. (1-tailed) | . | 0.392 |
| | | N | 97 | 97 |

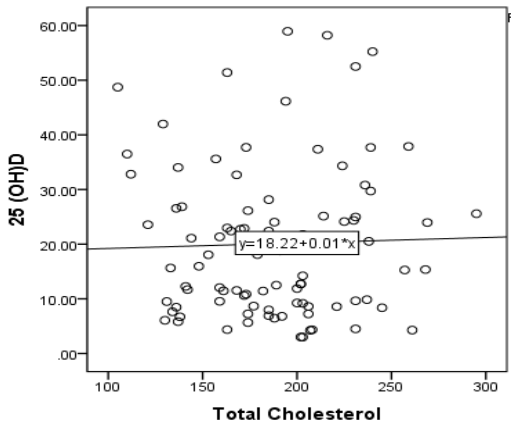


*Figure 6-11 X-Y Scatter Graph of 25(OH)D (ng/ml) and TC (mg/dL) concentrations*

## *6.4.4. Analysis of HDL cholesterol*

### *6.4.4.1. Descriptive analysis of HDL in the pooled sample and between sub-groups*

High-density lipoprotein cholesterol was measured in all 97 participants. Normal values of HDL concentrations for the age of the study group are 35 to 55 mg/dL (table 6-1). In the current study, the minimum value was 20 mg/dL and the maximum was 114 mg/dL. The majority of the participants had normal concentrations 62.9% (44.93 ± 6.1), 20.6% had high concentrations (67.1 ± 13), and only 16.5% had low HDL concentrations (29.4 ± 4) (figure 6-12).

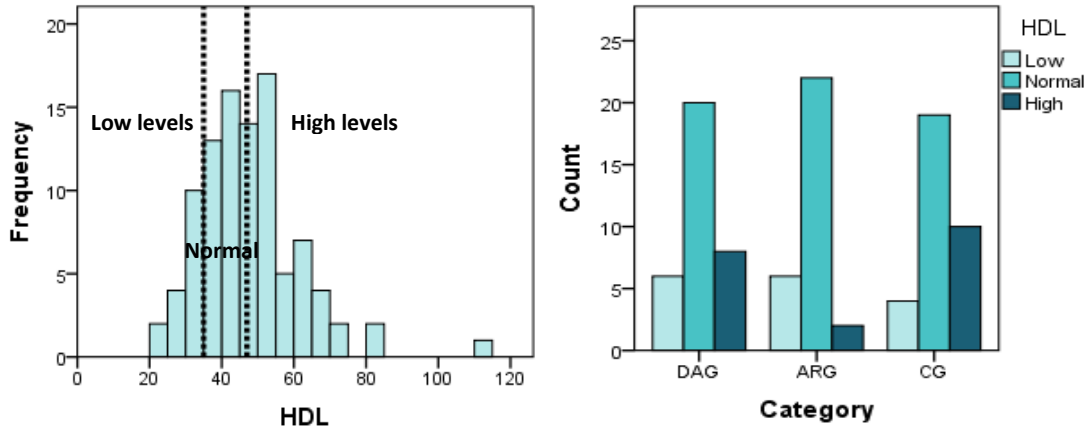


*Figure 6-12 Illustration of HDL concentrations (mg/dL) among the whole participants and the clinical groups. Normal concentrations are shown between the dotted lines.*

*Table 6-12 Descriptive and mean ranks of HDL concentrations (mg/dL) among the clinical groups and gender*

| | N | Mean ± SD | Median (IQR) | Mean Rank |
|---|---|---|---|---|
| **CG** | 33 | 48 ± 12.8 | 49 (19) | 52.30 |
| **ARG** | 30 | 44.23 ± 11.83 | 44 (12) | 44.60 |
| **DAG** | 34 | 48.35 ± 16.87 | 45 (19) | 49.68 |
| **Male** | 15 | 39.67 ± 9.83 | 39 (17) | 33.97 |
| **Female** | 82 | 48.29 ± 14.37 | 47 (16) | 51.75 |
| **Total** | 97 | 46.96 ± 14.08 | 45 (16) | |

### *6.4.4.2. HDL concentration and the difference between sub-groups*

Analysis of the differences between sub-groups in HDL concentrations was performed using Kruskal-Wallis and Mann-Whitney tests. The Kruskal-Wallis test revealed no significant difference in HDL level between the clinical groups (P=0.546). However, the Mann-Whitney test indicated a significant difference in HDL concentrations between male and female (U=389.5, P=0.024). Mean ranks are shown in table (6-7). Box and whiskers plots in (figure 6-13) shows the differences in HDL concentrations among the clinical groups and gender.

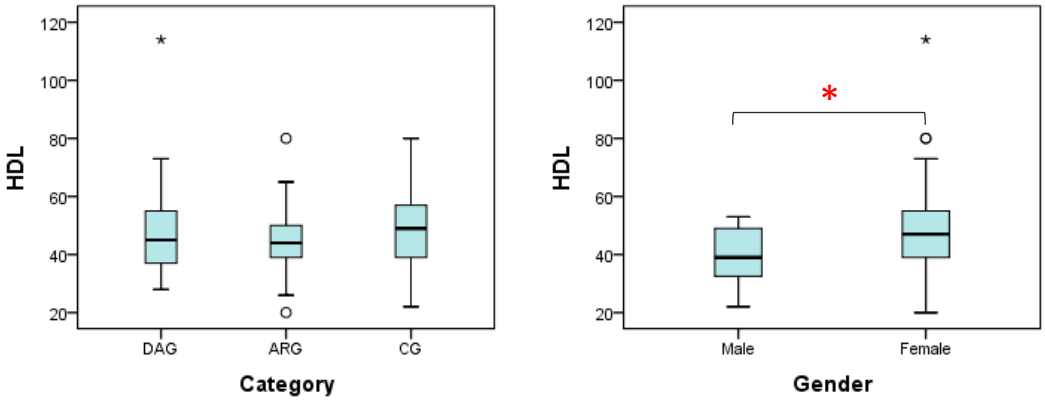


*Figure 6-13 Box and whiskers plots for HDL level (mg/dL) among the clinical groups and gender. Data shows medians, interquartile range, whiskers and outliers.* Indicates significant between group differences (P<0.05)*

### *6.4.4.3. Correlations between TC and 25(OH)D and the other blood parameters*

Additional analysis was performed to investigate relations between HDL and other blood markers. A Spearman rho test indicated no significant correlation between 25(OH)D status and HDL concentration in the total sample (P=0.181) (table 6-13). Further analysis using a scatter plot showed the lack of impact between 25(OH)D and HDL in the pooled sample (figure 6-14). However, there were significant correlations between HDL and FBG (rho= -0.221, P=0.015), TC (rho= 0.893, P<0.0001), TG (rho= -0.474, P<0.0001) (table 6-19).

*Table 6-13 Spearman's correlation between 25(OH)D (ng/mL) and HDL (mg/dl) concentrations*

| | | | 25 (OH)D | HDL |
|---|---|---|---|---|
| Spearman's rho | 25 (OH)D | Correlation Coefficient | 1.000 | 0.094 |
| | | Sig. (1-tailed) | . | 0.181 |
| | | N | 97 | 97 |

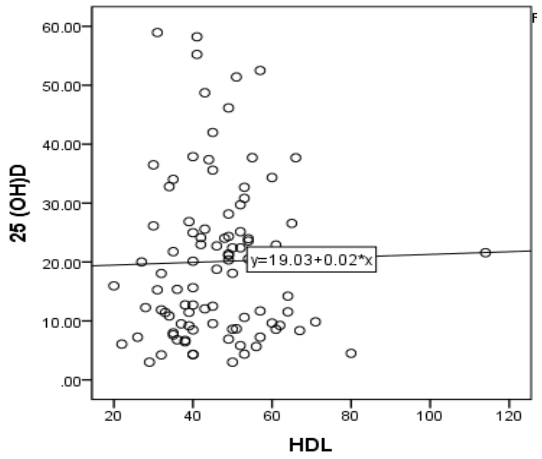


*Figure 6-14 X-Y Scatter graph of 25(OH)D (ng/ml) against HDL (mg/dL) concentrations*

## *6.4.5. Analysis of LDL cholesterol*

### *6.4.5.1. Descriptive analysis of LDL in the pooled sample and between sub-groups*

Low-density lipoprotein was measured in all 97 participants. Normal concentrations of LDL level for the target group are ≥ 0.001 and ≤ 150 mg/dL (table 6-1) .The majority of the participants had normal concentrations of LDL 81% (mean ± SD) (103.67 ± 25.89) whereas, 18.6% had high levels of LDL (168.0 ± 18.96) (figure 6-15). The minimum value of LDL was 46 mg/dL and the maximum was 220 mg/dL. Descriptive analysis of LDL levels among the clinical groups, gender in total sample and when consuming Statin medication are illustrated in (table 6-14).

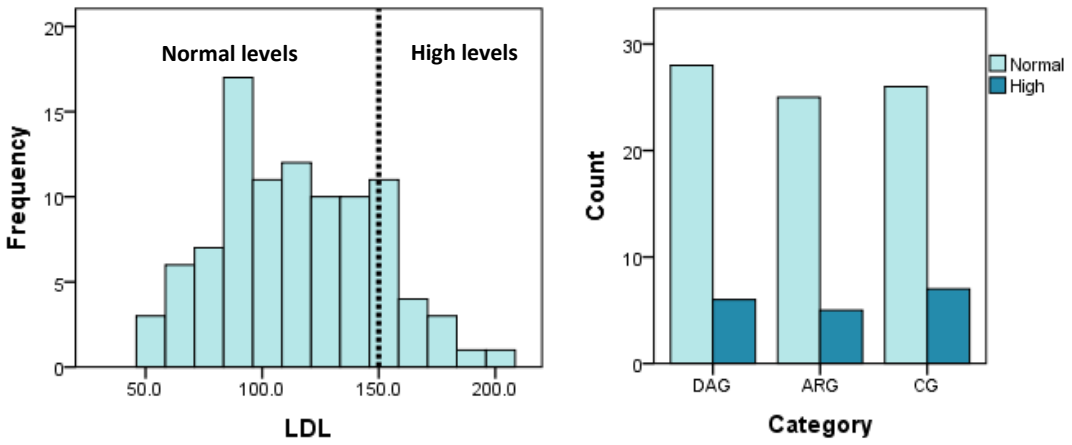


*Figure 6-15 LDL levels (mg/dL) among the whole sample and between the clinical groups. Cut off values are shown between the dotted lines.*

*Table 6-14 Distribution of participants LDL levels (mg/dL) among the study groups and gender when consume Statin medication or not*

| | Taking Statins | N | Mean ± SD | Median (IQR) | Mean Rank |
|---|---|---|---|---|---|
| **CG** | Yes | 0 | | | |
| | No | 33 | 129.48 ± 27.85 | 132 (38.5) | 48.08 |
| | Total | 33 | 129.48 ± 27.85 | 132 (38.5) | 61.58 |
| **ARG** | Yes | 10 | 103.64 ± 32.89 | 94.5 (61.1) | 9.70 |
| | No | 20 | 112.74 ± 41.41 | 98.50 (43.1) | 34.23 |
| | Total | 30 | 109.7 ± 38.44 | 97.50 (49.8) | 24.62 |
| **DAG** | Yes | 10 | 115.8 ± 45.15 | 113 (78.8) | 11.30 |
| | No | 24 | 103.83 ± 31.13 | 97.50 (49.8) | 30.50 |
| | Total | 34 | 107.35 ± 35.52 | 100.50 (48.5) | 42.43 |
| **Male** | Yes | 4 | 101.8 ± 20.07 | 103 (38.6) | 9.88 |
| | No | 11 | 127.45 ± 52.57 | 117 (87) | 10.66 |
| | Total | 15 | 120.6 ± 46.89 | 113 (73.8) | 49.20 |
| **Female** | Yes | 16 | 111.70 ± 42.66 | 10 (77.5) | 41.55 |
| | No | 66 | 115.42 ± 30.47 | 113.50 (47.3) | 38.58 |
| | Total | 82 | 114.69 ± 32.93 | 112.50 (48.3) | 48.96 |
| **Total** | | 97 | 115.6 ± 35.21 | 113 (53) | |

### *6.4.5.2. LDL concentration and the difference between sub-groups*

To investigate the difference within the sub-groups in LDL concentrations a series of non-parametric tests were performed. Analysis of the LDL levels indicated a significant statistical difference between the clinical groups ($\chi^2$ (2) =9.988 P=0.007) using Kruskal-Wallis test. The difference were between CG vs ARG (U=295.5, P=0.006), and CG vs DAG (U=345.5, P=0.007). However, a Mann-Whitney test revealed no significant statistical difference (P=0.976) between male and female. Additionally, a Mann-Whitney indicated no significant difference in LDL concentration when Statins were taken or not (P=0.545). Box and whiskers plots of the difference between the sub-groups in LDL concentration are shown in figure 6-16.

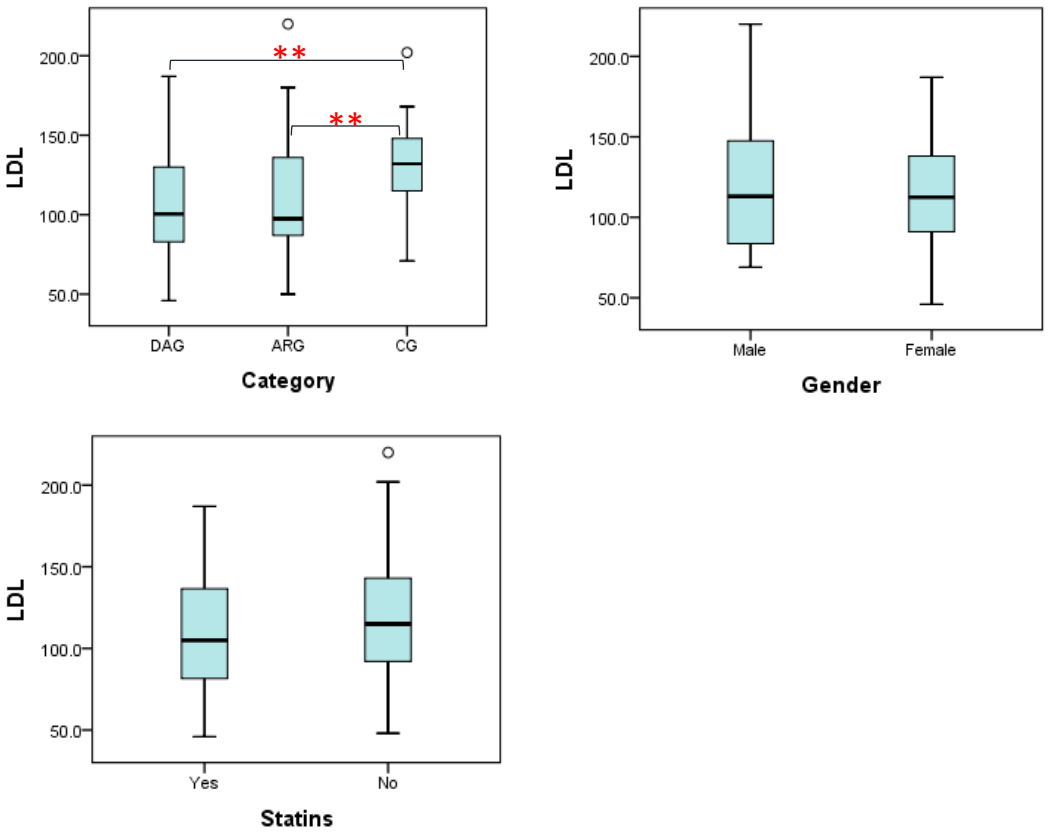


*Figure 6-16 Box and whiskers plots for LDL (mg/dL) in clinical groups, gender, usage of Statin. Data shows medians, interquartile range, whiskers and outliers. ** Indicates significant between group differences (P<0.01)*

### *6.4.5.3. Correlations between LDL and 25(OH)D and the other blood parameters*

Further investigations of the relationship between LDL concentration and the other blood parameters were performed. A Spearman rho test revealed no significant correlation between LDL and 25(OH)D concentrations (P=0.386) (table 6-15). A simple scatter plot between LDL and 25(OH)D illustrates the lack of relationship between the two variables in the pooled sample (figure 6-17). On the other hand, there was a significant correlation between LDL and TC (rho=0.893, P<0.0001) (table 6-19).

*Table 6-15 Spearman's correlation between 25(OH)D (ng/mL) and TC (mg/dl) concentrations*

| | | | 25 (OH)D | LDL |
|---|---|---|---|---|
| Spearman's rho | 25 (OH)D | Correlation Coefficient | 1.000 | 0.030 |
| | | Sig. (1-tailed) | . | 0.386 |
| | | N | 97 | 97 |

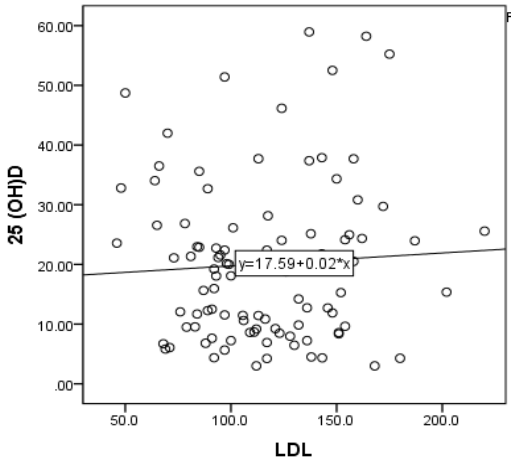


*Figure 6-17 X-Y Scatter graph of 25(OH)D (ng/mL) and LDL (mg/dL)*

## *6.4.6. Analysis of Triglycerides (TG)*

### *6.4.6.1. Descriptive analysis of TC in the pooled sample and between sub-groups*

Triglycerides were measured in all 97 participants. Normal concentrations of TG are between 30-200 mg/dL. In the current study, the minimum concentration of TG was 35 mg/dL and the maximum was 605 mg/dL. Seventy seven percent of the participants had normal TG values (mean± SD) (111.40 ± 39.55) and the rest 20.6% had high values (297.75 ± 98.75) (figure 6-18). Descriptive analysis of TG results are shown in table 6-16. There were four values of TG above 340 mg/dl considered outliers. Table 6-17 shows TG values without outliers. Figure 6-18 shows the distribution of the sample without TG outliers. The results were reviewed for validation. None of participants who had outliers' values in TG had outliers when the rest of the blood markers were analysed. This appears to indicate that these outliers are simply related to the TG data and have no common feature.

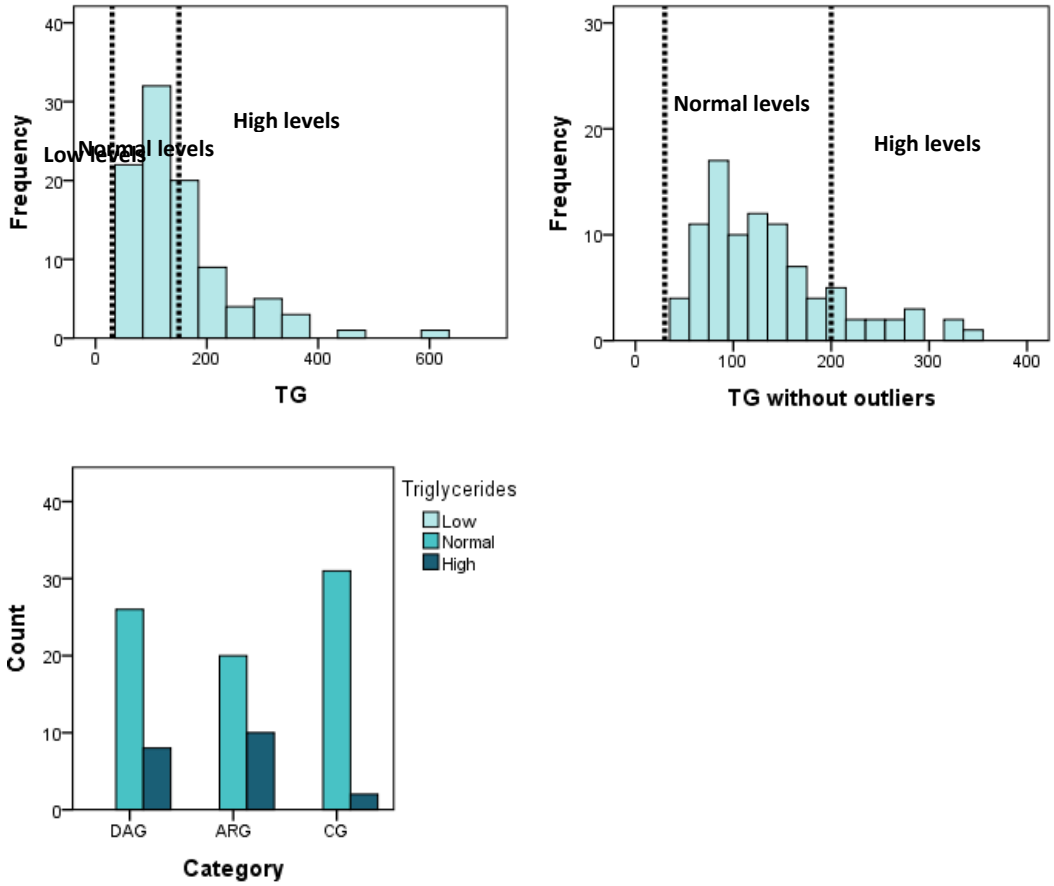


*Figure 6-18 Distribution of Triglycerides concentrations (mg/dL) in the total sample and without outliers. Cut off concentrations are shown between the dotted lines.*

*Table 6-16 Distribution of Triglycerides among the participants within the clinical groups and gender*

| | N | Mean ± SD | Median (IQR) | Mean Rank |
|---|---|---|---|---|
| **CG** | 33 | 108.7 ± 62.83 | 91 (65) | 33.79 |
| **ARG** | 30 | 182.37 ± 114.02 | 157 (97) | 60.27 |
| **DAG** | 34 | 161.03 ± 88.81 | 137 (111) | 53.82 |
| **Male** | 15 | 145.40 ± 82.58 | 123 (69) | 49.07 |
| **Female** | 82 | 150.63 ± 96.84 | 122.50 (106) | 48.99 |
| **Total** | 97 | 149.82 ± 94.4 | 123 (92) | |

*Table 6-17 Distribution of TG without outliers among the participants within the clinical groups and gender*

| | N | Mean ± SD | Median (IQR) | Mean Rank |
|---|---|---|---|---|
| **CG** | 33 | 108.7 ± 62.83 | 91 (65) | 33.79 |
| **ARG** | 28 | 157.46 ± 62.55 | 149.50 (92) | 57.68 |
| **DAG** | 32 | 147.72 ± 72.7 | 129.5 (91) | 51.28 |
| **Male** | 14 | 129.3 ± 56.45 | 121 (58) | 642 |
| **Female** | 79 | 138.1 ± 71.19 | 120 (87) | 3729 |
| **Total** | 93 | 136.8 ± 68.97 | 120 (81) | |

### *6.4.6.2. TG concentration and the difference between sub-groups*

To investigate the difference in TG levels between the sub-groups a series of non-parametric tests were performed. A Kruskal-Wallis test revealed a statistically significant main effect in clinical groups on TG levels ($\chi^2$ (2) =15.448, P<0.0001). The significant differences were between CG vs ARG (U=227, P<0.0001) and CG vs DAG (U=327, P=0.003). When the test performed without the outliers similar results were observed ($\chi^2$ (2) =13.09, P=0.001) in that the differences were also between CG vs ARG (U=227, P=0.001), and CG vs DAG (U=327, P=0.008) (figure 6-19). However, a Mann-Witney test indicated no significant difference in TG concentrations between male and female neither with (P=0.992) nor without the outliers (P=0.864). In addition, there was no difference in TG levels whether Statin was taken or not (P=0.711). Box and whiskers plots of TG concentrations between the study groups and gender are shown in figure 6-20.

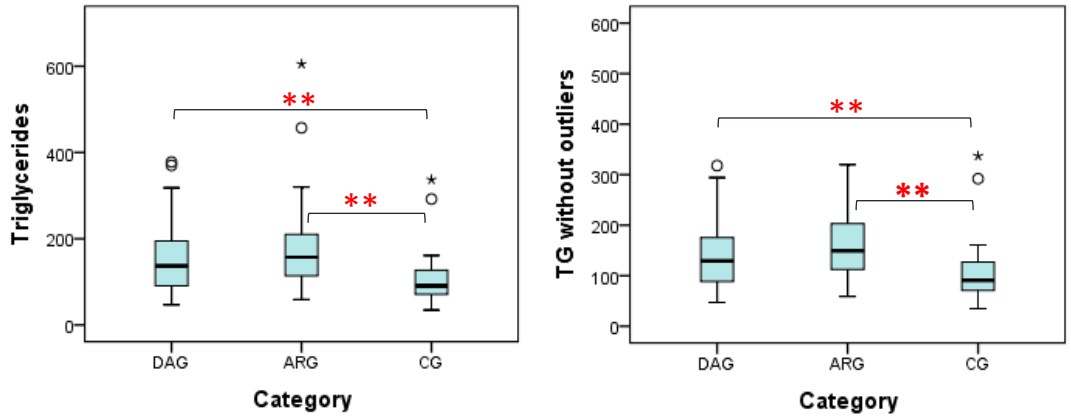


*Figure 6-19 Box and whiskers plots of TG (mg/dL) in clinical groups in the total sample and without outliers. Data shows medians, interquartile range, whiskers and outliers. ** Indicates significant between group differences (P<0.01).*

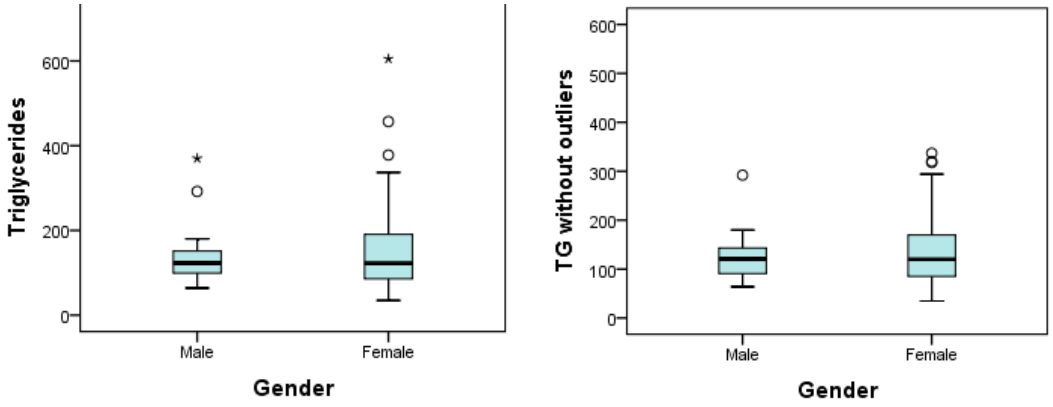


*Figure 6-20 Box and whiskers plots of TG (mg/dL) and gender. Data shows medians, interquartile range, whiskers and outliers.*

### *6.4.6.3. Correlations between LDL and 25(OH)D in addition to the other blood parameters*

For further investigation of the relationship between triglycerides concentrations and the other blood health markers, a sequence of non-parametric statistical tests were performed. A Spearman's rho indicated no significant correlation between 25(OH)D and TG concentrations either with (P=0.499) nor without outliers (P=0.497) (table 6-18). Figure 6-21 presents the scatter diagrams showing the lack of any relationship between 25(OH)D and TG. However, there was a significant negative correlation between 25(OH)D and TG, in the ARG in participants not taking vitamin D supplements (rho= -0.587, P=0.005). Similar results were obtained with TG without

outliers (rho= -0.585, P=0.009) (table 6-20). Moreover, there were statistically significant correlations between TG and FBG (rho=0.541 P<0.0001), CRP (rho=0.198 P=0.026), TC (rho=0.211 P=019), and HDL (rho=-0.474 P<0.0001) (table 6-19).

*Table 6-18 Spearman's correlation between 25(OH)D (ng/mL), TG and TG without outliers (mg/dl) concentrations*

| | | | 25 (OH)D | TG | TG without outliers |
|---|---|---|---|---|---|
| Spearman's rho | 25(OH)D | Correlation Coefficient | 1.000 | 0.000 | 0.001 |
| | | Sig. (1-tailed) | . | 0.499 | 0.497 |
| | | N | 97 | 97 | 93 |

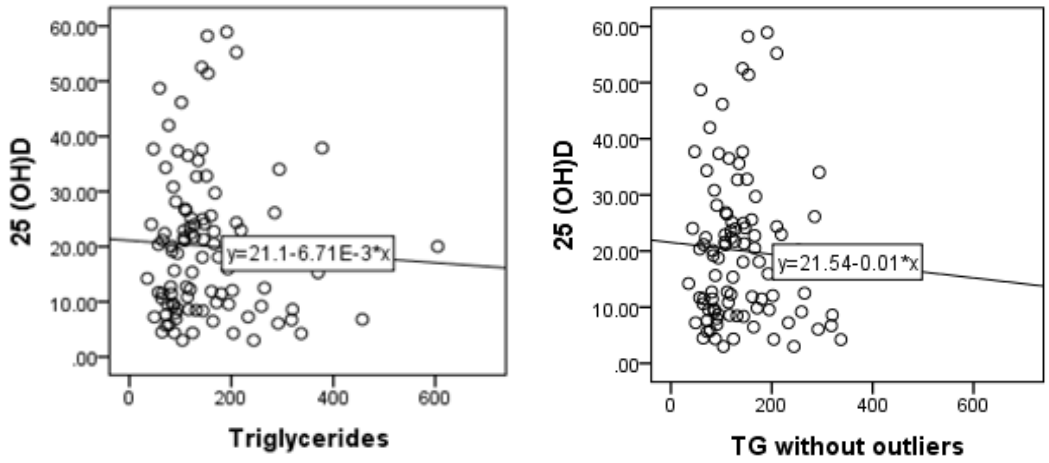


*Figure 6-21 X-Y Scatter graph of 25(OH)D (ng/mL) against Triglycerides (mg/dL) in total sample and without outliers.*

## *6.4.7. Correlations between 25(OH)D and other blood parameters, and multiple linear regression model*

A number of Spearman correlations were performed to investigate the relationship between 25(OH)D concentration and the rest of the blood markers. The test revealed no significant correlations between 25(OH)D concentration and the blood markers in the pooled sample (table 6-19). Thus, a multiple linear regression model was not appropriate to predict 25(OH)D from the blood health markers of interest in this chapter.

*Table 6-19 Spearman's rho correlations between all of the chapter blood parameterises in the pooled population*

| | | | 25 (OH)D | CRP | CRP without outliers | FBG | TC | HDL | LDL | TG | TG without outliers |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Spearman's rho | 25 (OH)D | Correlation Coefficient | 1.000 | 0.029 | 0.011 | 0.027 | 0.028 | 0.094 | 0.030 | 0.000 | 0.001 |
| | | Sig. (1-tailed) | . | 0.388 | 0.458 | 0.398 | 0.392 | 0.181 | 0.386 | 0.499 | 0.497 |
| | | N | 97 | 97 | 94 | 97 | 97 | 97 | 97 | 97 | 93 |
| | CRP | Correlation Coefficient | | 1.000 | 1.000** | 0.172* | -0.126 | -0.140 | -0.143 | 0.198* | 0.209* |
| | | Sig. (1-tailed) | | . | . | 0.046 | 0.109 | 0.085 | 0.082 | 0.026 | 0.022 |
| | | N | | 97 | 94 | 97 | 97 | 97 | 97 | 97 | 93 |
| | CRP No outliers | Correlation Coefficient | | | 1.000 | 0.221* | -0.084 | -0.109 | -0.111 | 0.181* | 0.187* |
| | | Sig. (1-tailed) | | | . | 0.016 | 0.210 | 0.148 | 0.144 | 0.040 | 0.039 |
| | | N | | | 94 | 94 | 94 | 94 | 94 | 94 | 90 |
| | FBG | Correlation Coefficient | | | | 1.000 | -0.003 | -0.221* | -0.148 | 0.541** | 0.501** |
| | | Sig. (1-tailed) | | | | . | 0.487 | 0.015 | 0.074 | 0.000 | 0.000 |
| | | N | | | | 97 | 97 | 97 | 97 | 97 | 93 |
| | TC | Correlation Coefficient | | | | | 1.000 | 0.215* | 0.893** | 0.211* | 0.153 |
| | | Sig. (1-tailed) | | | | | . | 0.017 | 0.000 | 0.019 | 0.072 |
| | | N | | | | | 97 | 97 | 97 | 97 | 93 |
| | HDL | Correlation Coefficient | | | | | | 1.000 | 0.078 | -0.474** | -0.433** |
| | | Sig. (1-tailed) | | | | | | . | 0.225 | 0.000 | 0.000 |
| | | N | | | | | | 97 | 97 | 97 | 93 |
| | LDL | Correlation Coefficient | | | | | | | 1.000 | 0.063 | 0.054 |
| | | Sig. (1-tailed) | | | | | | | . | 0.270 | 0.305 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | N | | | | | | | 97 | 97 | 93 |
| | TG | Correlation Coefficient | | | | | | | | 1.000 | 1.000** |
| | | Sig. (1-tailed) | | | | | | | | . | . |
| | | N | | | | | | | | 97 | 93 |
| | TG without outliers | Correlation Coefficient | | | | | | | | | 1.000 |
| | | Sig. (1-tailed) | | | | | | | | | . |
| | | N | | | | | | | | | 93 |
| **. Correlation is significant at the 0.01 level (1-tailed).<br>*. Correlation is significant at the 0.05 level (1-tailed). | | | | | | | | | | | |

*Table 6-20 Spearman's rho correlations between 25(OH)D and each of the blood parameterises within the clinical groups considering the consumption of vitamin D supplements*

| Take vitamin D supplements | Category | | | CRP | CRP No outliers | FBG | TC | HDL | LDL | TG | TG No outliers |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Yes | DAG | 25 (OH)D | Correlation Coefficient | -0.280 | -0.426 | -0.182 | -0.168 | -0.263 | -0.063 | -0.028 | -0.245 |
| | | | Sig. (1-tailed) | 0.189 | 0.096 | 0.286 | 0.301 | 0.204 | 0.423 | 0.466 | 0.233 |
| | | | N | 12 | 11 | 12 | 12 | 12 | 12 | 12 | 11 |
| | ARG | | Correlation Coefficient | -0.666** | -0.765** | -0.266 | -0.035 | 0.014 | 0.035 | -0.063 | -0.063 |
| | | | Sig. (1-tailed) | 0.009 | 0.003 | 0.201 | 0.457 | 0.483 | 0.457 | 0.423 | 0.423 |
| | | | N | 12 | 11 | 12 | 12 | 12 | 12 | 12 | 12 |
| | CG | | Correlation Coefficient | -0.348 | -0.348 | -0.086 | 0.086 | -0.029 | 0.371 | 0.667 | 0.667 |
| | | | Sig. (1-tailed) | 0.250 | 0.250 | 0.436 | 0.436 | 0.479 | 0.234 | 0.074 | 0.074 |
| | | | N | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| No | DAG | 25 (OH)D | Correlation Coefficient | -0.277 | -0.155 | 0.194 | 0.282 | -0.015 | 0.153 | 0.275 | 0.262 |
| | | | Sig. (1-tailed) | 0.106 | 0.252 | 0.193 | 0.102 | 0.474 | 0.248 | 0.108 | 0.125 |
| | | | N | 22 | 21 | 22 | 22 | 22 | 22 | 22 | 21 |
| | ARG | | Correlation Coefficient | 0.128 | 0.128 | -0.206 | -0.253 | 0.369 | -0.259 | -0.587** | -0.585** |
| | | | Sig. (1-tailed) | 0.307 | 0.307 | 0.207 | 0.156 | 0.066 | 0.150 | 0.005 | 0.009 |
| | | | N | 18 | 18 | 18 | 18 | 18 | 18 | 18 | 16 |
| | CG | | Correlation Coefficient | 0.035 | 0.035 | -0.047 | 0.106 | 0.041 | 0.175 | -0.168 | -0.168 |
| | | | Sig. (1-tailed) | 0.431 | 0.431 | 0.407 | 0.300 | 0.420 | 0.192 | 0.201 | 0.201 |
| | | | N | 27 | 27 | 27 | 27 | 27 | 27 | 27 | 27 |

*. Correlation is significant at the 0.05 level (1-tailed).
**. Correlation is significant at the 0.01 level (1-tailed).

## 6.5. Discussion

The current study hypothised an association between 25(OH)D concentrations and the indicators of blood health that are deemed to be markers or risk factors for atherosclerosis. The hypothesis was rejected in this chapter, as there was no association observed between the desired variables in the pooled sample. A detailed discussion of the results and the peer-reviewed previous studies are presented in this section.

### *6.5.1. Discussion of CRP and vitamin D status*

C-reactive protein is considered to be an important marker of cardiovascular disease and atherosclerosis (Sibley et al., 2014). In the present study, 41.2% of the participants had normal values of CRP (0.007 ± 0.047 mg/dl) and the rest had high concentrations (1.086 ± 1.186 mg/dl). There was a significant difference in CRP concentrations between the clinical groups (CG, ARG, DAG) $P<0.001$. It appears that the ARG had a mean rank of 60.27 mg/dL whilst the other two groups had the same mean rank of 41.75 mg/dL of CRP. The ARG were at risk of developing atherosclerosis and they were not currently undergoing any treatments for the disease, which explains the high concentrations of CRP (0.837 ± 0.804 mg/dl). This was unlike the DAG patients who were being treated for atherosclerosis (0.626 ± 1.52 mg/dl) and the CG who were free of atherosclerosis and its risk factors (0.479 ± 0.533).

In the current study, no statistically significant correlation was found between 25(OH)D and CRP in the pooled sample. However, there was a statistically significant correlation between 25(OH)D and CRP in the ARG when taking vitamin D supplements ($rho=0.666$, $P<0.05$). In this group (ARG), 40% of the participants were using supplements. This result indicates the positive impact of vitamin D supplementation in preventing the causes that leads to high production of CRP, which is one of the inflammatory markers of atherosclerosis (Targher et al., 2006; Lertratanakul et al., 2014).

These results are similar to findings by Lertratanakul et al. (2014) who studied the association between 25(OH)D deficiency and the increased risk of cardiovascular disease and its risk factors in the general population. Lertratanakul et al. (2014) found that participants with adequate 25(OH)D concentrations (31-91 ng/mL) had normal CRP concentrations <0.300 mg/dL. On the other hand, in the present study, the ARG had 86.7 % diabetic participants and that could be one of the reasons for the noticeable correlation with CRP. Targher et al., (2006) studied type 2 diabetic adults and found that those diabetic patients who were vitamin D deficient (11.2±2.8 ng/mL) had significantly (P<0.001) higher levels of CRP (0.498 ± 0.65mg/dL). The potential biological explanation for the inverse association between CRP and 25(OH)D could be because of the protective effect of vitamin D against inflammation and inflammation is one of the reasons that CRP is produced by the liver (van Wissen et al., 2002; Giovannucci, 2009). These findings confirm results from the current study of the inverse association between 25(OH)D and CRP in participants at risk of developing atherosclerosis.

### *6.5.2. Discussion of FBG and vitamin D status*

Fasting blood glucose is one of the key indicators of diabetes, which is a risk factor for further ill health including cardiovascular disease and atherosclerosis (Beckman et al., 2002; Witham et al., 2010). In the current study, high FBG concentrations were prevalent in the ARG and DAG but not in the CG P<0.001. In addition, 40% of the participants were diabetic and they were taking hypoglycaemic agents. The ARG included the highest number of diabetic participants, 86.7%, of any group in the current study. However, hyperglycaemia was observed in most of the diabetic participants in the current study, 43% of participants had FBG levels > 115 mg/dL even with the consumption of hyperglycaemic agents. This could be because their condition was poorly controlled or they had other diseases, which is an issue that needs to be considered (Beckman et al., 2002; Talaei et al., 2013).

The current study has found no significant relationship between 25(OH)D and FBG concentrations in the pooled sample or in the sub-groups regardless of whether

vitamin D supplements were taken nor not. This finding is supported by other studies such as (Jorde and Figenschau, 2009; Qadhi, 2016). In the study by Qadhi (2017), the impact of vitamin D supplementation in diabetic participants was tested for 16 weeks. There was a significant improvement in the HbA1c ($P<0.001$) but there was no significant impact on FBG concentration. HBA1c was a more accurate predictor than FBG for mean glucose concentration in serum for the previous three months (Qadhi, 2016). Additionally, Jorde and Figenschau (2009) also tested vitamin D supplementation in diabetic patients and found no association between 25(OH)D and FBG. Those studies and the current study observed no association between vitamin D status and FBG. However, high concentrations of FBG and diabetes are considered major risk factors for atherosclerosis (Beckman et al., 2002; Menezes et al., 2014) and some other studies have found an association between FBG and 25(OH)D in atherosclerosis risk factor participants. For instance, a study by Talaei et al. (2013) found a significant association between FBG and 25(OH)D in diabetic patients after treatment of vitamin D deficiency by vitamin D supplements for eight weeks ($P<.05$). Another recent study by Aljefree et al. (2016) examined participants with coronary heart disease and considered the measurement of FBG as one of the markers of the disease. They found a significant association between the markers and the prevalence of vitamin D deficiency ($P<0.001$). The association between FBG and 25(OH)D needs further examination to confirm whether such an association exists.

### *6.5.3. Discussion of lipids profiles and vitamin D status*

In the current study, lipid profiles included total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL), and triglycerides (TG). The majority of the participants had normal values of TC, HDL, LDL, and TG; 59%, 61%, 79%, and 77% respectively. In total, 39.2% of the participants had hyperlipidaemia (that is, the highest lipid concentrations in the current study). The results were certainly affected by other factors such as the use of Statin medications. All of the lipid measurements in the current study were significantly associated with each other ($P<0.05$).

There was no significant correlation between 25(OH)D concentrations and any of the lipids profile measurements in the pooled sample. These results were similar to observations by Gepner et al. (2012). They performed a double-blind, placebo-controlled trial to examine the impact of vitamin D supplementations on cardiovascular risk. They did not observe any significant correlation between 25(OH)D and lipids (TC, HDL, LDL, and TG) either in the placebo, or the supplement group. Another study tested the association between 25(OH)D and lipid profile measurements in vitamin D deficient participants (Tarcin et al., 2009). They also observed no significant association between 25(OH)D concentration and any of the measured lipids.

The association between 25(OH)D and lipids is not consistent; some studies observe associations and some do not. The current study observed an inverse correlation between 25(OH)D and TG in the ARG when vitamin D supplements were not consumed ($P<0.01$). Similar results were observed in a longitudinal community-based study by Faridi et al., (2017) in which participants at a high risk of developing atherosclerosis were tested for 25(OH)D and lipid profiles. The measurements were taken at baseline and twice more within 5 years. The study found that vitamin D deficiency was associated with lower HDL and higher TC, but it was not associated with TG and LDL. However, in another study (Qadhi, 2016) the impact of vitamin D supplementation for 16 weeks was examined in diabetic participants. Significant improvement in TC ($P<0.05$) and LDL ($P<0.001$) was observed, but no association was found with HDL and TG. Conversely, another study by Lertratanakul et al. (2014) did observe associations between 25(OH)D concentrations and all of the lipid profile measurements ($P<0.001$) (Lertratanakul et al., 2014). This indicates that the association between 25(OH)D concentration and hyperlipidaemia could be affected by other factors such as (medications and diseases). These factors could be the explanation of the un-clear association between 25(OH)D and lipids in the current study.

## 6.6. Summary and conclusion

The current study confirmed the significant differences in blood markers of cardiovascular disease between the study's clinical groups. The control group had normal concentrations of CRP, FBG, and lipid profiles when compared with the at-risk group and the diagnosed with atherosclerosis group. Additionally, the use of hypoglycaemic agents, statins and vitamin D supplements had an impact on the blood markers. However, 25(OH)D concentrations did not correlate with any of the other measured blood-health markers in the pooled sample.

The use of vitamin D supplements had an impact on the results only in the ARG. Correlations were observed between 25(OH)D and CRP in the ARG when using vitamin D supplements. In addition, there was an association between 25(OH)D and TG in the ARG who were not using vitamin D supplements. The majority of the participants (86.7%) in the ARG were diabetic patients and many studies have observed an association between vitamin D status and diabetes (Targher et al., 2006; Faridi et al., 2017), which probably provides an explanation for the correlation seen in this group between taking supplements and blood markers.

In conclusion, the use of vitamin D supplements tends to be a factor that affects the association between blood markers of cardiovascular disease and 25(OH)D concentration only in the group at-risk of developing atherosclerosis. Further investigation may be needed to clarify the impact of 25(OH)D concentration on blood markers of cardiovascular disease and atherosclerosis risk factors.

# Chapter 7 - Association between circulating 25(OH)D levels and vascular structural and functional characteristics

## 7.1. Introduction

The details of the association between 25(OH)D concentrations and atherosclerosis incidence and risk factors are still under investigation (Menezes et al., 2014; Adamczak, 2017). Assessment of atherosclerosis can be performed by testing vascular structural and functional characteristics. The status of carotid-radial pulse wave velocity (PWV), central blood pressure (cBP), peripheral blood pressure (pBP) carotid intima-media thickness (IMT), carotid artery inter-adventitial diameter (IAD), and other markers were therefore assessed. The measurements were determined on the pooled study sample and in each of the sub-groups, clinical groups (control group (CG), at-risk group (ARG), and participants diagnosed with atherosclerosis group (DAG)), and between the sexes. Analysis based on the clinical groups was considered an indicator of the association between the measurement and the participants' clinical condition. Analysis between the sexes determined any male vs female variation in each of the measurements. The associations between these vascular structural and functional characteristics and 25(OH)D status were investigated to determine any association between these markers of health.

### *7.1.1. Chapter aim*

- To determine if there is a relationship between circulating 25(OH)D levels and vascular structural and functional characteristics.

### *7.1.2. Chapter objectives*

1- To assess each of the vascular structural and functional characteristics (PWV, cBP, pBP, IMT, and IAD) in the pooled sample and in each of the study's sub-groups.
2- To investigate any association between the measurements monitored and the concentration of 25(OH)D in the pooled sample and in each of the study's sub-groups.
3- To elucidate any observable relationship between the monitored measurements.

## 7.2. Methods

### *7.2.1. Measurements of arterial stiffness using pulse wave velocity (PWV) and central blood pressure (cBP) measurement*

PWV and cBP were performed using a non-invasive pulse-wave velocity device (Complior, Alam Medical, Vincennes, France) and software (V1.9.4, Alam Medical, Vincennes, France). These were provided for the purpose of the study by Manchester Metropolitan University. Following training in the use of the equipment, and systematic assessment of the intra-day reliability of the results obtained, the study proper was resumed. The reliability pilot study was performed on 11 different participants (age 35 -51 years; seven females and four males; seven CG, three ARG, and one DAG) who were otherwise not included in the current study. Coefficient of variation (CV) [CV= standard deviation/mean) was below 5% on 3-5 repetitive tests for all outcome variables.

PWV and cBP assessments were performed in a quiet room where the temperature was controlled at 23° C (Ikonomidis et al., 2013). Prior to testing, the participants lay in a supine position, without a pillow or reclining seatback, on the examination bed for 10 minutes to allow for fluid equilibrium. Then, the relevant sensors were placed on the key anatomical sites, previously marked on the participant's skin at the neck and the wrist (for the carotid and radial arteries respectively). The correct positions to place the sensors were defined by palpation using the index and middle fingers to feel a pulse signal (Sztrymf et al., 2013).

The Complior software (v1.9.4, Vincennes, France) had a login username and password that were known only to the researcher. The programme asked for the participant's gender, identification name or ID (the current study used ID). Peripheral systolic blood pressure (pSBP) and diastolic blood pressure (DBP) would then be entered, prior to the measurement, which was taken using an automated electronic sphygmomanometer (CARESCAPE V100 Monitor, GE Healthcare, St. Louis, USA) (Appendix 12). Total body height (in centimetres) and mass (in kilogrammes), were assessed using a stadiometer (Doran Scales, Charles, IL, USA) and an electronic scale

(Doran Scales, Charles, IL, USA), respectively and these data were also inputted. The distance between the carotid and radial arteries was measured (in metres) and then inputted. After that, the sensors were positioned on the respective arteries at the pre-selected locations. The red sensor was used for the carotid artery using the neck, hands-free holder. The blue sensor was used for the radial artery using the wrist, hands-free holder. During the measurements, the participant's arm was not allowed to come in contact with the body, and the legs were kept sufficiently apart to avoid any possible damping of the signal (figure 7-2) (Sztrymf et al., 2013). The Complior screen displayed the pulse waves in the monitored arteries.

*Figure 7-1 The pulse wave analysis test being performed on a participant*

The PWV was calculated by the Complier software as the distance between the two measured points divided by the transit time between the waves (m/s) (figure 7-2). Once 10 valid pulses had been obtained, the acquisition could be processed, preferably only when the signal of quality score was above 80% (Complior analyser operator's manual, 2016).

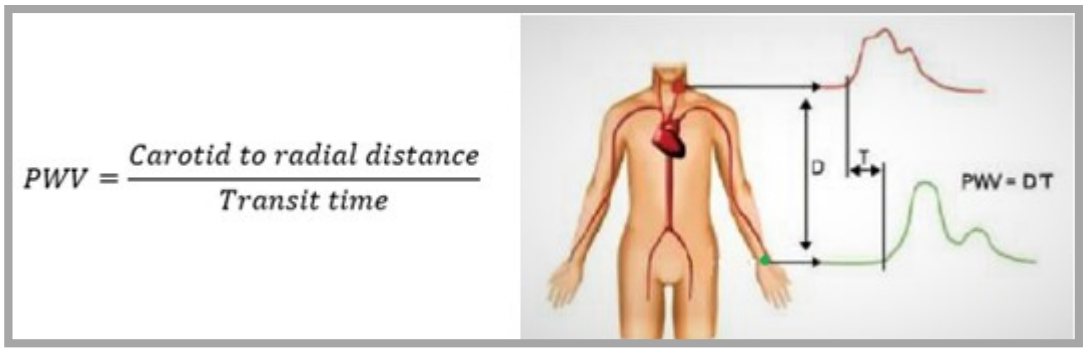


*Figure 7-2 Illustration of the method used to measure PWV but in the current study the arteries used were carotid- brachial- edited from the (Complior analyse operator's manual, 2016)*

The central blood pressure (cBP) is the pressure in the main large arteries (aorta and carotid). cBP is a better indicator of blood pressure than the standard peripheral blood pressure and it is more related to cardiovascular events (Pini et al., 2008; Sztrymf et al., 2013). It can be determined by measuring the central systolic blood pressure (cSBP). The diastolic blood pressure is the same by definition in the carotid and peripheral arteries (Pereira et al., 2014; Complior analyse operator's manual, 2016).

The test was repeated 2 to 3 times for validation. For accurate and fast results, the participant needed to be relaxed. Out of the 97 participants on the study it was not possible to obtain readings using the Complior software (v1.9.4, Vincennes, France) from six (6) patients. It is believed that this was due to the volunteers failing to relax because they were in a rush to leave or distracted by someone waiting for them. In each of the six cases, the researcher was unable to relieve the stress of the participants despite the attempts to do so. Thus, results from only 91 participants were included in the study from the pulse-wave velocity device.

The software displayed the result and saved it under the participant's profile. The data from the highest quality score recording (i.e. the one closest to 100% signal strength) was used in the study. An additional copy of the results was saved in an Excel file as a backup (Pereira et al., 2014; Complior analyse operator's manual, 2016). Figure 7-3 illustrate an example of the result and analysis sheet details of the pulse wave analysis software, Complior.

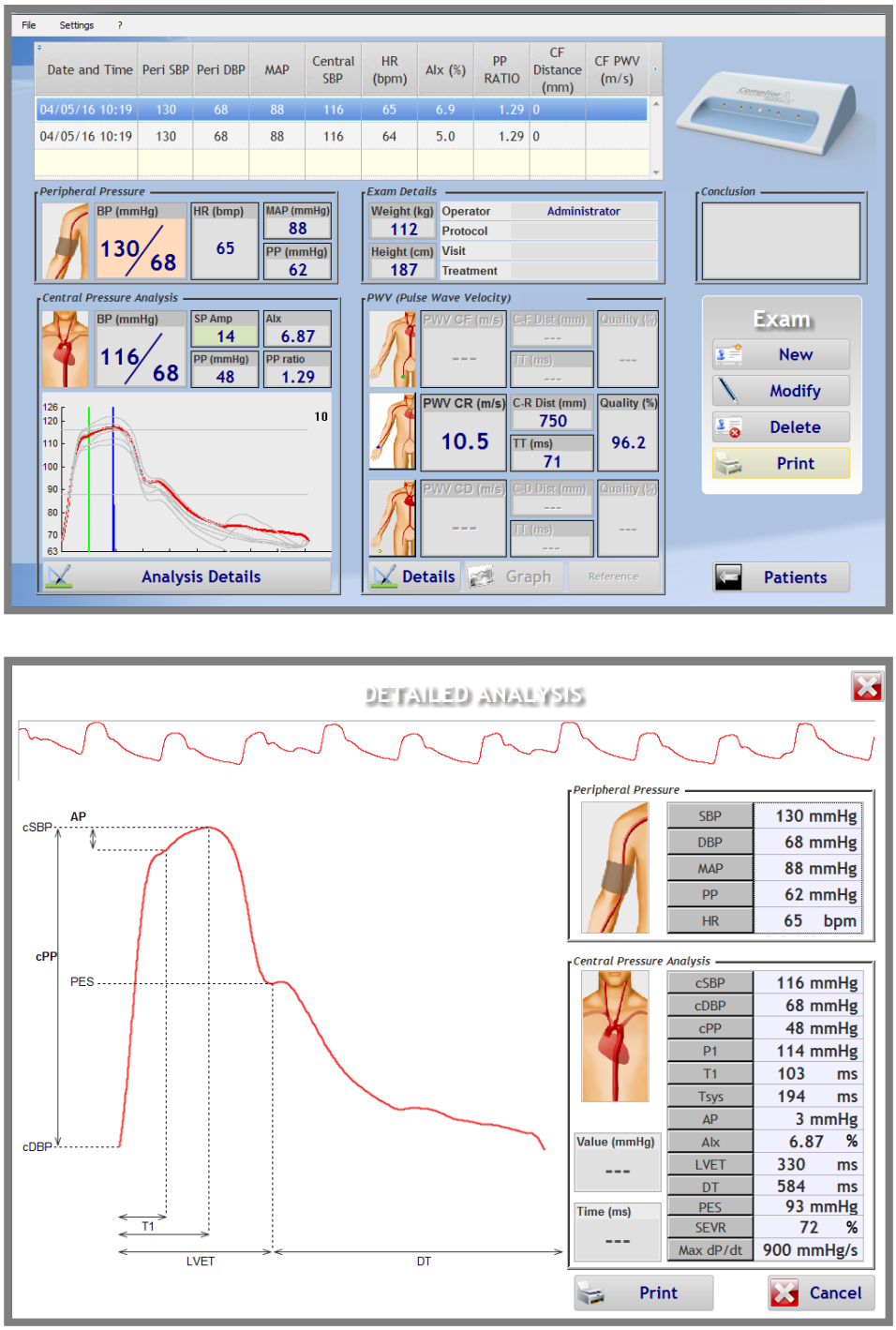





*Figure 7-3 Examples of the result and analysis sheet details of the pulse wave analysis software, Complior*

### *7.2.2. Carotid artery ultrasound measurements*

Carotid artery examinations were used to assess participants' arterial structure status. The examination was performed using an ultrasound (MyLab 70, Esaote, Genoa, Italy) that had a brightness mode as well as colour Doppler facilities. A high frequency, 10 MHz, linear array transducer was used for the examination. The participant had to be lying down flat with their head in a straight position (figure 7-4). The right side of the common carotid artery (CCA) was visualised first in the coronal view and then the probe is twisted along the sagittal plane. As a safety precaution, the colour Doppler mode was used for only up to 10 seconds maximum to ensure the right position on the CCA (Corretti et al., 2002). The angle of insonation was at 60° to eliminate the error in velocity measurements (Roman et al., 2006). Measurements of the carotid intima media thickness (IMT), carotid artery inter-adventitial diameter (IAD), heart rate (HR), resistance index (RI) and blood flow integral (BFI (total average velocity (TAV) + blood flow (BF)) were taken. Before starting data collection, intense training on the use of the ultrasound was performed. Risk assessment forms were written and signed. A pilot study was undertaken to assess the reliability of the results was performed on 11 different participants (see details above in section 7.2.1). The coefficient of variation (CV) [CV= standard deviation/mean) was < 5 % on three repeated readings of each parameters.


*Figure 7-4 The ultrasound test being performed on a participant*

#### *7.2.2.1. Carotid intima media thickness (IMT)*

To measure IMT, a clear ultrasound image had to be taken. The image had to show the carotid node (as an anatomical reference and standardised position point) and had to be taken at the end of the systolic phase, which is when the artery had its largest diameter. Measurements were taken in three different positions along the vessel, 10.00 Millimetres (mm) away from the carotid node. The distance from the lumen-intima interface to the media-adventitial interface is the measurement of IMT as shown in figure 7-5. IMT is considered normal <0.75 mm; at risk of atherosclerosis between the 0.75 mm and 1.00 mm; and as having atherosclerosis at > 1.00 mm (Polak et al., 2013; Taskiran et al., 2017).

#### *7.2.2.2. Carotid artery inter-adventitial diameter (IAD)*

The carotid artery diameter is the measurement of the inter-adventitial diameter (IAD). IAD is defined as the distance between the medial-adventitial interfaces on the near wall to the medial-adventitial interface on the far wall on the CCA as shown in figure 7-5 (Roman et al., 2006; Lloyd et al., 2012). The measurements were performed on a scan image at three different points 10 mm away from the node. The ultrasound images were taken during the systolic phase.

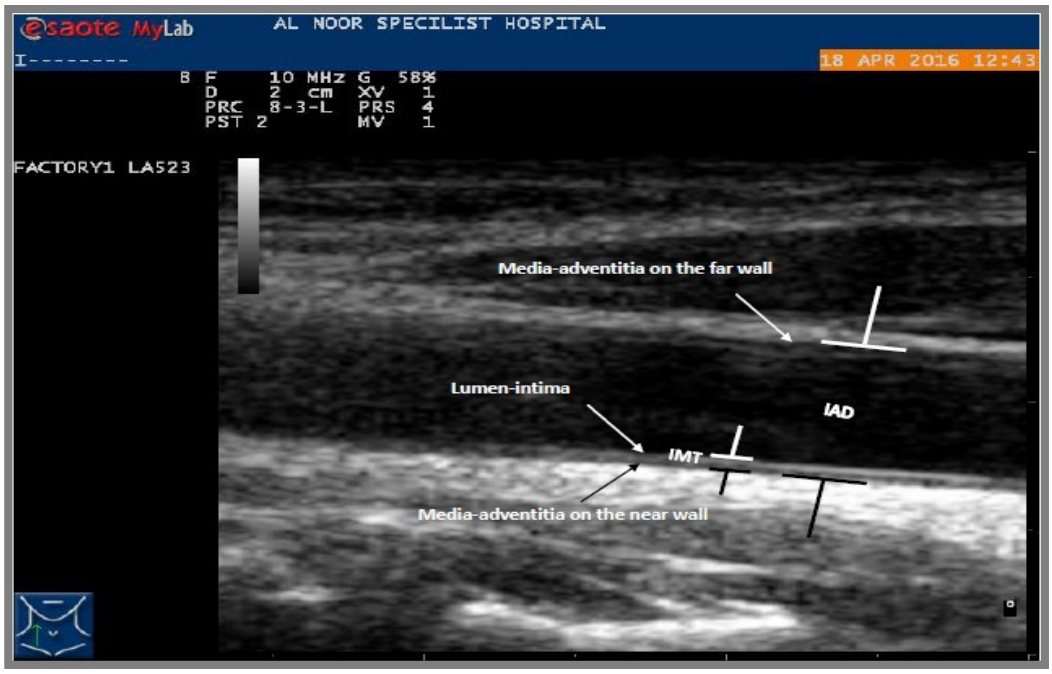


*Figure 7-5 Illustration of IMT and IAD measurements on a participant's CCA ultrasound image*

### *7.2.2.3. Additional vascular measurements*

Resistive Index (RI)

Resistive index is the measurement between the peak of the systolic velocity wave and the trough of the diastolic velocity wave as illustrated in figure 7-6. After recording at least ten clear waveforms, the measurement was taken on three different waves along the waveform. The average of the three measurements was recorded for each participant.

Blood flow integral (BFI)

Blood flow integral is the total average velocity (TAV) + blood flow (BF). The measurement was taken on three different waves along the waveforms. The measurement can be taken by measuring the velocity wave from the peak of the systolic velocity wave to the trough of the diastolic velocity wave, as illustrated in figure 7-6.

Heart Rate (HR)

Heart rate was measured using two methods in the current study. The first one was by using an electronic sphygmomanometer (CARESCAPE V100 Monitor, GE Healthcare, St. Louis, USA), which gives an automatic reading. The second method was through using the Doppler ultrasound in the CCA. At least ten clear waveforms were recorded. The measurement between two systolic velocity waves is the measure of heart rate as shown in figure 7-6. At least three measurements were taken, and the averages were counted to indicate the heart rate. A coefficient of variation of 95% was confirmed between the two methods, and then the average was calculated.

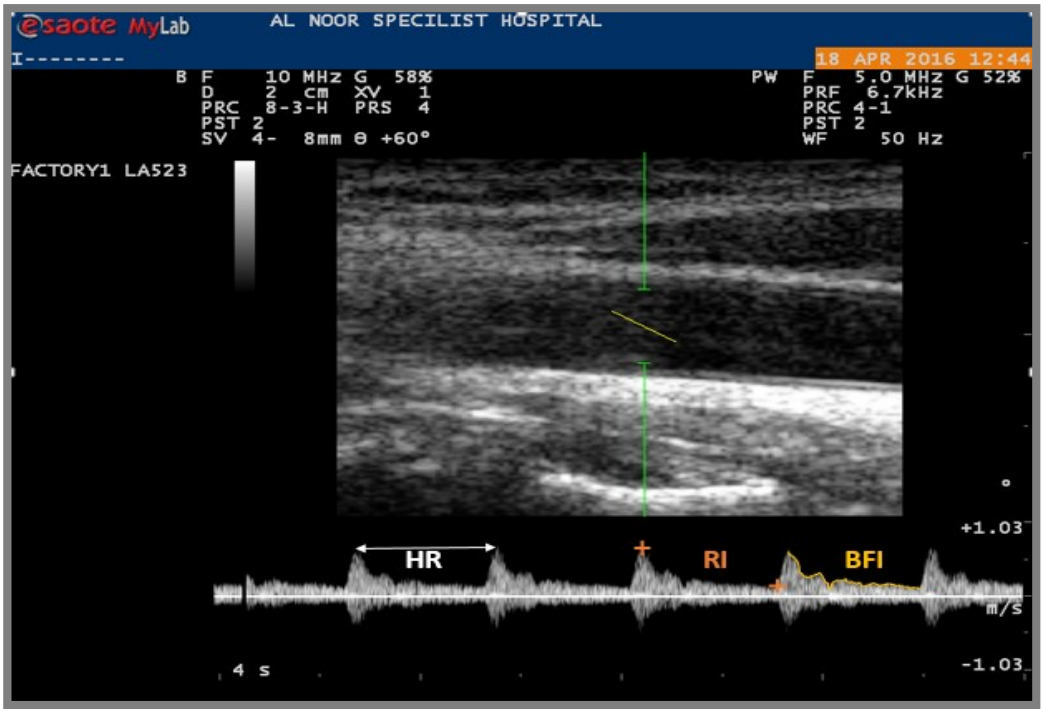


*Figure 7-6 Illustration of HR, RI, and BFI measurements on a participant's CCA ultrasound image*

## *7.2.3. Measurement of Serum 25 (OH) D*

Measurement of 25(OH)D was accomplished using an electrochemiluminescence binding assay. Details of the measurement are provided in section 4.3.1. of the current study.

## 7.3. Statistical analysis of the data

Statistical analysis was performed using SPSS v24 (Inc., Chicago, IL, USA). Data were tested for normal distribution using the Kolgomorov-Smirnov test. Similarly, homogeneity of variance was assessed using the Levene's test. Descriptive analysis is presented as mean ± standard deviation, median (interquartile range), and mean rank. In the current chapter, only data from IAD were normally distributed. One-way ANOVA, with Bonferroni corrected *post-hoc* pairwise mean comparisons, were carried out where there were 3 groups to compare (clinical groups). Independent t-test was used to compare two groups (i.e. gender). The rest of the chapter measurements were not normally distributed, as such data was deemed suitable for non-parametric statistical tests. Therefore, Kruskal-Wallis tests, with appropriate *post-hoc* Mann-Whitney tests were carried out where there were three groups, or simply Mann-Whitney tests for simple pairwise mean comparisons. Wilcoxon Signed test was carried out to compare the difference in SBP in peripheral and central arteries. Confidence Interval percentage (CI) was set at 95% for all of the data. Box and whiskers plots were used to illustrate medians, interquartile range, whiskers, outliers, and indicates significant between group differences.

Additionally, where data was continuous rather than grouped, Spearman Rho correlations were carried out. Multiple linear regression model was applied to try and ascertain whether 25(OH)D levels can be predicted from a composite of all the significant confounders if any. Statistical significance was set at $p \leq 0.05$.

## 7.4. Results

In this section, descriptive analysis of the vascular structural and functional characteristics were performed. Additionally, the relationships between 25(OH)D concentrations and the monitored characteristics were examined. It was not possible to obtain measurements using the pulse wave analysis device and the peripheral blood pressure monitor from six participants who were not able to relax for the procedure. Thus the total complete set samples (PWV, cSBP, pSBP, and DBP) was derived from 91 participants. It was possible to obtain ultrasound measurements (IMT, IAD, RI, TAV, BF) from all 97 participants. Data were analysed as a pooled sample and as sub-groups. First sub-group was the clinical groups ((control group (CG), at-risk group (ARG), and diagnosed with atherosclerosis group (DAG)). The second sub-group was gender (male, female). Table 7-1 shows the statistical mean and standard deviation of the chapter measurements within each of the clinical groups and the pooled study sample.

*Table 7-1 Descriptive analysis of the chapter parameters in each of the clinical groups and in the pooled sample. Data were presented as mean ± standard deviation. Parameters included carotid-radial pulse wave velocity (PWV), central systolic blood pressure (cSBP), Peripheral systolic blood pressure (pSBP), Diastolic blood pressure (DBP )-represent both peripheral and central-, Carotid intima-media thickness (IMT), Carotid artery inter-adventitial diameter (IAD), Resistive index (RI), total average velocity (TAV) & blood flow (BF), and Heart rate (HR).*

| Measurements | CG | ARG | DAG | Total sample |
|---|---|---|---|---|
| **PWV (m/s)** | 7.35 ± 4.81 | 9.87 ± 5.39 | 8.58 ± 3.58 | 8.57 ± 4.66 |
| **cSBP (mmHg)** | 105.4 ± 10.24 | 119.82 ± 18.9 | 125.64 ± 19.2 | 117.2 ± 18.62 |
| **pSBP (mmHg)** | 123.0 ± 15.5 | 133.17 ± 12.57 | 139.88 ± 18.26 | 132.06 ± 17.12 |
| **DBP (mmHg)** | 68.76 ± 11.15 | 73.0 ± 9.06 | 75.76 ± 9.74 | 72.53 ± 10.37 |
| **IMT (mm)** | 0.508 ± 0.121 | 0.603 ± 0.161 | 0.749 ± 0.261 | 0.622 ± 0.215 |
| **IAD (mm)** | 6.306 ± 0.602 | 6.64 ± 0.756 | 6.94 ± 0.914 | 6.63 ± 0.807 |
| **RI (cm/s)** | 0.745 ± 0.053 | 0.734 ± 0.062 | 0.783 ± 0.203 | 0.755 ± 0.129 |
| **TAV (cm/s)** | 19.366 ± 6.817 | 16.703 ± 6.01 | 16.072 ± 6.843 | 17.388 ± 6.678 |
| **BF (ml/min)** | 348.5 ± 121.3 | 335.8 ± 123.7 | 346.9 ± 146.6 | 344.03 ± 130.2 |
| **HR (bpm)** | 70.16 ± 10.2 | 78.9 ± 11.04 | 73.85 ± 14.77 | 74.16 ± 12.6 |

### *7.4.1. Analysis arterial stiffness by pulse wave velocity (PWV)*

#### *7.4.1.1. Descriptive analysis of PWV in the pooled sample and between sub-groups*

The carotid-radial measurements were performed in 91 participants with an average quality of the PWV measurement of 83.8 ± 14.19 %. Descriptive analysis of PWV analysis among the pooled sample and in sub-groups are shown in table 7-2. The minimum PWV value was 2.1 m/s and the maximum was 26.2 m/s. figures 7-7 illustrate the distribution of PWV in the pooled sample.

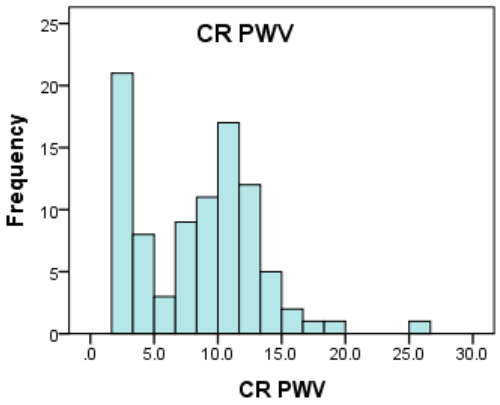


*Figure 7-7 Distribution of carotid-radial PWV among the pooled sample*

*Table 7-2 Descriptive statistic of CR PWV (m/s) in the pooled sample and in Sub-groups*

| | N | Mean ± SD | Median (IQR) | Mean rank |
|---|---|---|---|---|
| **CG** | 30 | 7.35 ± 4.8 | 7.1 (7.6) | 37.68 |
| **ARG** | 28 | 9.56 ± 5.39 | 10.9 (8) | 53.59 |
| **DAG** | 33 | 8.58 ± 3.58 | 9.3 (5.8) | 47.12 |
| **Male** | 15 | 9.88 ± 5.43 | 10.5 (8.4) | 53.60 |
| **Female** | 76 | 8.31 ± 4.496 | 9.2 (7.9) | 44.50 |
| **Total** | 91 | 8.57 ± 4.66 | 9.3 (7.9) | |

### *7.4.1.2. PWV and the difference between sub-groups*

To investigate the differences between the sub-groups in their PWV, a series of statistical tests were performed. A Kruskal-Wallis test indicates no significant difference in PWV among the clinical groups CG, ARG, and DAG (P=0.069). Similarly, there was no variation in PWV between sex (P=0.223). Box and whiskers plots are illustrated in figure 7-8 show no significant variation

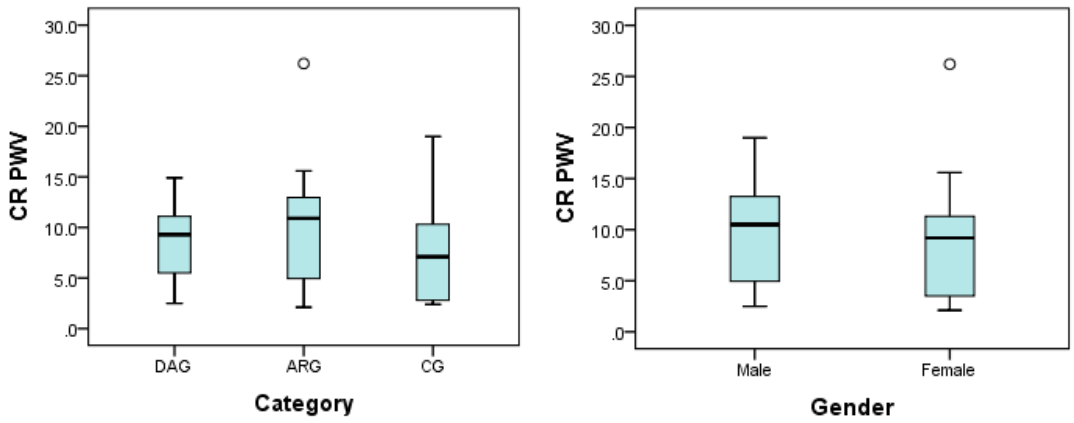


*Figure 7-8 A box and whiskers plot of PWV (m/s) among the clinical groups. Data shows medians, interquartile range, whiskers and outliers.*

### *7.4.1.3. Correlations between PWV and 25(OH)D and the rest of the vascular structural and functional characteristics.*

To examine the relationship between PWV and 25(OH)D concentrations Spearman correlation was performed and indicated no significant correlation (P=0.201) (table 7-3). Additionally, simple linear X-Y scatter plots were used to illustrate the lack of association between 25(OH)D status and PWV (figure 7-9).

*Table 7-3 Spearman correlations between 25(OH)D (ng/mL) and PWV (m/s)*

| | | | 25 (OH)D | CR PWV |
|---|---|---|---|---|
| Spearman's rho | 25 (OH)D | Correlation Coefficient | 1.000 | -0.089 |
| | | Sig. (1-tailed) | . | 0.201 |
| | | N | 97 | 91 |

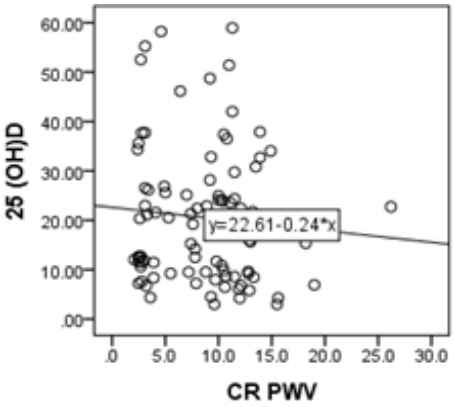


*Figure 7-9 X-Y scatter plot between PWV and 25(OH)D ng/mL.*

## *7.4.2. Analysis of peripheral and central blood pressure*

### *7.4.2.1. Descriptive analysis of peripheral and central blood pressure in the pooled sample and between sub-groups*

The pulse wave analysis software takes measurements of the central blood pressure as the central systolic blood pressure (cSBP). The average observation in the study sample was (mean ± SD) 132.06 ± 17.12 mmHg (table 7-5). The average diastolic blood pressure (DBP) was 72.53 ± 10.37 mmHg, which is the same in Peripheral and central assessments (table 7-1). The average peripheral systolic blood pressure (pSBP) was 132.06 ± 17.12 mmHg measured by the electronic sphygmomanometer (CARESCAPE V100 Monitor, GE Healthcare, St. Louis, USA) (table 7-4). The measurements were performed in 91 participants. Figures 7-10 illustrate distribution and descriptive statistic of pSBP and cSBP in the pooled sample.

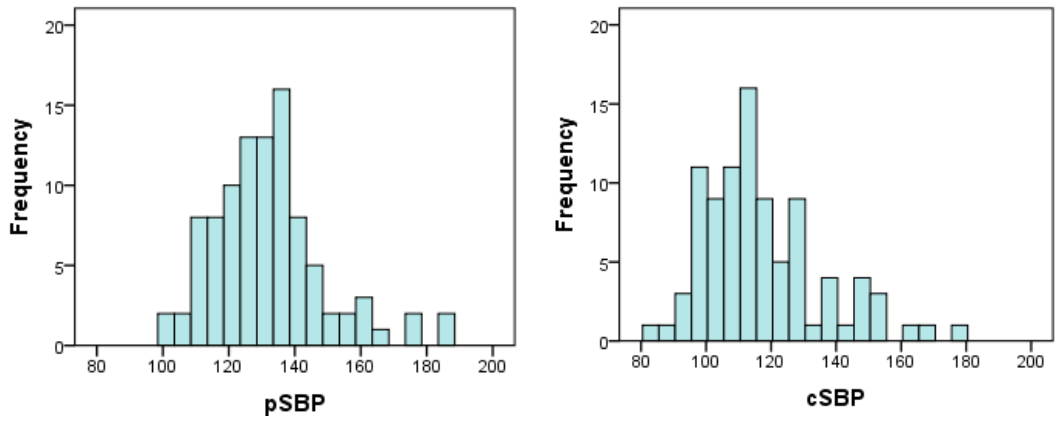

*Figure 7-10 Distribution of pSBP and cSBP among the pooled sample*

*Table 7-4 Descriptive statistic of pSBP in the pooled sample and in Sub-groups*

| pSBP | N | Mean ± SD | Median (IQR) | Mean Rank |
|---|---|---|---|---|
| **CG** | 30 | 121.4 ± 12.65 | 121.2 (20) | 29.35 |
| **ARG** | 28 | 133.6 ± 12.9 | 130 (15) | 49.88 |
| **DAG** | 33 | 139.2 ± 18.07 | 137 (18) | 57.85 |
| **Male** | 15 | 129.6 ± 10.369 | 130.2 (18) | 45.97 |
| **Female** | 76 | 131.97 ± 17.5 | 131 (18) | 46.01 |
| **Total** | 91 | 131.59 ± 16.58 | 131 (18) | |

*Table 7-5 Descriptive statistic of cSBP in the pooled sample and in Sub-groups*

| cSBP | N | Mean ± SD | Median (IQR) | Mean Rank |
|---|---|---|---|---|
| **CG** | 30 | 105.4 ± 10.24 | 106 (15) | 28.50 |
| **ARG** | 28 | 119.8 ± 18.9 | 117.5 (22) | 50.70 |
| **DAG** | 33 | 125.6 ± 19.2 | 123 (31) | 57.92 |
| **Male** | 15 | 115.6 ± 16.2 | 116 (20) | 47.10 |
| **Female** | 76 | 117.5 ± 19.140 | 112 (24) | 45.78 |
| **Total** | 91 | 117.18 ± 18.62 | 113 (22) | |

### *7.4.2.2. pSBP, cSBP and the difference between sub-groups*

To investigate the results further, a series of non-parametric tests were performed. The Kruskal-Wallis test indicates a significant difference between the clinical groups in pSBP ($\chi^2$ (2) = 19.28, P<0.0001). The differences were significant between CG, ARG (U=263, P=0.001) and CG, DAG (U=247.5, P<0.0001). However, there was no variation in pSBP between sexes (P=0.996). Box and whiskers plots of the pSBP between the clinical groups and sex are illustrated in (figure 7-11).

On the other hand, there was a significant difference between the clinical groups in cSBP ($\chi^2$ (2) = 20.79, P<0.0001). The differences were significant between CG, ARG (U=207.5, P=0.001) and CG, DAG (U= 182.5, P<0.0001). Additionally, there was no difference between sexes in cSPB (P=0.860). Box and whiskers plots of the cSBP between the clinical groups and sex are illustrated in (figure 7-12).

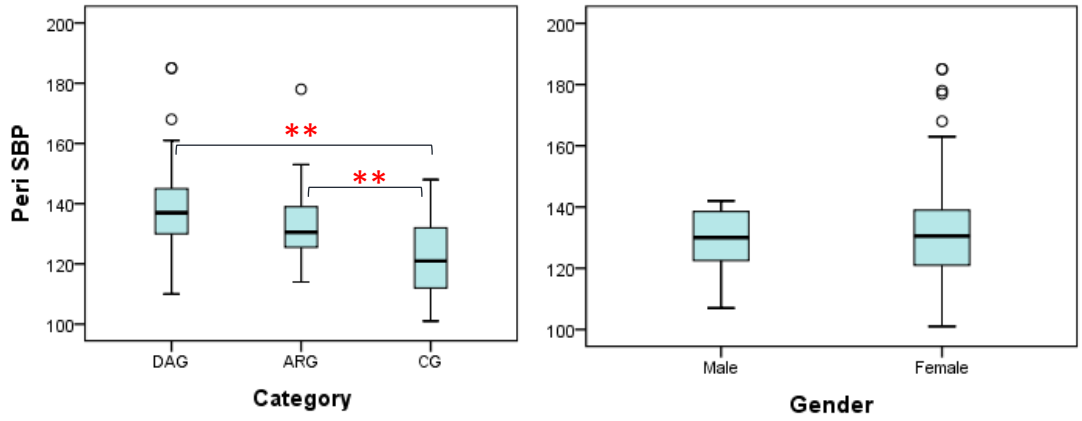


*Figure 7-11 Box and whiskers plots of pSBP, (mmHg) among the clinical groups and sexes. Data shows medians, interquartile range, whiskers and outliers. ** Indicates significant between group differences (P<0.01)*

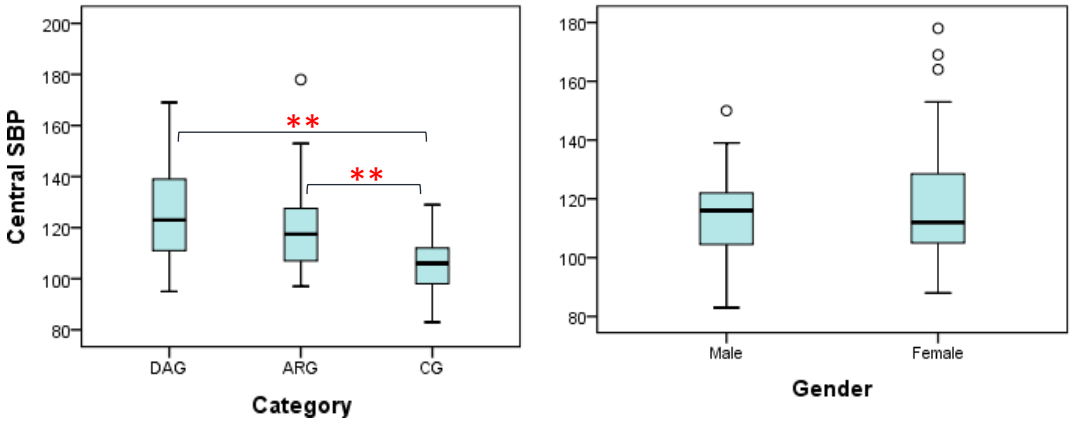


*Figure 7-12 Box and whiskers plots of cSBP, (mmHg) among the clinical groups and sexes. Data shows medians, interquartile range, whiskers and outliers. ** Indicates significant between group differences (P<0.01)*

For further investigation in the difference between peripheral SBP and central SBP a Wilcoxon Signed Rank test was performed. There was a significant difference between the pSBP and cSBP in the pooled sample (Z= -6.836, P< 0.0001). The test was performed again, after splitting the sample by the clinical groups, to further mine the data. The significant differences between pSBP and cSBP occurred within the CG (Z= -4.705, p< 0.0001), ARG (Z= -3.372, P< 0.0001), and DAG (Z= -4.003, P< 0.0001). Box and whiskers plots are shown in (figure 7-13).

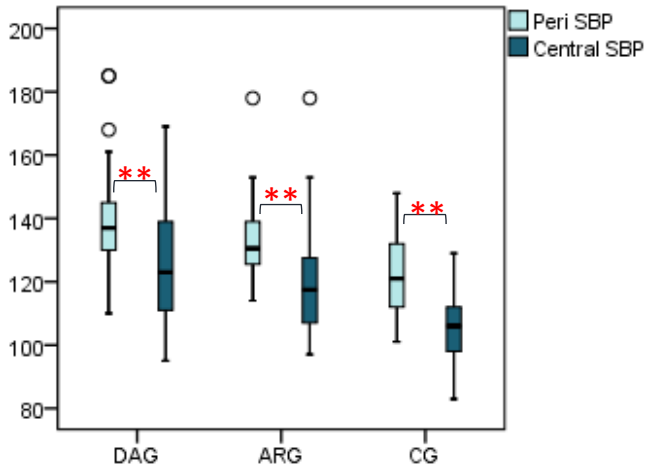


*Figure 7-13 Box and whiskers plot of pSBP and cSBP (mmHg) within the clinical groups. Data shows medians, interquartile range, whiskers and outliers. ** Indicates significant between group differences (P<0.01)*

### *7.4.2.3. Correlations between pSBP, cSBP and 25(OH)D and the rest of the vascular structural and functional characteristics.*

There was no significant Spearman's association between pSBP, cSBP and 25(OH)D in the pooled sample (table 7-6). Interestingly however, pSBP was significantly correlated to IMT (rho=0.390, P<0.0001) and IAD (rho= 0.279, P=0.003). cSBP was significantly correlated to IMT (rho=0.327, P=0.001) and IAD (rho=0.340, P<0.0001) (table 7-11). Scatter plot of 25(OH)D against pSBP and against cSBP are illustrated in figure 7-14.

When the sample was separated by the clinical groups and consumption of vitamin D supplements, a strong negative significant Spearman's correlation was observed between 25(OH)D and pSBP only in the DAG when taking vitamin D supplements (rho= -0.642, P=0.017, $R^2$=0.41). Conversely, cSBP was moderately correlated with 25(OH)D Also in the DAG when vitamin D supplements are not taken (rho= 0.360, P=0.050, $R^2$=0.31) (table 7-12).

*Table 7-6 Spearman correlations between 25(OH)D (ng/mL) and pSBP, cSBP (mmHg)*

| | | | 25 (OH)D | pSBP | cSBP |
|---|---|---|---|---|---|
| Spearman's rho | 25 (OH)D | Correlation Coefficient | 1.000 | -0.028 | 0.139 |
| | | Sig. (1-tailed) | . | 0.393 | 0.095 |
| | | N | 97 | 97 | 91 |
| | pSBP | Correlation Coefficient | | 1.000 | 0.690** |
| | | Sig. (1-tailed) | | . | 0.000 |
| | | N | | 97 | 91 |
| | cSBP | Correlation Coefficient | | | 1.000 |
| | | Sig. (1-tailed) | | | . |
| | | N | | | 91 |

*. Correlation is significant at the 0.05 level (1-tailed).
**. Correlation is significant at the 0.01 level (1-tailed).

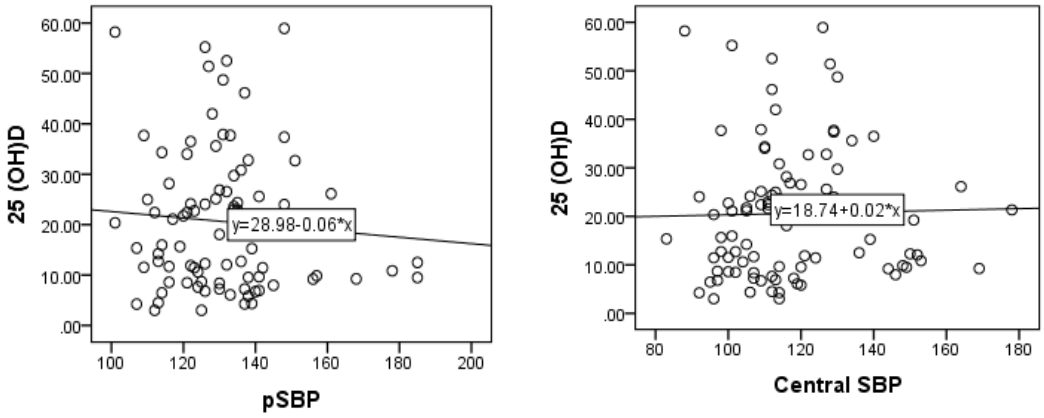


*Figure 7-14 X-Y scatter plots between pSBP, cSBP (mmHg) and 25(OH)D ng/mL.*

## *7.4.3 Analysis of carotid artery intima-media thickness (IMT) using ultrasound*

### *7.4.3.1. Descriptive analysis of IMT in the Pooled sample and between sub-groups*

Measurement of carotid artery intima-media thickness provided continuous data for 97 participants using MyLab 70 ultrasound (Esaote, Genoa, Italy). The minimum IMT in the current study was 0.32 mm and the maximum was 1.43 mm. Descriptive analysis of IMT values among the pooled sample and in sub-groups are presented in table 7-7. Distribution of IMT values among the pooled sample are represented in (figure 7-15). IMT is considered normal <0.75mm; at risk of atherosclerosis between

the 0.75mm and 1.00 mm; and atherosclerosis patients at > 1.00 mm (Polak et al., 2013; Taskiran et al., 2017). Based on the IMT measurements 83.5% of the participants had normal values of IMT (0.547 ± 0.1 mm), 9.3% were at risk (0.85 ± 0.06 mm), and 7.2% were atherosclerosis patients (1.19±0.16 mm) (figure 7-15)

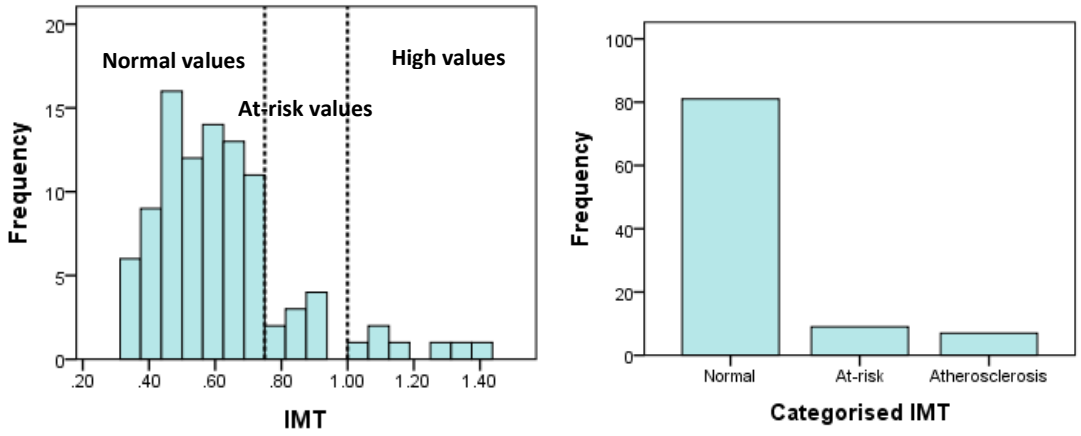


*Figure 7-15 Distribution of IMT values (mm) among the whole sample and categorised based on the IMT values. The dotted lines illustrate the cut off values for IMT.*

*Table 7-7 Descriptive analysis of IMT (mm) among the pooled sample and in sub-groups.*

| | N | Mean ± SD | Median (IQR) | Mean Rank |
|---|---|---|---|---|
| **CG** | 33 | 0.508 ± 0.12 | 0.49 (0.19) | 32.68 |
| **ARG** | 30 | 0.603 ± 0.16 | 0.57 (0.19) | 49.35 |
| **DAG** | 34 | 0.749 ± 0.26 | 0.658 (0.32) | 64.53 |
| **Male** | 15 | 0.64 ± 0.23 | 0.59 (0.39) | 51.87 |
| **Female** | 82 | 0.61 ± 0.21 | 0.57 (0.21) | 48.48 |
| **Total** | 97 | 0.622 ± 0.215 | 0.58 (0.21) | |

### *7.4.3.2. IMT values and the difference between sub-groups*

The difference in IMT values within the sub-groups has been tested. Analysis showed significant differences in IMT between the study clinical groups CG, ARG, and DAG using a Kruskal-Wallis test ($\chi^2$ (2) = 21.456, P<0.0001). The significant differences were between CG, ARG (U=304, P=0.009), CG, DAG (U=213.5, P<0.0001), and ARG, DAG (U= 329.5, P=0.015). However, a Mann-Whitney test on IMT by sex revealed no significant difference (P=0.668). Box and whiskers plots are illustrated in figure 7-16

shows the significant differences in FBG levels between the clinical groups and shows no sex bias.

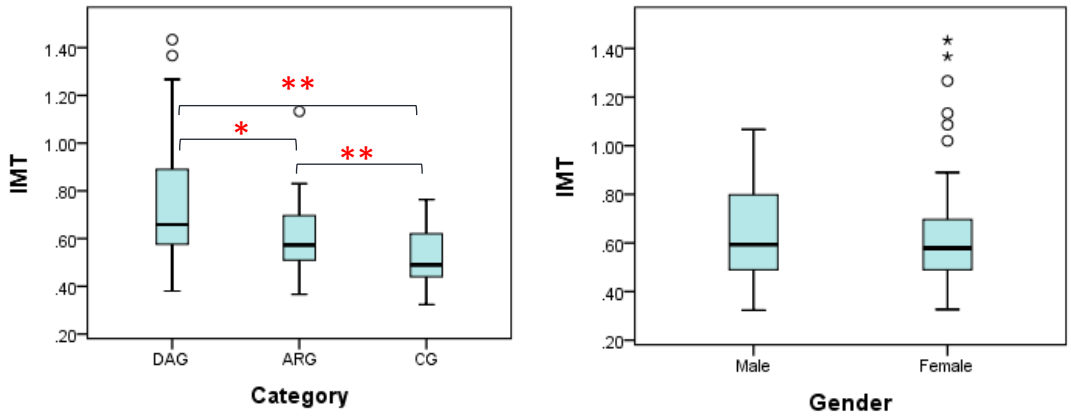


*Figure 7-16 A box and whiskers plot of IMT (mm) among the clinical groups and gender. Data shows medians, interquartile range, whiskers and outliers. Significant differences between groups indicated by (* = P<0.05) and (** = P<0.01).*

### *7.4.3.3. Correlations between IMT and 25(OH)D and the rest of the vascular structural and functional characteristics.*

No significant correlation was observed between 25(OH)D and IMT (P=0.414) (table 7-8). Similarly, there was no effect of 25(OH)D concentration on IMT levels (P=0.87) as illustrated in the simple X-Y scatter graph line in figure 7-17. However, IMT was significantly correlated to pSBP (rho=0.390, P<0.0001), cSBP (rho= 0.327, P=0.001), and IAD (rho=0.431, P<0.0001) (table 7-10).

When the sample was separated by the clinical groups and consumption of vitamin D supplements, a moderate negative significant Spearman's correlation was observed between 25(OH)D and IMT only in the CG when vitamin D supplements are not taken (rho= -0.358, P=0.033, $R^2$=0.13) (table 7-12).

*Table 7-8 Spearman correlations between 25(OH)D (ng/mL) and IMT (mm)*

| | | | 25 (OH)D | IMT |
|---|---|---|---|---|
| Spearman's rho | 25 (OH)D | Correlation Coefficient | 1.000 | -0.022 |
| | | Sig. (1-tailed) | . | 0.414 |
| | | N | 97 | 97 |

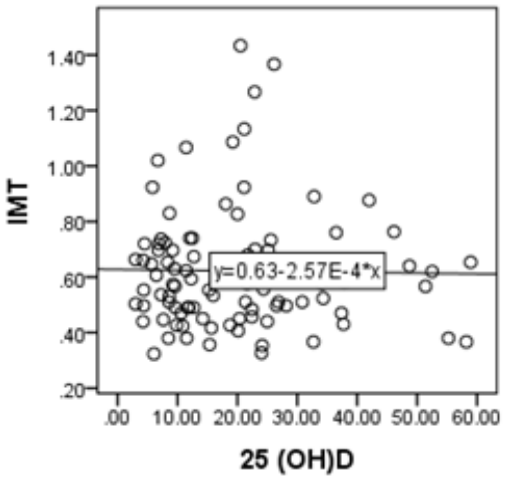


*Figure 7-17 Simple X-Y scatter plot between IMT (mm) and 25(OH)D ng/mL*

## *7.4.4. Analysis of carotid artery inter-adventitial Diameter (IAD) using ultrasound*

### *7.4.4.1. Descriptive analysis of IAD in the Pooled sample and between sub-groups*

IAD was measured in all 97 participants 6.63 ± 0.807 mm. The minimum value of IAD was 5.17 mm and the maximum was 8.73 mm. Figure 7-18 shows the distribution of IAD among the participants. Descriptive analysis of IAD levels among the clinical groups, gender in the total sample and are illustrated in (table 7-8)

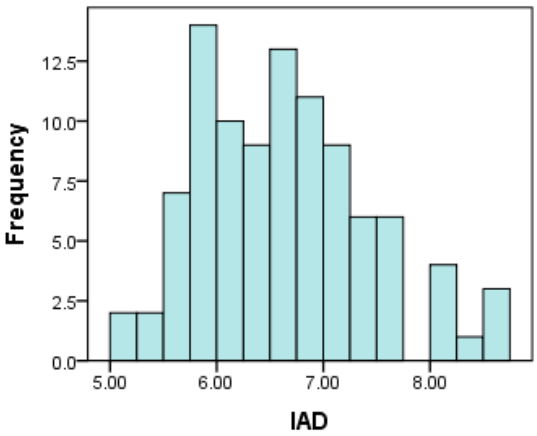


*Figure 7-18 Distribution of IAD values (mm) among the whole sample*

*Table 7-9 Descriptive analysis of IAD (mm) among the pooled sample and in sub-groups.*

| | N | Mean ± SD | Median |
|---|---|---|---|
| **CG** | 33 | 6.30 ± 0.6 | 6.13 (1) |
| **ARG** | 30 | 6.64 ± 0.756 | 6.58 (1.01) |
| **DAG** | 34 | 6.94 ± 0.91 | 6.93 (1.21) |
| **Male** | 15 | 7.03 ± 0.92 | 6.86 (1.33) |
| **Female** | 82 | 6.56 ± 0.766 | 6.5 (1.15) |
| **Total** | 97 | 6.63 ± 0.807 | 6.56 (1.20) |

### *7.4.4.2. IAD values and the difference between sub-groups*

To investigate the difference between the sub-groups in IAD a series of parametric statistical tests were performed. One-way ANOVA revealed significant mean differences between the clinical groups [F (2, 94) = 5.68, P=0.005]. The *post-hoc* test indicates that the mean difference was only between CG and DAG (df= -0.63, P=0.003). Additionally, there was a significant difference between males and females in their IAD values when independent t-test was performed (T=0.47, P=0.036). Figure 7-19 illustrates box and whiskers plots of the difference in IAD within the sub-groups.

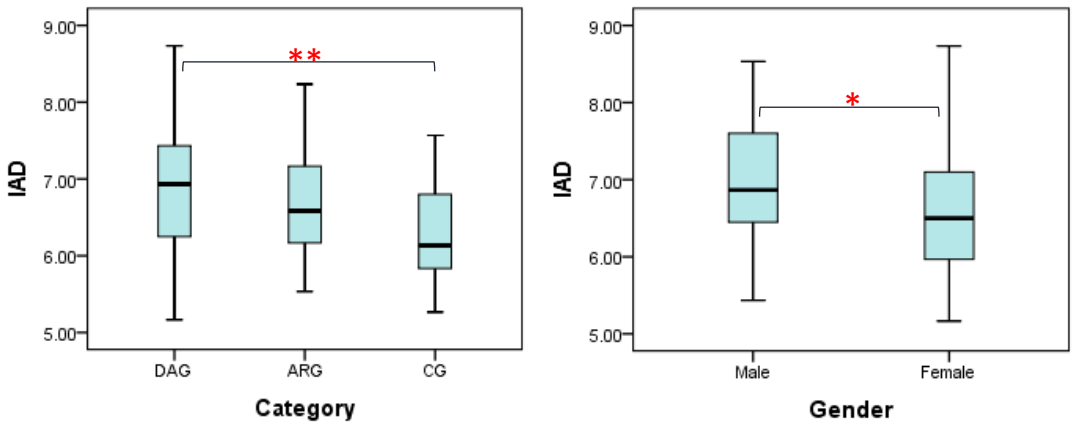


*Figure 7-19 Box and whiskers plots for IAD values (mm) within clinical groups and gender. Data shows medians, interquartile range, whiskers and outliers. Significant differences between groups indicated by (* = P<0.05) and (** = P<0.01).*

*7.4.4.3. Correlations between IAD and 25(OH)D and the rest of the vascular structural and functional characteristics.*

No significant correlation was observed between 25(OH)D and IAD (P=0.164) (table 7-10). Similarly, there was no effect of 25(OH)D concentration on IAD levels (P=0.258) as illustrated in the X-Y scatter graph line in figure 7-20. Conversely, IAD were significantly correlated to pSBP (rho=0.279, P=0.003), cSBP (rho=0.340, P<0.0001), and IMT (rho=0.431, P<0.0001) (table 7-10).

*Table 7-10 Spearman correlations between 25(OH)D (ng/mL) and IAD (mm)*

| | | | 25 (OH)D | IAD |
|---|---|---|---|---|
| Spearman's rho | 25 (OH)D | Correlation Coefficient | 1.000 | -0.100 |
| | | Sig. (1-tailed) | . | 0.164 |
| | | N | 97 | 97 |

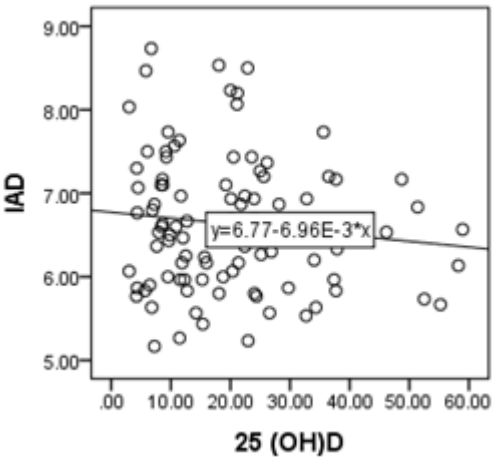


*Figure 7-20 Simple X-Y scatter graph between IAD (mm) and 25(OH)D ng/mL*

### *7.4.5. Additional vascular measurements RI, TVA, BF, and HR*

A preview of the mean ± SD of RI, TVA, BF and HR in the pooled sample and in each of the clinical groups is illustrated in table 7-1. The primary analysis of those measurements has shown similar results to results from the main indicator of vascular function measurement IMT and IAD. In addition, Spearman's correlation shows no significant correlation between 25(OH)D concentration and those vascular measurements. Thus, further detailed analysis was not performed.

### *7.4.6. Correlations between 25(OH)D and the study vascular structural and functional characteristics, and multiple linear regression model*

Spearman correlations were performed to investigate the relationship between 25(OH)D concentration the vascular structural and functional characteristics. The test revealed no significant correlations between 25(OH)D concentration and any of the measurements in the chapter (table 7-11). Given the lack of significant association between vascular structure/ function and 25(OH)D, the multiple linear regression model was not performed in this chapter.

*Table 7-11 Spearman's rho between the vascular structural and functional characteristics and 25(OH)D levels.*

| | | | 25 (OH)D | CR PWV | pSBP | DBP | cSBP | IMT | IAD | RI | TVA | BF | HR |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Spearman's rho | 25 (OH)D | Correlation Coefficient | 1.000 | -0.089 | -0.028 | -0.083 | 0.139 | -0.022 | -0.100 | -0.119 | -0.028 | -0.106 | -0.006 |
| | | Sig. (1-tailed) | . | 0.201 | 0.397 | 0.217 | 0.095 | 0.414 | 0.164 | 0.124 | 0.392 | 0.150 | 0.478 |
| | | N | 97 | 91 | 91 | 91 | 91 | 97 | 97 | 97 | 97 | 97 | 97 |
| | CR PWV | Correlation Coefficient | | 1.000 | 0.027 | 0.056 | -0.080 | -0.067 | 0.103 | 0.065 | -0.087 | -0.039 | 0.118 |
| | | Sig. (1-tailed) | | . | 0.401 | 0.299 | 0.226 | 0.264 | 0.167 | 0.271 | 0.207 | 0.356 | 0.134 |
| | | N | | | 91 | 91 | 91 | 91 | 91 | 91 | 91 | 91 | 91 |
| | pSBP | Correlation Coefficient | | | 1.000 | 0.513** | 0.690** | 0.393** | 0.268** | 0.043 | -0.238* | -0.142 | 0.214* |
| | | Sig. (1-tailed) | | | . | 0.000 | 0.000 | 0.000 | 0.005 | 0.344 | 0.011 | 0.090 | 0.021 |
| | | N | | | 91 | 91 | 91 | 91 | 91 | 91 | 91 | 91 | 91 |
| | DBP | Correlation Coefficient | | | | 1.000 | 0.416** | 0.129 | 0.128 | -0.172 | -0.072 | -0.081 | 0.272** |
| | | Sig. (1-tailed) | | | | . | 0.000 | 0.111 | 0.114 | 0.052 | 0.250 | 0.221 | 0.004 |
| | | N | | | | 91 | 91 | 91 | 91 | 91 | 91 | 91 | 91 |
| | cSBP | Correlation Coefficient | | | | | 1.000 | 0.327** | 0.340** | -0.100 | -0.096 | 0.075 | 0.098 |
| | | Sig. (1-tailed) | | | | | . | 0.001 | 0.000 | 0.173 | 0.183 | 0.238 | 0.178 |
| | | N | | | | | 91 | 91 | 91 | 91 | 91 | 91 | 91 |
| | IMT | Correlation Coefficient | | | | | | 1.000 | 0.431** | 0.278** | -0.275** | -0.053 | 0.006 |
| | | Sig. (1-tailed) | | | | | | . | 0.000 | 0.003 | 0.003 | 0.303 | 0.475 |
| | | N | | | | | | 97 | 97 | 97 | 97 | 97 | 97 |
| | IAD | Correlation Coefficient | | | | | | | 1.000 | 0.101 | -0.259** | 0.254** | -0.101 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Sig. (1-tailed) | | | | | | | . | 0.163 | 0.005 | 0.006 | 0.163 |
| | | N | | | | | | | 97 | 97 | 97 | 97 | 97 |
| | RI | Correlation Coefficient | | | | | | | | 1.000 | -0.397** | -0.354** | -0.075 |
| | | Sig. (1-tailed) | | | | | | | | . | 0.000 | 0.000 | 0.233 |
| | | N | | | | | | | | 97 | 97 | 97 | 97 |
| | TVA | Correlation Coefficient | | | | | | | | | 1.000 | 0.785** | 0.028 |
| | | Sig. (1-tailed) | | | | | | | | | . | 0.000 | 0.392 |
| | | N | | | | | | | | | 97 | 97 | 97 |
| | BF | Correlation Coefficient | | | | | | | | | | 1.000 | 0.004 |
| | | Sig. (1-tailed) | | | | | | | | | | . | 0.485 |
| | | N | | | | | | | | | | 97 | 97 |
| | HR | Correlation Coefficient | | | | | | | | | | | 1.000 |
| | | Sig. (1-tailed) | | | | | | | | | | | . |
| | | N | | | | | | | | | | | 97 |

**. Correlation is significant at the 0.01 level (1-tailed).
*. Correlation is significant at the 0.05 level (1-tailed).

*Table 7-12 Spearman's rho corelations between the vascular structural and functional characteristics and 25(OH)D levels in consideration of the consumption of vitamin D supplements among the clinical groups*

| Take VitD supplements | Category | | | CR PWV | pSBP | DBP | cSBP | IMT | IAD | RI | TVA | BF | HR |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Yes | DAG | 25 (OH)D | Correlation Coefficient | 0.264 | -0.642* | -0.521 | -0.284 | 0.042 | -0.289 | 0.294 | 0.322 | 0.203 | 0.049 |
| | | | Sig. (1-tailed) | 0.216 | 0.017 | 0.050 | 0.199 | 0.448 | 0.181 | 0.177 | 0.154 | 0.264 | 0.440 |
| | | | N | 11 | 11 | 11 | 11 | 12 | 12 | 12 | 12 | 12 | 12 |
| | ARG | | Correlation Coefficient | -0.049 | 0.326 | 0.283 | 0.428 | 0.070 | -0.021 | 0.042 | 0.301 | 0.007 | 0.308 |
| | | | Sig. (1-tailed) | 0.440 | 0.151 | 0.187 | 0.083 | 0.414 | 0.474 | 0.448 | 0.171 | 0.491 | 0.165 |
| | | | N | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 |
| | CG | | Correlation Coefficient | 0.551 | -0.086 | -0.029 | 0.000 | -0.371 | 0.377 | -0.371 | -0.371 | -0.429 | -0.143 |
| | | | Sig. (1-tailed) | 0.129 | 0.436 | 0.479 | 0.500 | 0.234 | 0.231 | 0.234 | 0.234 | 0.198 | 0.394 |
| | | | N | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| No | DAG | 25 (OH)D | Correlation Coefficient | -0.211 | 0.057 | 0.113 | 0.360* | 0.360 | 0.069 | -0.437* | 0.339 | 0.238 | -0.071 |
| | | | Sig. (1-tailed) | 0.173 | 0.401 | 0.308 | 0.050 | 0.050 | 0.379 | 0.021 | 0.061 | 0.143 | 0.377 |
| | | | N | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 |
| | ARG | | Correlation Coefficient | -0.206 | 0.150 | -0.108 | 0.192 | -0.342 | -0.338 | -0.061 | -0.350 | -0.494* | -0.047 |
| | | | Sig. (1-tailed) | 0.222 | 0.289 | 0.346 | 0.238 | 0.082 | 0.085 | 0.405 | 0.077 | 0.019 | 0.426 |
| | | | N | 16 | 16 | 16 | 16 | 18 | 18 | 18 | 18 | 18 | 18 |
| | CG | | Correlation Coefficient | 0.018 | -0.070 | -0.239 | 0.044 | -0.358* | -0.040 | 0.078 | -0.021 | -0.149 | 0.075 |
| | | | Sig. (1-tailed) | 0.466 | 0.373 | 0.130 | 0.419 | 0.033 | 0.421 | 0.350 | 0.458 | 0.230 | 0.354 |
| | | | N | 24 | 24 | 24 | 24 | 27 | 27 | 27 | 27 | 27 | 27 |

*. Correlation is significant at the 0.05 level (1-tailed).
**. Correlation is significant at the 0.01 level (1-tailed).

## 7.5. Discussion

### *7.5.1 Discussion of Arterial stiffness using pulse wave velocity (PWV)*

PWV was performed between the carotid-radial arteries. The average for signal quality reached in this study was 83.8%, which is above 80% (the desired signal) (Pereira et al., 2014; Complior analyse operator's manual, 2016). PWV has been proposed to be an independent indicator for atherosclerosis (Collaboration, 2010; Pereira et al., 2014). However, the current study did not find significant differences between the clinical groups for PWV. Mean values for PWV were 7.35 ± 4.81 for the CG, 9.87 ± 5.39 for the ARG, and 8.58 ± 3.58 m/s for the DAG. Additionally, there was no significant correlation observed between 25(OH)D concentration and PWV measurements. The current data are similar to observations by Arora and Rehan (2015), who also tested the association between 25(OH)D and cardiovascular disease risk and observed no significant association between 25(OH)D and PWV (P=0.93). However, the findings of the current study are unlike another study by Al Mheid et al., (2011), who examined the relationship between 25(OH)D and PWV measurement and found that low 25(OH)D concentrations were inversely associated with PWV values (adjusted $R^2$=0.42, P=0.04).

The gold standard for PWV measurement is the carotid-femoral arterial measurement. However, in the current study, the carotid-radial arteries were used as the participants were from a conservative society that is not comfortable participating in a study if the thigh area is one of the points where the sensor is to be placed. The use of carotid-to-radial arteries instead of the carotid-to-femoral arteries could be an explanation for the absence of difference between the clinical groups and the absence of a correlation with 25(OH)D. Another issue that could affect the PWV results is the signal quality. Despite numerous attempts to improve the signal quality by insuring the position of the sensors, encouraging the participants to relax, removing the source of stress if it is possible, and explaining any worries they have from the examination. Out of all of the participants, 27.5% had PWV measurements exhibited a below 80% signal quality.

### *7.5.2. Discussion peripheral and central blood pressure*

Hypertension is considered to be one of the major risk factors for atherosclerosis and cardiovascular disease (Hamburg et al., 2008; Sibley et al., 2014). In the current study, that association was clearly evident. Both peripheral and central blood pressure were significantly different between the study groups (P<0.0001). The control group (CG) had the lowest pSBP and cSBP (123.0 ± 15.5 and 105.4 ± 10.24 mmHg respectively) compared to the at-risk group (ARG) (133.17 ± 12.57 and 119.82 ± 18.9 mmHg respectively) and the diagnosed with atherosclerosis group (DAG) (139.88 ± 18.26 and 125.64 ± 19.2 mmHg respectively). The difference was significant (P<0.001) in both pSBP and cSBP between the CG and ARG. Additionally, the difference was similarly significant (P<0.0001) in both pSBP and cSBP between the CG and DAG. The current study indicates that both pSBP and cSBP are associated with the incidence and progression of atherosclerosis and cardiovascular disease. In addition, both pSBP and cSBP were positively correlated with the other markers of atherosclerosis, IMT (P<0.0001, P<0.001 respectively) and IAD (P<0.01, P<0.0001 respectively).

Conversely, both pSBP and cSBP did not show correlations with 25(OH)D concentration in the pooled sample. This is similar to observations in a study by Gepner et al. (2012) who examined the association between 25(OH)D and cardiovascular risk factors in a randomised, double-blind, placebo-controlled study. They did not observe any correlation between pSBP and 25(OH)D (P=0.976) nor between cSBP and 25(OH)D (P=0.66) in neither the supplemented nor the non-supplemented groups. Since the current study is an observational study, the absence of the correlations between the factors can be expected.

On the other hand, other correlations were observed only in the DAG. The pSBP was correlated with 25(OH)D in the DAG only in the group who took vitamin D supplements (P<0.05). Additionally, when vitamin D supplements were not consumed, there was a significant correlation between cSBP and 25(OH)D in the DAG (P<0.05) as well. There are many studies that have observed associations between blood pressure and 25(OH)D concentration (Forman et al., 2007; Reis et al., 2009; A. Zittermann, 2014). Since there are conflicting results between these studies, more

research is needed to confirm the relationship between blood pressure and 25(OH)D concentration.

### *7.5.3. Discussion of carotid artery intima-media thickness (IMT)*

IMT is an important risk marker of atherosclerosis (Constantinescu et al., 2012; Simova, 2015). A common artery IMT thicker than above 1.00 mm is an indicator of atherosclerosis and endothelial dysfunction (Flammer et al., 2012; Awad and Abbas, 2017). In the current study, the measurements were performed using a colour Doppler ultrasound (MyLab 70, Esaote, Genoa, Italy) in a quiet, temperature controlled room as illustrated in section 7.2.2. of the thesis. The results on N= 97 participant show that there were significant differences in IMT measurement between the clinical groups, as expected. The differences were between the CG and ARG (U=304, P=0.009), the CG and DAG (U=213.5, P<0.0001), and the ARG and DAG (U= 329.5, P=0.015). The CG had the lowest IMT values 0.508 ± 0.121 mm, then the ARG 0.603 ± 0.161 mm, and the highest IMT values were in the DAG 0.749 ± 0.261 mm. Additionally, IMT was significantly correlated with the other vascular structural and functional characteristics pSBP (P<0.0001), cSBP (P=0.001), and IAD (P<0.0001). These observations confirmed the clinical condition of the participants.

However, IMT was not significantly correlated to 25(OH)D concentration in the pooled sample, which supports what has previously been observed in other studies by Taskiran et al. (2017) and Reis et al. (2009). Taskiran et al. (2017) tested the relationship between IMT and 25(OH)D concentration in 93 diabetes sufferers in an observational study and found no association. Reis et al. (2009) tested the association between IMT and $1,25(OH)_2D$ in older adults without a history or incidence of cardiovascular disease. The study was cross-sectional and did not observe any significant correlation in the pooled sample. However, they observed an association in a sub-group of hypertensive patients (P=0.036); for each 1 SD increase in $1,25(OH)_2D$, the IMT decreased by 0.050 mm. Additionally, Targher et al. (2006) found a negative correlation between 25(OH)D and IMT in diabetic patients, but did not observe any association in healthy participants. In contrast to a sub-group of

healthy participants (CG) in the current study, where there was a significant correlation between 25(OH)D and IMT ($P<0.05$) in the group that did not take vitamin D supplements. These results from all the studies discussed show that the relationship between vitamin D status and intima-media thickness could be affected by many factors and it is not a direct association.

### *7.5.4. Discussion of carotid artery inter-adventitial diameter (IAD)*

The common carotid artery inter-adventitial diameter (IAD) is another risk marker for atherosclerosis (British Heart Foundation, 2017). Tt is an arterial structure measurement that was assessed using an ultrasound (MyLab 70, Esaote, Genoa, Italy) of the carotid artery section 7.2.2. A larger IAD is associated with a higher risk and incidence of cardiovascular diseases (Krejza et al., 2006; Lloyd et al., 2012). In the current study there was a significant difference in IAD measurements between the control group (CG) and the diagnosed with atherosclerosis group (DAG) ($P<0.01$). This indicates that atherosclerosis patients have a larger IAD compared to healthy people. The CG had an IAD measurement of 6.306 ± 0.602 mm which was the lowest compared to the other clinical groups, 6.64 ± 0.756 mm in the ARG and 6.94 ± 0.914 mm in the DAG, as expected. Additionally, there was an expected significant gender variation ($P<0.05$). Males had a larger IAD than females, 7.03 ± 0.92mm, and 6.56 ± 0.807 mm respectively. Males naturally have larger arteries than females (British Heart foundation, 2017). Furthermore, the IAD was significantly correlated with the other vascular structural and functional characteristics, pSBP ($P=0.003$), cSBP ($P<0.0001$), and IMT ($P<0.0001$). This indicates that there is an association between IAD and the clinical status of the participants.

As with the other vascular structural and functional characteristics in this study, IAD was not significantly correlated with 25(OH)D concentration. Additionally, to our knowledge, no study has previously examined the association between 25(OH)D and common carotid artery inter-adventitial diameter (IAD).

## 7.6. Summary and conclusion

In the current study, with the exception of the PWV outcome measures, all of the vascular structural and functional characteristics (pSBP, cSBP, IMT, and IAD) were good indicators of the participants' cardiovascular health condition. The parameters were significantly different between the study clinical groups. In addition, they were significantly correlated with each other. However, none of the measurements was significantly correlated with circulating 25(OH)D concentrations in the pooled sample. In the sub-groups some correlations were observed, but these were influenced by the consumption of vitamin D supplements. A consensus on the presence or absence of correlations, bearing in mind both our data and previous research, has yet to be reached.

In conclusion, the study indicates that pSBP, cSBP, IMT, and IAD are good indicators of atherosclerosis risk factors and incidence. Nevertheless, the parameters were not correlated to 25(OH)D concentrations. The relationship between vitamin D status and atherosclerosis risk factors and incidence needs further investigation.

# Chapter 8 - General discussion, final conclusion, study limitations and future work

## 8.1. General discussion

The current study has investigated the association between vitamin D status and atherosclerosis risk factors and incidence in a sample of Saudi Arabia dwellers. The current study agreed with many other studies that found a relatively high prevalence of vitamin D deficiency among adults living in Saudi Arabia (Al Faraj and Al Mutairi, 2003; Ardawi et al., 2012; Aljefree et al., 2017a). The results show that 51.5% of the participants were vitamin D deficient (25(OH)D < 20 ng/mL) with a mean value of 9.88 ± 4.2 ng/mL, and 28.9% had an insufficient concentration of vitamin D in their blood plasma (25(OH)D 20 ≤ 30 ng/mL) (23.37 ± 2.57 ng/mL). Only 19.6% of the participants had adequate concentrations of vitamin D (25(OH)D > 30 ng/mL) (42.13 ± 9.28 ng/mL). 25(OH)D status was significantly correlated with the use of vitamin D supplements, dietary intake of vitamin D (DI-VitD), total intake of vitamin D (TI-VitD), exposure to sunlight, and age in the pooled sample of the study.

The associations between 25(OH)D concentrations and all of the sources of the vitamin (vitamin D supplements, diet, and sun exposure) were indicators of the importance of all of these sources in obtaining optimal concentrations of vitamin D (Lehmann and Meurer, 2010; Holick et al., 2011). Further analysis of the data in this thesis indicates that only vitamin D supplementation and diet are reliable predictors of the vitamin status, as suggested by the multiple linear regression model.

The final multiple linear regression stable model that predicts serum 25(OH)D concentration for the current study is the model that incorporated only the consumption of vitamin D supplements and dietary intake of vitamin D (DI-VitD) (out of a possible 27 potential modulators investigated). The stable model explains 39.6% of the variance in 25(OH)D and is statistically significant and has low shrinkage of R (adjusted $R^2$= 0.383), thereby indicating good statistical power. The predictors of this model are all significant, consumption of vitamin D supplements (P=0.0001) and DI-VitD (P=0.019) (Table 4-17). Thus, in summary, 25(OH)D blood concentration can be predicted as was discussed in section 4.2.6 using the following equation:

| $\boldsymbol{Y = (B1 * -0.019) + (B2 * -17.579) + 54.433 \pm 10.55}$ |
| --- |
| Y= 25(OH)D concentrations (ng/mL)<br>B1= DI-VitD in IU<br>B2= 1 if using vitamin D supplements or 2 if not using vitamin D supplements |

On the other hand, observation of the data in consideration of the use of vitamin D supplements and the clinical groups (control group (CG), at-risk group (ARG), and the diagnosed with atherosclerosis group (DAG)) provided interesting insights in the inter-relationship between atherosclerosis markers. When vitamin D supplements were used, 25(OH)D levels were associated with CRP only in the ARG, and pSBP in the DAG group. In contrast, in participants who were not using vitamin D supplements, 25(OH)D concentrations were correlated to TG in the ARG, cSBP in the DAG, and IMT, in the CG. On the other hand, the participants' health status, as represented by the study's clinical groups were significantly different in terms of age, WHR, CRP, FBG, LDL, TG, pSBP, cSBP, IMT, and IAD measurements. This indicates an association between those markers and the incidence and development of atherosclerosis.

Previous studies have not agreed on a consistent outcome that illustrates the relationship between 25(OH)D and atherosclerosis risk factors and markers. In a study by Al Mheid et al. (2011) on healthy participants, 25(OH)D was associated only with the LDL levels from the lipids profile test and with PWV and FMD from the arterial measurements. No association was observed with the rest of the blood profile measurements. Another double-blind study examined the impact of vitamin D supplementations on cardiovascular disease markers in healthy participants (Zittermann et al., 2009). The study observed a significant difference between the placebo and supplement groups on TG and inflammation markers of cardiovascular disease, but there was no association with body weight and the rest of the blood profile measurements. The inconsistent association could be because of the substantial number of factors that affect the association such as the form of measured vitamin D, the participants' health condition, and the study design.

Furthermore, in the studies that focus on of the cardiovascular disease risk factors, such as diabetes or hypertension, there were always associations between vitamin D status and cardiovascular disease (Targher et al., 2006; Zittermann et al., 2009; Yiu et al., 2011) that could be because when the damage is there, the difference can be easily distinguished. The association between vitamin D and these major risk factors of disease have been observed by many previous studies (Talaei et al., 2013; Al-Daghri, 2016; Qadhi, 2016); as such, vitamin D status is considered as providing a valid and reliable early warning system in the prevention of these risk-factor diseases such as diabetes, hypertension, and hyperlipidaemia.

Moreover, it has been observed that in order for the association between vitamin D status and cardiovascular disease to be observed, longitudinal (as opposed to the cross-sectional design in the present study and/or in the majority of the published literature) studies need to be performed. Indeed, the studies that were longitudinal observed the change before and after giving vitamin D supplements, thereby reporting a significant impact of vitamin D on cardiovascular health markers (Reis et al., 2009; A. Zittermann, 2014; Aljefree et al., 2016). In support of the longitudinal design's importance, whilst even in observational studies such as the one by Degerud (2016), the association between 25(OH)D and atherosclerosis progression was not observed after one year of follow-up. After 12 years of follow up, there was an inverse association between low 25(OH)D and cardiovascular mortality.

The issue of vitamin D deficiency is still occurring in Saudi Arabia in addition to the increasing number of atherosclerosis disease cases and deaths. Some healthcare professionals are aware of the situation, but decisive action is needed to control the prevalence of vitamin D deficiency in Saudi Arabia.

### *8.1.1. Contribution of the thesis*

The current study is the first, to our knowledge, to evaluate the effects of vitamin D from a multitude of sources and to hence systematically examine the association between serum 25(OH)D and atherosclerotic disease and its risk factors in Saudi Arabia. The present study developed a model to measure sun exposure based on the weather, cultural and social factors in Saudi Arabia, with consideration of the guidelines by the PMCO. In addition, the current study developed a dietary vitamin D FFQ, based on the diet used by Saudi people, and an analysis program to determine total intake of vitamin D. Moreover, the study developed an equation to assess vitamin D status from dietary intake and use of vitamin D supplements. The current study additionally used a novel assessment method of markers of atherosclerosis which could be used in studies that examine the impact of any potential dietary habits in atherosclerosis incidence and development. This programme of studies is the first, to our knowledge, to use the pulse wave velocity (PWV) and central blood pressure (cBP) measurements of arterial stiffness in Saudi Arabia and the Middle East. The vascular structural and functional characteristic measurements were novel for the current study. This study investigated the gaps in research in the area and contributed to a number of novel findings. As for the remainder of the gaps in knowledge in the area of vitamin D deficiency impact on cardiovascular health, suggestions have been provided to inform future research.

## 8.2. Final conclusion

The current study confirms the continued prevalence of vitamin D deficiency in Saudi Arabia. The sources of vitamin D that showed the biggest impact on vitamin D status were the use of vitamin D supplements and diet. On the other hand, the measured markers of atherosclerosis and its risk factors were significantly correlated with one another except for the PWV. Nevertheless, this study did not observe associations between vitamin D status and atherosclerosis risk factors and incidence in the pooled sample. 25(OH)D was observed to be associated with some markers and this, only in sub-groups of vitamin D supplements users. Additionally some of the study measurements such as of age, WHR, CRP, FBG, LDL, TG, pSBP, cSBP, IMT, and IAD showed desired values with the participants' health condition that defines their atherosclerosis status. This observational programme of studies investigated the bivariate associations without accounting for the multitude of other potential modulators of the relationships under investigation. Further research, such as longitudinal experimental studies, is needed to clarify the point at which vitamin D levels become critical to markers of atherosclerosis.

## 8.3. Study limitations

The results of the current programme of studies might have been affected by some limitations. First is the study design, as an observational cross-sectional set of studies, there was an advantage in examining the cases without any additional factors that could affect the data. However, this made it difficult to distinguish any causal nature in any of the associations. One of the reasons for limiting the study to this design was that the hospital where the data was collected would not approve a longitudinal design. Additionally, a longitudinal design would have reqired at least six to eight months extra time for data collection and time was limited for this study. The use of medication (such as Statins) by some of the participants will have affected the results of the study. Moreover, the self-reporting questionnaire could have errors linked to responses affected by the social desirability effects. The use of carotid-radial instead of carotid-femoral PWV to assess arterial stiffness was one of the study limitations. Finally, the unequal number of participant between the sexes may have influenced the study's power to determine any sex variations.

## 8.4. Recommendations for future work

- Recommendations on sun exposure and vitamin D fortification and supplementation in Saudi Arabia have been published recently (in 2016) by the PMCO. However, the current study has highlighted the need for awareness and additional studies to test the best methods to reach optimal vitamin D levels.
- The current study developed a model to measure sun exposure and total intake of vitamin D. Both models could be used in an awareness intervention study to test the effectiveness of sun exposure and dietary vitamin D intake.
- The current study was an observational study that was free from any intervention that could affect the study results. The next step should be taking the novel design of the current study and use it in experimental, longitudinal studies by adding vitamin D supplements to the diet of the three clinical groups and observing the impact of these on the atherosclerosis disease markers used in the current study. In fact, a long duration (up to 12 months) impact of sun exposure, diet, and use of supplements on atherosclerosis patients appears to be the preferable option for future work.
- The current study tested serum 25(OH)D status, however, the availability of the vitamin D receptors needs to be tested on the endothelial function to better assess the association between circulating, free vitamin D, and its function through receptors on endothelial cells.
- Assessment of vitamin D content in food items consumed in Saudi Arabia such as Camel's liver and certain types of mushrooms should be conducted to cover the gap in literature regarding this data.

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# Appendices

*Appendix 1- Ethical approval from the Faculty of Research Degrees Committee at Manchester Metropolitan University*

**FACULTY OF HOLLINGS**

Manchester Metropolitan University

**MEMORANDUM**

**TO** Wedad Azhar

**FROM** Megan Schofield

**DATE** 23 February 2016

**SUBJECT** Application for Ethical Approval **(HOLL151601)**

---

On the 23 February 2016 the Head of Ethics for Hollings considered your application for Ethical Approval (HOLL151601) entitled "the effect of vitamin D deficiency on atherosclerosis patients living in Saudi Arabia". The application has been granted Favourable Opinion and you may now commence the project.

MMU requires that you report any Adverse Event during this study immediately to the Head of Ethics (Dr David Tyler) and the Administrator (Megan Schofield). Adverse Events are adverse reactions to any modality, drug or dietary supplement administered to subjects or any trauma resulting from procedures in the protocol of a study.

An Adverse Event may also be accidental loss of data or loss of sample, particularly human tissue. Loss of human tissue or cells must also be reported to the designated individual for the Human Tissue Authority licence. Please notify Professor Craig Banks of any issues relating to this.

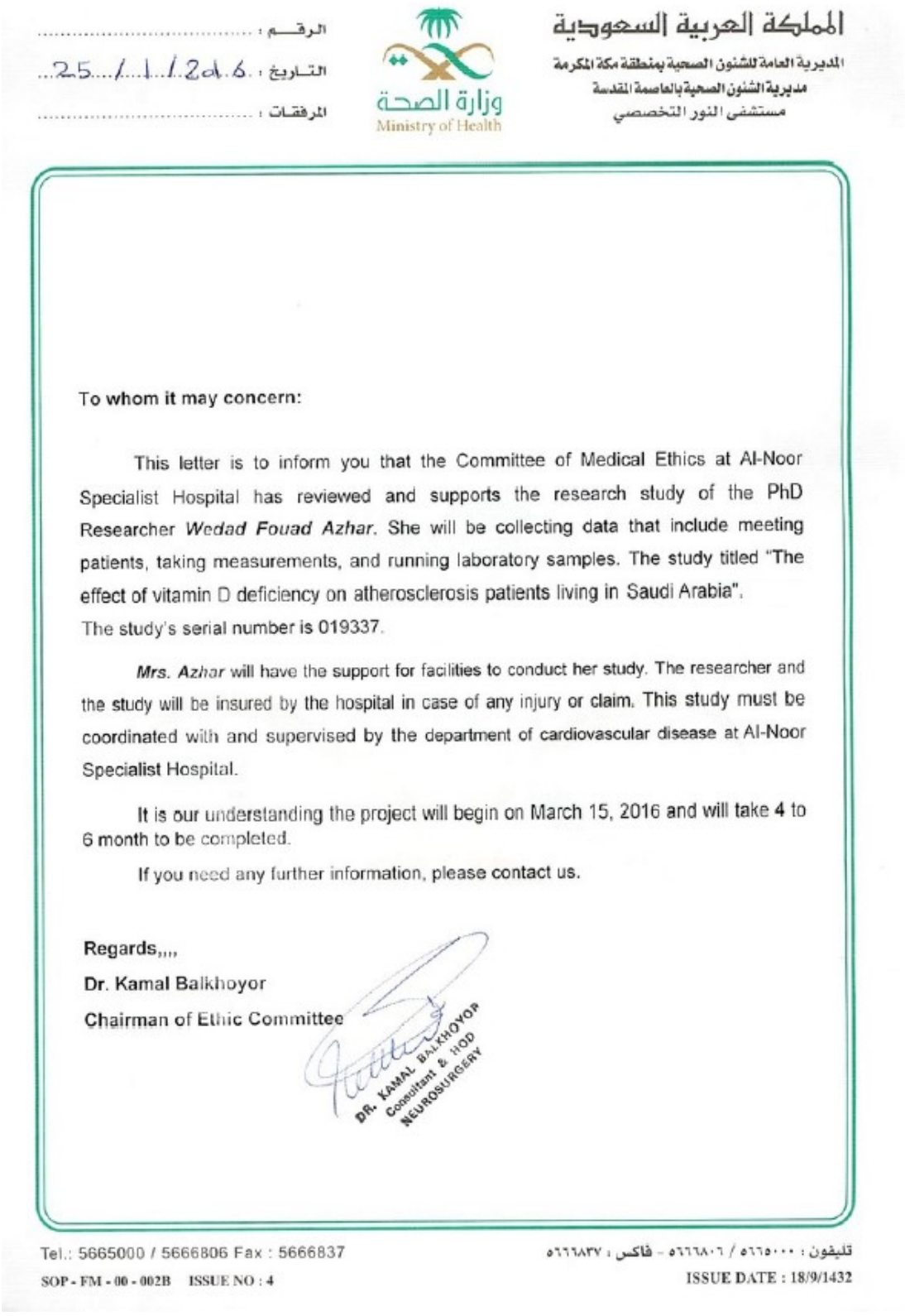
If you make any changes to the approved protocol these must be approved by the Faculty Head of Ethics. If amendments are required you should complete the MMU Request for Amendment form (found on the Graduate School website) and submit it to the Administrator.

Regards

Megan Schofield
Research Degrees Group Officer

Page **1** of **1**

*Appendix 2- Ethical Approval from the Committee of Medical Ethics at Al-Noor Specialist Hospital in Saudi Arabia*



المملكة العربية السعودية
المديرية العامة للشئون الصحية بمنطقة مكة المكرمة
مديرية الشئون الصحية بالعاصمة المقدسة
مستشفى النور التخصصي

وزارة الصحة
Ministry of Health

الرقم : .....
التاريخ : 25/1/2016
المرفقات : .....

To whom it may concern:

This letter is to inform you that the Committee of Medical Ethics at Al-Noor Specialist Hospital has reviewed and supports the research study of the PhD Researcher *Wedad Fouad Azhar*. She will be collecting data that include meeting patients, taking measurements, and running laboratory samples. The study titled "The effect of vitamin D deficiency on atherosclerosis patients living in Saudi Arabia".
The study's serial number is 019337.

*Mrs. Azhar* will have the support for facilities to conduct her study. The researcher and the study will be insured by the hospital in case of any injury or claim. This study must be coordinated with and supervised by the department of cardiovascular disease at Al-Noor Specialist Hospital.

It is our understanding the project will begin on March 15, 2016 and will take 4 to 6 month to be completed.

If you need any further information, please contact us.

Regards,,,,
Dr. Kamal Balkhoyor
Chairman of Ethic Committee

DR. KAMAL BALKHOYOR
Consultant & HOD
NEUROSURGERY

Tel.: 5665000 / 5666806 Fax : 5666837
SOP - FM - 00 - 002B ISSUE NO : 4

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ISSUE DATE : 18/9/1432

*Appendix 3 Information sheet -English*

Wedad Azhar
Food & Nutrition
Faculty of Health
Manchester Metropolitan University
Tel: 07

Participant Information Sheet

**The effect of vitamin D deficiency on atherosclerosis patients living in Saudi Arabia**

You have been invited to participate in a research study that is designed to identify the best markers of vitamin D and its impact on atherosclerosis prevention and treatment.

Vitamin D is an essential vitamin that is required for the maintenance of good health. It is obtained either through exposure to sun light or dietary sources.

Vitamin D have an important role in bone health and musculoskeletal outcomes such as increasing muscle strength and performance. In addition, it has been recently proposed to play a critical role in the immune system and a broad range of organ functions such as the cardiovascular system.

The study you are being asked to participate in will require you to answer questions on your social and economic status, education level, daily exposure to sunlight, smoking status, consumption of food items that has vitamin D and consumption of supplements and medication.

The researcher will take ten ml of blood sample from you by needle to be analysed for your vitamin D status, lipids profile, and glucose level. The researcher have a phlebotomy certificate and she is well trained to take blood samples.

In addition, the researcher will use non-intensive methods to assess your cardiovascular status. She will measure your blood pressure from your left hand using a blood pressure monitor, which will apply a slight pressure for approximately 30 seconds. The Researcher will also apply ultrasonography measurements on your right carotid artery to assist your cardiovascular status. She will take measurements on your vessel diameter, intima Media thickness, blood flow integral, heart rate and

resistance index. She will need to use gel on the right side of you neck and she will apply very slight pressure using the linear probe.

The researcher will use another device to measure central blood pressure and arterial stiffness. The device is connected to sensors that will be placed above your Carotid to the Radial arteries (on the neck to the forearm).

The researcher is appropriately trained to take all of the measurements and to use all of the equipment's for this study.

The data will be collected and stored in accordance with the Data Protection Act 1998 and will be disposed of in accordance of Human Tissue Act (2004). All Information that we obtain during this study will be maintained confidentially in a locked cabinet that is maintained in a locked office that only the investigator will have access too. Your name will not be associated with this research and all data will be maintained using confidential code numbers. Results of this study will be presented at a national forum in Saudi Arabia/United Kingdom and will be submitted for publication in an internationally recognized, peer reviewed health publication. All published results will present only group data. Subject names will not be used in any presentations/publications.

Your participation in this study is voluntary and you may withdraw at any time without negative consequences regarding your health care.

Thank you…

Wedad Azhar

PhD Researcher

Manchester Metropolitan University

Faculty member at Um Al-Quraa University

Saudi phone number: 050

*Appendix 4- Information sheet Arabic*

Wedad Azhar
Food & Nutrition
Department of Health Professions
Manchester Metropolitan University

Manchester Metropolitan University

**وثيقة معلومات**

**موضوع البحث / مدى تأثير نقص فيتامين د على مرضى تصلب الشرايين الذين يعيشون في المملكة العربية السعودية**

لقد تم إختيارك للمشاركة في هذا البحث الذي صمم لدراسة أفضل الطرق لتحديد حالة فيتامين د ومدى تأثيرة في الوقاية من مرض تصلب الشرايين وعلاجة.

يعتبر فيتامين د من الفيتامينات الضرورية للحفاظ على صحة جيدة وكذالك للحفاظ على قوة وسلامة العظام والعضلات في الجسم. علاوة على ذالك، أكتشف حديثاً أن لفيتامين د دور أساسي في تقوية جهاز المناعة كما أن له تأثير فعال في صحة العديد من أعضاء الجسم منها القلب والأوعية الدموية.

للمشاركة في هذا البحث نرجو منك التكرم بتعبئة الإستبيان الملحق والذي يتضمن أسئلة عامة عن حالتك الإجتماعية و الإقتصادية و مستوى التعليم وكذالك بعض الأسئلة المتعلقة بمدى التعرض لأشعة الشمس، التدخين، وعن إستهلاك الغذاء و المكملات الغذائية والأدوية.

للمشاركة في هذا البحث ستؤخذ منك عينة دم لتحليل مستوى لفيتامين د , والدهون, والسكر في الدم. بالإضافة إلى ذالك سيقوم الباحث بأخذ قياسات للشريان السباتي بواسطة جهاز الموجات فوق صوتيه (الألتراساوند). وسيتم قياس تصلب التدابن والضغط المركزي بجهاز أخر بوضع حساسات على الرقبة والساعد لدقائق.

عزيزي المشارك، نفيدك بخصوصية مشاركتك في هذا البحث بحيث لن يستخدام إسمك في البحث نهائياً بل سيتم الإشاره إليك برموز سرية، كما سيتم التعامل مع جميع المعلومات والبيانات المعطاة بشكل سري، وسيتم حفظها في خزنة مغلقة في مكتب مغلق بحيث لايمكن لأحد غير الباحث الإطلاع عليها.

إن نتائج هذا البحث سوف تُعرض وتنشر في مؤتمرات و ندوات ومجلات علمية داخل المملكة العربية السعودية وخارجها مع الحفاظ على خصوصية المعلومات.

نشكر لكم تعاونكم ...

وداد فؤاد أزهر
باحثة دكتوراة في جامعة مانشستر متروبوليتان
عضو هيئة تدريس في جامعة أم القرى - قسم العلوم الطبية التطبيقية
جوال: 05[redacted]

*Appendix 5- Consent form English*

Date
Wedad Azhar
Food & Nutrition
Faculty of Health
Manchester Metropolitan University

Tel: 07

Manchester Metropolitan University

**Consent Form**

**Title of Project:** the effect of vitamin D deficiency on atherosclerosis patients living in Saudi Arabia

**Name of Researcher:**
Wedad Azhar

**Participant Identification Code for this project:**

| | **Please initial box** |
|---|---|
| 1. I confirm that I have read and understood the information sheet dated …. for the above project and have had the opportunity to ask questions about the interview procedure. | ☐ |
| 2. I understand that my participation is voluntary and that I am free to withdraw at any time. | ☐ |
| 3. I understand that my responses will be recorded and used for analysis for this research project. | ☐ |
| 4. I understand that my responses will remain anonymous. | ☐ |
| 5. I give permission for my medical record to be inspected by the researcher. | ☐ |
| 6. I agree to take part in the above research project. | ☐ |

| | | |
|---|---|---|
| _____ | _____ | |
| Name of Participant | Date | Signature |
| _____ | _____ | |
| Researcher | Date | Signature |

*To be signed and dated in presence of the participant*

*Once this has been signed, you will receive a copy of your signed and dated consent form and information sheet at the same time.*

*Appendix 6- consent form Arabic*

**رمز تعريف للمشاركين وإقرار المشاركة في البحث**

إسم المشارك/ .....

رقم الجوال/ .....

رقم الملف/ .....

**إقرار**

1- أقر بأني قرأت و فهمت وثيقة المعلومات المرفقة بهذا البحث وأنه كان لدي الفرصة للإستفسار عن أي شئ في البحث. ☐

2- أقر بأن مشاركتي في هذا البحث تطوعية وأنه بإمكاني الإنسحاب في أي وقت. ☐

3- أقر بأن معلوماتي المعطاة ستستخدم في البحث وفي تحليل النتائج. ☐

4- أقر بأن مشاركتي سوف تُمثل برمز بدلاً عن إسمي. ☐

5- أوافق على المشاركة في هذا البحث. ☐

| | | |
|---|---|---|
| إسم المشارك ..... | التاريخ ..... | التوقيع ..... |
| إسم الباحثة ..... | التاريخ ..... | التوقيع ..... |

## نموذج إقرار بالموافقة على المشاركة بدراسة بحث/دراسة سريرية

**Confidential**

**بتوقيعي على هذا الإقرار أقر بأني لم أتنازل عن أي من حقوقي القانونية أو أخلي أي طرف من المسؤولية عن التقصير.**

| | | |
|---|---|---|
| اسم المشارك طباعة | التوقيع | التاريخ |
| اسم الوصي القانوني/طباعة<br>إذا كان المريض قاصر ا | التوقيع | التاريخ |
| أسم الشاهد /طباعة<br>إذا وافق المريض شفهيا ولم يوقع الموافقة | التوقيع | التاريخ |
| اسم الباحث الرئيسي/طباعة | التوقيع | التاريخ |
| اسم المقدم/طباعة<br>الذي قام بشرح/تقديم الوثيقة | التوقيع | التاريخ |

لا يجوز استخدامه أو إفشاؤه أو نشره أو كشفه بأي طريقة
بدون موافقة مستشفى النور التخصصي

## *Appendix 7- Questionnaire English*

**Questionnaire**

**The effect of vitamin D deficiency on atherosclerosis patients living in Saudi Arabia**

**PART1: PERSONAL INFORMATION**

**File Number:** ……..

**Phone number**:……

1) **What is your gender?**
   ☐ Male ☐ Female

2) **Year of birth / age:** ……

3) **City of Birth:** ……

4) **Place of living:** ……..

5) **What is the category of your employment?**

   ☐ Education ☐ Health ☐ Public sector ☐ Private sector ☐ Housewife

   ☐ Retired ☐ Other ……

6) **Which best describes your main occupation?**
   ☐ Mainly indoors
   ☐ Half indoor and half outdoor
   ☐ Mainly outdoor

7) **Income position (according to family income) by SR**
   ☐ Less than 5,000 ☐ 5,000 to 10,000
   ☐ 10,000 to 15,000 ☐ 15,000 to 20,000 ☐ 20,000 and above

8) **Number of family members (who are sharing the income with you):** ……

9) **Education level:**
   ☐ Primary ☐ Secondary ☐ High School ☐ Diploma
   ☐ Bachelor ☐ Postgraduate ☐ Other ……

Next Page

Please make sure you have given an answer for every questions before leaving this page

## PART 2: SUN EXPOSURE

**10) How often are you exposed directly to the sun (not through glass, window, or umbrella)?**

**a) During weekdays:**

☐ Daily ☐ 5-6 times per week ☐ 3-4 times per week
☐ 1-2 times per week ☐ 3-4 times per month ☐ 1-2 times per month ☐ Rarely

**11) What time of the day are you exposed directly to the sun (not through glass, window, or umbrella)?**

**a) During the weekdays:**

☐ 7-9 am ☐ 9-11 am ☐ 11-1 pm
☐ 1-3 pm ☐ 3-5 pm ☐ 5-7 pm ☐ None

**b) During the weekends:**

☐ 7-9 am ☐ 9-11 am ☐ 11-1 pm
☐ 1-3 pm ☐ 3-5 pm ☐ 5-7 pm ☐ None

**12) How much time do you usually outdoors in daylight?**

| **Typical weekdays** | **Typical weekends** |
|---|---|
| ☐ <10 min | ☐ <10 min |
| ☐ 10-20 mins | ☐ 10-20 mins |
| ☐ 20-30 mins | ☐ 20-30 mins |
| ☐ 30 mins - 1 hours | ☐ 30 mins - 1 hours |
| ☐ 1-2 hours | ☐ 1-2 hours |
| ☐ 2-4 hours | ☐ 2-4 hours |
| ☐ 4-6 hours | ☐ 4-6 hours |
| ☐ 8 or more hours | ☐ 8 or more hours |
| ☐ None | ☐ None |

**13) What areas of your body are often exposed to sunlight?**

☐Face ☐ Hands ☐Full arms ☐Half arms
☐Feet ☐Half legs ☐Full legs ☐Other …….

**14) What kind of sun protection do you usually use?**

☐ Sunscreen ☐ Umbrella ☐ Hat ☐ Shomak or Qutra
☐ head cover ☐ other……..

**15) How often do you use sun protection?**

☐ Daily ☐ 3-4 times per week ☐ 1-2 times per week
☐ 3-4 times per month ☐ 1-2 times per month ☐ Never

**16) If you use sunscreen, what is the sun protection factor (SPF) number of the sunscreen that you use most often?**

☐ 10 ☐ 15 ☐ 20 ☐ 30 ☐ 40 ☐ 50 ☐ 60

Please make sure you have given an answer for every questions before leaving this page

2

## PART 3: HEALTH INFORMATION

**17) Do you smoke (now)?**

☐ Yes (go to question 19) ☐ No

**18) Have you ever been a smoker?**

☐ Yes ☐ No (go to question 21)

**19) How do you describe your smoking habits (now or in the past)?**

☐ Heavy smoker ☐ Moderate smoker ☐ Light smoker
☐ Rarely smoke

**20) What type of smoking?**

☐ Cigarettes ☐ Electronic cigarettes ☐ Hookah ☐ Shisha ☐ other……

**If you are a female: (If not apply go to question 23)**

**21) Are you currently pregnant?**

☐ Yes ☐ No

**22) Are you currently breastfeeding?**

☐ Yes ☐ No

Please make sure you have given an answer for every questions before leaving this page

## PART 4: SUPPLEMENT and MEDICATIONS

**23) Do you take any vitamins, minerals, fish oils or other food supplement?**

☐ Yes ☐ No (go to question 25)

**24) If yes, please complete the table below:**

| Name and brand | Dose (please state number of pills or capsules or teaspoons consumed) | How often do you take these? | | | |
|---|---|---|---|---|---|
| | | Daily | Weekly | Monthly | Less often |
| | | | | | |
| | | | | | |
| | | | | | |

**25) What are the reasons for taking supplements?**

☐ Preventative ☐ Therapeutic (prescribed by your doctor)

**26) Do you take any medication?**

☐ Yes ☐ No

**27) If yes, please complete the table below:**

| Name and brand | Dose please state number of pills or capsules or teaspoons consumed | How often do you take these? | | | |
|---|---|---|---|---|---|
| | | Daily | Weekly | Monthly | Less often |
| | | | | | |
| | | | | | |
| | | | | | |

Please make sure you have given an answer for every questions before leaving this page

**28) Over the last 3 months, how often have you eaten the foods in the table below?**

| Food items (unit) | Amount | Never | Less than once a month | 1-3 times a month | Once a week | 2-4 times a week | 5-6 times a week | Once a day | Twice a day | Three or more times a day |
|---|---|---|---|---|---|---|---|---|---|---|
| Cow's liver (gram) | | | | | | | | | | |
| Lamb's liver (gram) | | | | | | | | | | |
| Camel's liver (gram) | | | | | | | | | | |
| Chicken's liver (gram) | | | | | | | | | | |
| Fresh Salmon (Piece / gram) | | | | | | | | | | |
| Salmon canned (can / gram) | | | | | | | | | | |
| Fresh Sardines (Piece / gram) | | | | | | | | | | |
| Sardines Canned (can / gram) | | | | | | | | | | |
| Tuna, canned (can / gram) | | | | | | | | | | |
| Shrimp (gram) | | | | | | | | | | |
| Mushrooms fresh (gram) | | | | | | | | | | |
| Sun dried Mushroom (gram) | | | | | | | | | | |

Please make sure you have given an answer for every questions before leaving this page

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Egg yolk | | | | | | | | | | |
| Fortified milk (cup) | | | | | | | | | | |
| Fortified orange juice (cup) | | | | | | | | | | |
| Fortified yogurts (cup) | | | | | | | | | | |
| Fortified butter (teaspoon) | | | | | | | | | | |
| Fortified margarine (teaspoon) | | | | | | | | | | |
| Fortified chees (tablespoon/ slice) | | | | | | | | | | |
| Fortified breakfast cereals (cup) | | | | | | | | | | |

**Thank you very much for completing this questionnaire.**

**Researcher: Wedad Azhar**

Please make sure you have given an answer for every questions before leaving this page

**The Researcher will fill this part:**

1) **Does the patient have any of the following disease?**

☐ Hart disease ……..

☐ Hypertension

☐ Diabetes ☐ type I ☐ type II

☐ Other Factors……..

2) **Weight**: ……..**kg**

3) **Height:** ……..**cm**

4) **Waist:** ……..……cm

5) **Hip** ……..cm

6) **The skin colour?**

I. ☐ Light, pale white (Always burns never tans)

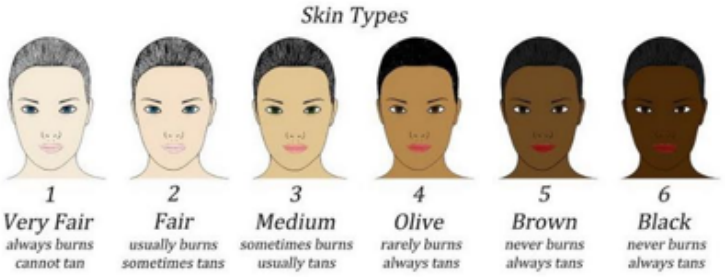
II. ☐ White, fair (usually burns tans with difficulty)

III. ☐ Medium, white to olive (sometimes mild burn, gradually tans to olive)

IV. ☐ Olive, moderate brown (Rarely burns, tans with ease to a moderate brown

V. ☐ Brown, dark brown (very rarely burns, tans very easily)

VI. ☐ Black, very dark brown to black ( never burns, tans very easily, deeply pigmented)




Please make sure you have given an answer for every questions before leaving this page

## *Appendix 8- Questionnaire Arabic*

**إستبيانة مشاركة في بحث**

***عنوان البحث/ مدى تأثير نقص فيتامين د على مرضى تصلب الشرايين الذين يعيشون في المملكة العربية السعودية***

**الجزء الأول: معلومات خاصة**

**رمز المشارك .....**

**1- الجنس**
☐ ذكر ☐ أنثى

**2- تاريخ الميلاد ..... العمر .....**

**3- مكان الميلاد.....**

**4- مكان الإقامة (المدينة).....**

**5- في أي مجال تعمل:**
☐ التعليم ☐ الصحة ☐ وظيفة حكومية ☐ القطاع الخاص

**6- أي من التالي يناسب وصف وظيفتك الأساسية:**
☐ تقضي أغلب الوقت في مكان مغلق
☐ تقضي نصف الوقت في مكان مغلق والنصف الأخر في الخارج
☐ تقضي أغلب الوقت في الخارج

**7- الدخل الشهري للعائلة بالريال السعودي:**
☐ أقل من 5,000 ☐ من 5,000 إلى 10,000 ☐ من 10,000 إلى 15,000
☐ من 15,000 إلى 20,000 ☐ أكثر من 20,000

**8- عدد أفراد الأسرة الذين يتشاركون في الدخل الشهري .....**

**9- مستوى التعليم:**
☐ إبتدائي ☐ متوسط ☐ ثانوي ☐ دبلوم
☐ جامعي ☐ تعليم عالي ☐ أخرى .....

1

الرجاء التأكد من الإجابة على جميع الأسئلة قبل الإنتقال للصفحة التالية

**الجزء الثاني: التعرض لأشعة الشمس**

**10- أي من الإجابات التالية يصف تعرضك المباشر لأشعة الشمس (ليس من خلاج الزجاج أو الناقذة):**

☐ يومياً ☐ 5-6 مرات في الأسبوع ☐ 3-4 مرات في الأسبوع
☐ 1-2 مرات في الأسبوع ☐ 3-4 مرات في الشهر ☐ 1-2 في الشهر ☐ أقل من ذالك

**11- أي من الأوقات التالية خلال النهار تتعرض قية لأشعة الشمس (ليس من خلال الزجاج أو الناقذه):**

**أ-خلال ايام الأسبوع (يمكن إختيار أكثر من خيار):**

☐ 6-9 ص ☐ 9-11 ص ☐ 11-1 م
☐ 1-3 م ☐ 3-5 م ☐ 5-7 م ☐ لا ينطبق

**ب- خلال نهاية الأسبوع (يمكن إختيار أكثر من خيار)**

☐ 6-9 ص ☐ 9-11 ص ☐ 11-1 م
☐ 1-3 م ☐ 3-5 م ☐ 5-7 م ☐ لا ينطبق

**12- كم من الوقت تقضي في التعرض المباشر لأشعة الشمس (ليس من خلال الزجاج أو الناقذه):**

| أيام الأسبوع | نهاية الأسبوع |
|---|---|
| ☐ أقل من 10 دقائق | ☐ أقل من 10 دقائق |
| ☐ من 10- 20 دقيقة | ☐ من 10- 20 دقيقة |
| ☐ من 20- 30 دقيقة | ☐ من 20- 30 دقيقة |
| ☐ من 30- 60 دقيقة | ☐ من 30- 60 دقيقة |
| ☐ من 1-2 ساعة | ☐ من 1-2 ساعة |
| ☐ من 2-4 ساعات | ☐ من 2-4 ساعات |
| ☐ من 4-6 ساعات | ☐ من 4-6 ساعات |
| ☐ 7 ساعات أو أكثر | ☐ 7 ساعات أو أكثر |
| ☐ لا ينطبق | ☐ لا ينطبق |

**13- أي من أجزاء جسمك تتعرض غالباً للشمس (يمكن إختيار أكثر من خيار):**

☐ الوجه ☐ الكفين ☐ الساعد ☐ الذراع كاملة ☐ القدم
☐ نصف الساق ☐ الساق كاملة ☐ لا ينطبق ☐ أخرى .....

**14- أي من الإجابات التالية يصف مدى إستخدامك لواقي الشمس**

☐ يومياً ☐ 5-6 مرات في الأسبوع ☐ 3-4 مرات في الإسبوع
☐ 1-2 مرات في الأسبوع ☐ 3-4 مرات في الشهر ☐ 1-2 في الشهر ☐ لا ينطبق

**15- إذا كنت تستخدم واقي الشمس ما هي نسبة الوقاية ( SPF ) التي تستخدمها**

☐ 10% ☐ 15% ☐ 20% ☐ 30% ☐ 40% ☐ 50% ☐ 60%

2

الرجاء التأكد من الإجابة على جميع الأسئلة قبل الإنتقال للصفحة التالية

**الجزء الثالث: معلومات صحية**

**16- هل تدخن (حالياً)؟**

☐ نعم (إذهب للسؤال 19) ☐ لا

**17- هل كنت تدخن سابقاً؟**

☐ نعم ☐ لا (إذهب للسؤال رقم 21)

**18- أي من الإجابات التالية يصف مدى تدخينك؟**

☐ يومياً ☐ 5-6 مرات في الأسبوع ☐ 3-4 مرات في الإسبوع

☐ 1-2 مرات فس الأسبوع ☐ 3-4 مرات في الشهر ☐ 1-2 في الشهر ☐ أقل من ذلك

**19- أي من أنواع التدخين تمارس ( يمكن إختيار أكثر من إجابة)**

☐ سجائر ☐ سجائر إلكترونية ☐ أرقيلة (معسل) ☐ شيشة جراك ☐ أخرى .....

**فقط للنساء ( إذا لا ينطبق إذهب للسؤال رقم 23)**

**20- هل أنت حامل؟**

☐ نعم ☐ لا

**21- هل ترضعين رضاعة طبيعية؟**

☐ نعم ☐ لا



الرجاء التأكد من الإجابة على جميع الأسئلة قبل الإنتقال للصفحة التالية

**الجزء الخامس: إستخدام المكملات الغذائية والأدوية**

**22- هل تستخدم أي مكملات غذائية مثل فيتاميتات، معادن، زيت كبد الحوت، الخ؟**

☐ نعم ☐ لا (إذهب للسؤال رقم 26)

**23- إذا أجبت بنعم أرجو إكمال الجدول التالي:**

| إسم المنتج والشركة المصنعة | الكمية المستهلكة بالملجم / مل | ما مدى الاستهلاك | | | |
|---|---|---|---|---|---|
| | | يومياً | أسبوعياً | شهرياً | أقل من ذالك |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |

**24- لماذا تقوم بأخذ المكملات الغذائية؟**

☐ للوقاية ☐ للعلاج ( موصوفة من قبل الطبيب)

**25- هل تتناول أي علاج موصوف أو غير موصوف من قبل الطبيب؟**

☐ نعم ☐ لا

**26- إذا أجبت بنعم أرجوا إكمال الجدول التالي:**

| إسم المنتج والشركة المصنعة | الكمية المستهلكة بالملجم / مل | ما مدى الاستهلاك | | | |
|---|---|---|---|---|---|
| | | يومياً | أسبوعياً | شهرياً | أقل من ذالك |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |

تعليقات

.....

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.....

.....

**أشكرك على تعاونك بتعبئة هذه الإستبانة**
**الباحثة / وداد فؤاد أزهـر**

4

الرجاء التأكد من الإجابة على جميع الأسئلة قبل الإنتقال للصفحة التالية

في خلال الثلاث شهور الماضية، ما مدى تكرار استهلاكك للمواد الغذائية المذكورة في الجداول أدناه؟

| المواد الغذائية (الوحدة) | الكمية | أبداً | أقل من مره في الشهر | 1 إلى 3 مرات في الشهر | مره في الاسبوع | 2 الى 4 مرات في الاسبوع | 5 الى 6 مرات في الاسبوع | مره في اليوم | مرتين في اليوم | ثلاث مرات أو أكثر في اليوم |
|---|---|---|---|---|---|---|---|---|---|---|
| كبد البقر (جرام) | | | | | | | | | | |
| كبد الخروف (جرام) | | | | | | | | | | |
| كبد الجمل (جرام) | | | | | | | | | | |
| كبد الدجاج (جرام) | | | | | | | | | | |
| سمك السالمون الطازج (قطعه / جرام) | | | | | | | | | | |
| سمك السالمون المعلب (علب / جرام) | | | | | | | | | | |
| ساردين طازج (قطعه / جرام) | | | | | | | | | | |
| ساردين معلب (علب / جرام) | | | | | | | | | | |
| تونا معلب (علب / جرام) | | | | | | | | | | |
| ربيان (جرام) | | | | | | | | | | |
| فطر طازج (جرام) | | | | | | | | | | |
| فطر مجفف (جرام) | | | | | | | | | | |
| صفار البيض | | | | | | | | | | |
| حليب مدعم (كوب) | | | | | | | | | | |
| لبن مدعم (كوب) | | | | | | | | | | |
| عصير البرتقال المدعم (كوب) | | | | | | | | | | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| زبده مدعمه (ملعقة شاي) | | | | | | | | | | |
| سمن نباتي (ملعقة شاي) | | | | | | | | | | |
| جبن مدعم (ملعقة شاي/ شريحة) | | | | | | | | | | |
| رقائق الإفطار المدعمة (كوب) | | | | | | | | | | |

*Appendix 9 text message sent to participants*


السلام عليكم ورحمة الله وبركاتة
أنا الباحثة وداد أزهر أحضر درجة الدكتوراة في التغذية الإكلينيكية. أقوم بجمع بيانات بحث عن تأثير فيتامين د على أمراض القلب وتصلب الشرايين في مستشفى النور التخصصي بمكة في عيادة بقسم القلب بالمستشفى.
لمن يرغب في التطوع للأشتراك بالبحث سنقوم بعمل سونار للشريان الرئيسي في الرقبة بالاضافة الى بعض الأسئلة وتحاليل الدم التي تشمل فيتامين د، دهون ، سكر ، والغدة الدرقية
اذا كنت ترغب في الاشتراك يجب ان تكون ما بين ٣٥ الى ٦٠ سنة. كذالك يجب أن تكون صائم لمدة ١٢ ساعة عن الطعام والشراب ماعدا الماء.
أذا كنت جاد في الرغبة بالإشتراك الرجاء التواصل مع الباحثة وداد أزهر من الساعة ٨:٣٠ ص الى الساعة ٨ م
جوال رقم 0[redacted]

## *Appendix 10 – Pilot study of the study questionnaire (part of the transfer report from MPhil to PhD)*

**Chapter 3. Refinement of methodologies:**

***3.1. Development of qualitative data capture (questionnaires)***

***3.1.1. Pilot study***

A pilot study was undertaken to examine the validity of a questionnaire before it was used in PhD studies. Piloting the questionnaire ensures that the questions are clear and understandable for the participants, as they were initially intended from their design.

***3.1.2 Questionnaire design***

A questionnaire was designed by the researcher based on the review of literature (Appendix 1) (McCarty, 2008; Gould et al., 2015). The questions were designed based on many factors: the purpose of the questions, the target population, and the climate of the target country (Saudi Arabia) (Ardawi et al., 2012). Most of the questions were close-ended questions in order to simplify answering, coding, and analysis. The questionnaire was divided into five parts.

- Firstly, personal information included: age, gender, socio-economic status, and educational level.
- Secondly, sun exposure covered questions in relation to time, length, and frequency of exposure to sunlight, in addition to the parts of the body that were exposed, as well as the use of sunscreen.
- Thirdly, health information included: smoking status, pregnancy, and breastfeeding.
- Fourthly, the questionnaire covered questions about the use of supplements and medications.
- A final section, completed by the researcher, covered the participants' weights, heights, and skin colours. However, the weights and heights were self-recorded in this pilot study (even though the researcher will take the measurements during the PhD study). Participants were categorised according to BMI as underweight (BMI < 18.5 $kg/m^2$), healthy weight (18.5 - 24.9 $kg/m^2$), overweight (25 - 29.9 $kg/m^2$), and obese (BMI > 30 $kg/m^2$). Skin colour was determined by the researcher using the Fitzpatrick scale in figure 2.


*Figure 1 The Fitzpatrick skin colour classification*

Ultimately, the questionnaire, study participant information sheet, and study participant consent form were translated into the Arabic language and then checked for accuracy to ensure no loss of meaning during translation, as Arabic is the native language used in Saudi Arabia. The final versions of the translated documents are in Appendix 2.

### *3.1.3. Participants and recruitment:*

This cross-sectional study was carried out by using convenient sampling. Twelve subjects were recruited from a specific age group (40-60). There was an even number of male and female individuals in the study. Additionally, most of the participants in the study live in Makkah city (21.4° N) , where the main study will take place, or in Jeddah city (21.5° N), ), a city that is geographically close to Makkah and has a similar climate (AccuWeather, 2015). All of the participants were initially provided with an information sheet and signed a consent form prior to their participation in the study (Appendices 3, 4)

### *3.1.4. Result and discussion of the pilot study*

#### *3.1.4.1. Statistical analysis:*

A descriptive analysis was used for data analysis at this point. (Pallant and Manual, 2010). Participants were aged 49.9 ± 6.7 and 75% of them lived in Makkah. Mean BMI was 27.7 ± 4 $kg/m^2$ x, which is above the normal weight. Half of the participants were overweight and the rest were equally divided between healthy weights and obese. Invariably, being overweight or obese is one of the main factors of vitamin D deficiency and cardiovascular disease, as discussed in the literature (Alyami et al., 2014). In total, half of the participants held postgraduate degrees. Moreover, fifty percent of the females were housewives whereas, all of the male participants

worked in different careers. Furthermore, most of the participants had medium to high income. However, there was a match in the economic answers because there were three couples in the sample. Table 3 exhibits additional details in regards to the general characteristic of the participants.

*Table 3 Descriptive statistics of the general characteristic*

| Variable | Gender | | Total (n=12) |
|---|---|---|---|
| | *Male (n=6)* | *Female (n=6)* | |
| **Age** | 50.6±8* | 49±5.8* | 49.9 ± 6.7* |
| **BMI (kg/m²)** | 27.9±4* | 27.6±4.7* | 27.7 ±4* |
| **Healthy weight** | 1 (16.7%) | 2(33.3%) | 3(25.0%) |
| **Overweight** | 4 (66.7%) | 2(33.3%) | 6 (50.0%) |
| **Obese** | 1 (16.7%) | 2(33.3%) | 3(25.0%) |
| *Place of living* | | | |
| **Makkah** | 4 (66.7%) | 5 (83.3%) | 9 (75.0%) |
| **Jeddah** | 2(33.3%) | 1 (16.7%) | 3(25.0%) |
| *Education level* | | | |
| **High school** | 1 (16.7%) | 2 (33.3%) | 3(25.0%) |
| **Diploma** | 1 (16.7%) | 1 (16.7%) | 2 (16.7%) |
| **Bachelor** | 0 | 1 (16.7%) | 1 (8.3%) |
| **Postgraduate** | 4 (66.7%) | 2 (33.3%) | 6 (50.0%) |
| *Occupation* | | | |
| **Education** | 1 (16.7%) | 2 (33.3%) | 3(25.0%) |
| **Health care** | 0 | 1 (16.7%) | 1 (8.3%) |
| **Public sector** | 1 (16.7%) | 0 | 1 (8.3%) |
| **Private sector** | 4 (66.7%) | 0 | 4 (33.3%) |
| **housewife** | 0 | 3(50.0%) | 3(25.0%) |
| *Income by SR* | | | |
| **5000-10000** | 0 | 1 (16.7%) | 1 (8.3%) |
| **10000-15000** | 1 (16.7%) | 1 (16.7%) | 2 (16.7%) |
| **15000-20000** | 2 (33.3%) | 2 (33.3%) | 4 (33.3%) |
| **>20000** | 3(50.0%) | 2 (33.3%) | 5 (41.7%) |

*** = data are calculated as mean ± standard deviation (SD)**

In regards to determining sun exposure, many factors were duly measured. The majority of the participants had medium or olive skin colour as shown in figure 2. Studies shown that darker skin colours is one of the factors behind of vitamin D deficiency (Lai et al., 2010).

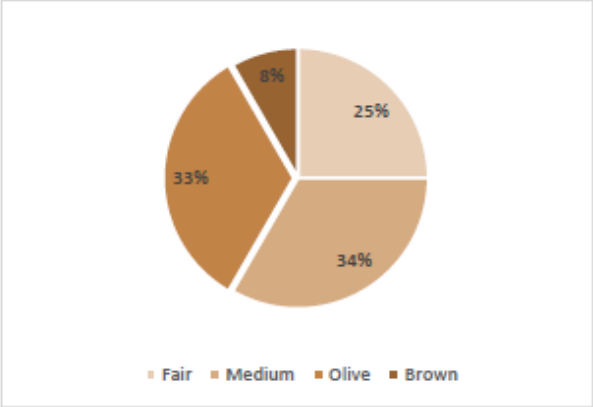



*Figure 2 distribution of sample using Fitzpatrick skin colour classification*

In general, the results demonstrated that men expose more parts of their bodies and spend more time directly under sunlight. When the participants are outdoors, they all expose their hands and face to sun light, although 33.3% of the female cover their faces (Figure 3). In fact, the traditional clothing in Saudi Arabia is one of the barriers to sunlight so that only hands and face are habitually exposed to the sun. Indeed, when measuring the frequency of exposure to sunlight, 66.7% of males exposed to sunlight on a daily occurrence. Whereas, 50% of the females are rarely exposed to sunlight or 1 to 2 times per month (figure 4).

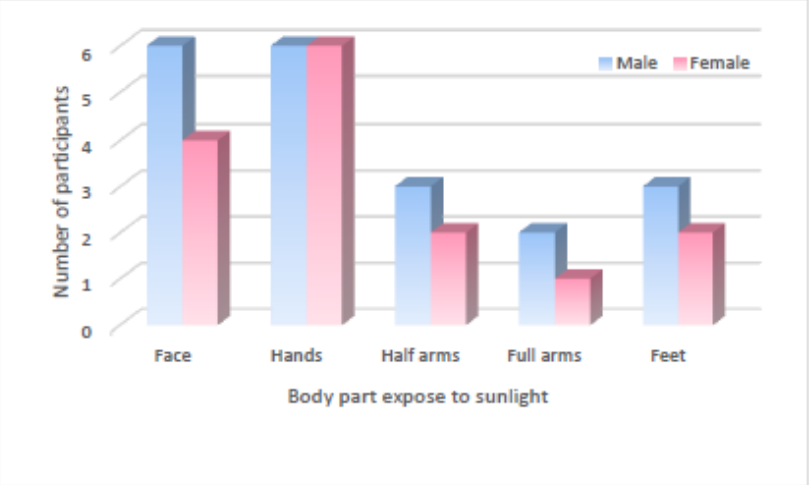



*Figure 3 demonstration of body parts exposed to sunlight among gender.*

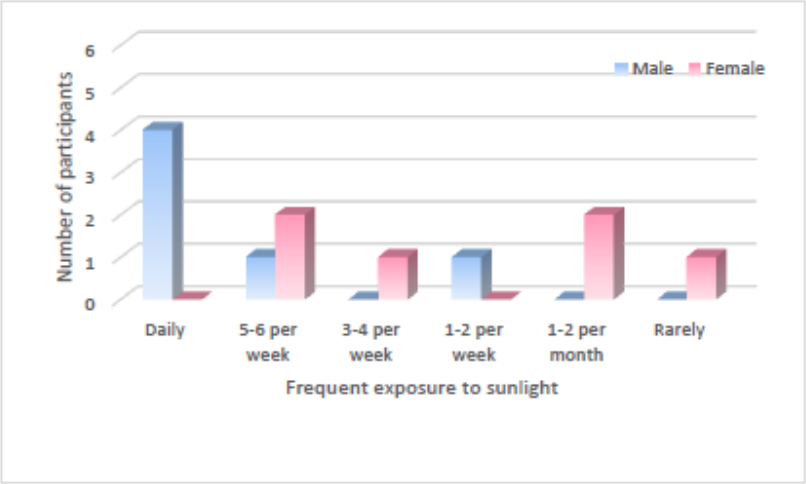



*Figure 4 frequency of exposure to sunlight by gender*

On the other hand, when studying the duration of time in sunlight during the weekdays, females are more likely to spend time outdoors in the morning, as shown in Figure 5. Indeed, only half of the participants expose themselves to sunlight during the weekend (Figure 6).

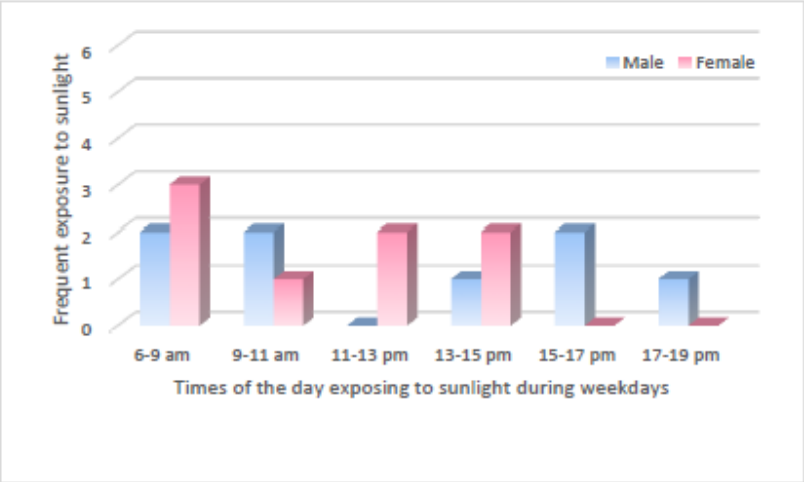



*Figure 5 distribution to time spent outside during weekdays*

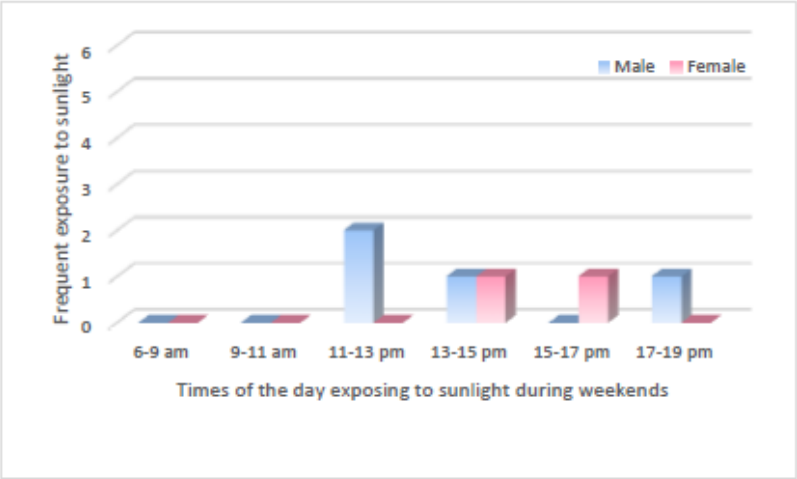



*Figure 6 distribution to time spent outside during weekend*

Additionally, most of the participants 66.7% emphasised that in their jobs they are mainly situated in places that are indoors. Observations of employed participants show that they tend to be exposed to sunlight during two periods of the day: morning and afternoon (Figure 7). That emphasises that they are mainly exposed to the sun during their commute to/from work. However, the females' limited exposure to sunlight was expected, since 50% of the current sample were housewives, hence have limited opportunity/cause (in this culture) to go outside during the daytime.

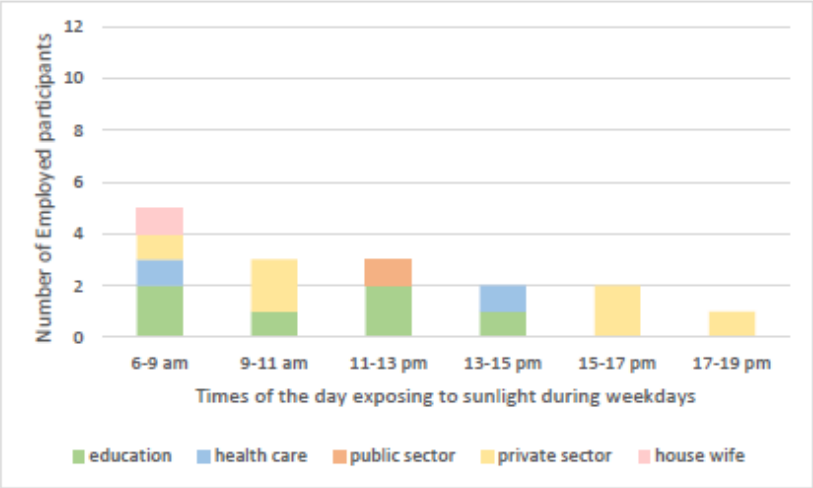



*Figure 7 distribution of times of the day employed spend exposing to sunlight*

The length of time spent outdoors signified that the participants are not spending sufficient time in the presence of sunlight (figure 8). Invariantly, most of the subjects, 66.7% during weekdays and 75% during weekend, spend 0 to 10 minutes under the sunlight daily. Nevertheless, avoidance of sun exposure in Saudi Arabia has been expected in the literature, mainly due to the extreme high temperature in the country. During the pilot study, collection of high temperature were 46° ± 1.47 and the low temperature were 32.5° ± 1.2 in Makkah city. Overall, the main feedback that has been produced from the pilot study is that Saudis from both genders are not been adequately exposed to sunlight. Consequently, as sunlight is the main source of vitamin D, Saudis are suffering from vitamin D deficiency and inefficiency (Siddiqui and Kamfar, 2007; Ardawi et al., 2012).

Only two out of the 12 participants were using sunscreen factor 10 or 30 at a frequency of 1-2 times per month. Notably also, two participants commented that they use an umbrella as a sun protector.


*Figure 8 Length of time participants spent outdoor during the day in weekdays and weekend*

For the smoking status, 50% of the participants were smokers of which 66.7% were males. The majority of these smokers smoked on a daily basis. Additionally, more men smoke shisha, which is a traditional type of hookah (water pipe), as shown in table 4. Comparatively, only 33.3% of the participants consumed food supplements, and these supplement all contained vitamin D.

*Table 4 distribution of smoking statues among the participants*

| Variable | Gender | | Total (n=12) |
|---|---|---|---|
| | *Male (n=6)* | *Female (n=6)* | |
| **Current Smokers** | 4 (66.7%) | 2 (33.3%) | 6 (50%) |
| **Previous smokers** | 2 (33.3%) | 1 (16.7%) | 3(25.0%) |
| *Frequent smoking* | | | |
| **Daily** | 5 (83.4%) | 1 (16.7%) | 6 (50.0%) |
| **5-6 per week** | 1 (16.7%) | 0 | 1 (8.3%) |
| **3-4 per month** | 0 | 1 (16.7%) | 1 (8.3%) |
| *Type of Tobacco* | | | |
| **Cigarettes** | 2 (33.3%) | 1 (16.7%) | 3(25.0%) |
| **Hookah** | 1 (16.7%) | 1 (16.7%) | 2 (16.7%) |
| **Shisha** | 3(50.0%) | 0 | 3(25.0%) |
| **Consumption of supplements** | 1 (16.7%) | 3(50.0%) | 2 (33.3%) |

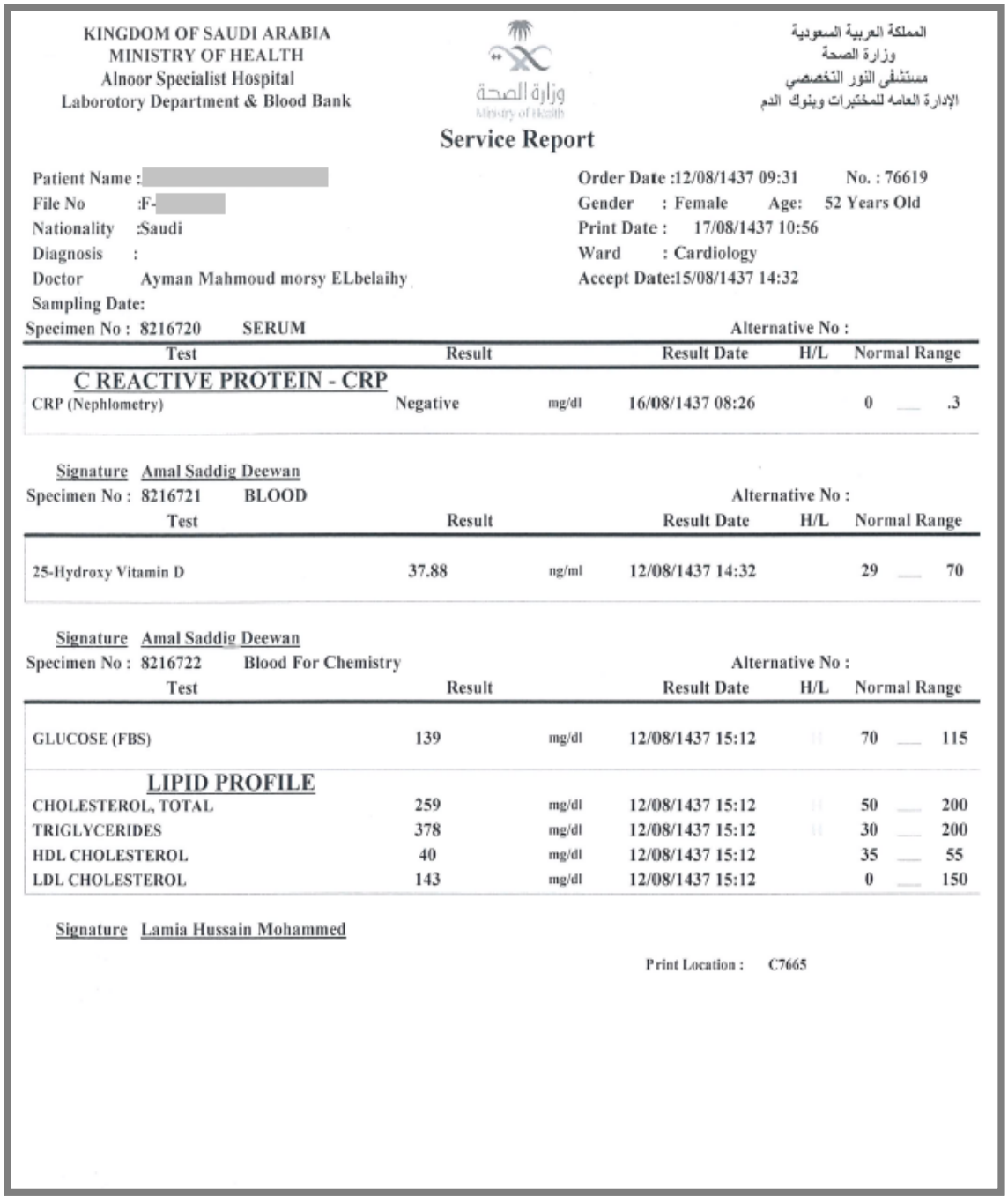
4.1.4.2. Feedback from the questionnaire:

The majority of the participants have recorded that the questionnaire was clear and easy to fill in. Most of the comments were about questions that do not fit the specific habits of Saudi Arabia such as the use of sunscreen. Table 6 provides details about each question that was subsequently modified. The modified version will be used within the program of PhD studies (Appendices 5, 6).

**TABLE 6 THE CHANGES IN THE QUESTIONNAIRE AFTER THE PILOT STUDY**

| The question | Comments or mistake in answering | Modification |
|---|---|---|
| Do you have a job? | It was confusing and the next question about occupation could cover it. | The question was deleted |
| Occupation | Need to be categorised | What is the category of your employment? 1- Education 2- Health 3-Public sector 4- Private sector 5- Housewife 6- Retired 7- Other |
| How often are you exposed directly to the sun (not through glass or window)? | The use of umbrella should be included | (not through glass, window, or umbrella) |
| How often do you use sunscreen? | The use of umbrella or hat should be included | *A question is added:<br>What kind of sun protection do you usually use? 1-sunscreen 2-umbrella 3-hat 4-Shomak or Qutra 5-head cover 6-other<br><br>*The question was modified:<br>How often do you use sun protection?<br><br>*A question was modified:<br>If you use sunscreen, what is the sun protection factor (SPF) number of the sunscreen that you use must often? |
| How often do you (or used to) smoke? | Smoking should be count by the amount of smoke | How do you describe your smoking habits (now or in the past)?<br>1-Heavy smoker 2-moderate smoker 3-light smoker 4-Rarely smoke |
| Are you currently breastfeeding? | It was confusing in the Arabic version | The word currently was emphasised in Arabic |
| Do you take any medication described or not described by the physician? | It was confusing | Do you take any medication? |

*Appendix 11- Example of the blood test report*



KINGDOM OF SAUDI ARABIA
MINISTRY OF HEALTH
Alnoor Specialist Hospital
Laborotory Department & Blood Bank

وزارة الصحة
Ministry of Health

المملكة العربية السعودية
وزارة الصحة
مستشفى النور التخصصي
الإدارة العامه للمختبرات وبنوك الدم

## Service Report

Patient Name : [redacted]
File No :F-[redacted]
Nationality :Saudi
Diagnosis :
Doctor Ayman Mahmoud morsy ELbelaihy
Sampling Date:

Order Date :12/08/1437 09:31 No. : 76619
Gender : Female Age: 52 Years Old
Print Date : 17/08/1437 10:56
Ward : Cardiology
Accept Date:15/08/1437 14:32

Specimen No : 8216720 SERUM Alternative No :

| Test | Result | | Result Date | H/L | Normal Range |
|---|---|---|---|---|---|
| **C REACTIVE PROTEIN - CRP** | | | | | |
| CRP (Nephlometry) | Negative | mg/dl | 16/08/1437 08:26 | | 0 ___ .3 |

Signature Amal Saddig Deewan

Specimen No : 8216721 BLOOD Alternative No :

| Test | Result | | Result Date | H/L | Normal Range |
|---|---|---|---|---|---|
| 25-Hydroxy Vitamin D | 37.88 | ng/ml | 12/08/1437 14:32 | | 29 ___ 70 |

Signature Amal Saddig Deewan

Specimen No : 8216722 Blood For Chemistry Alternative No :

| Test | Result | | Result Date | H/L | Normal Range |
|---|---|---|---|---|---|
| GLUCOSE (FBS) | 139 | mg/dl | 12/08/1437 15:12 | H | 70 ___ 115 |
| **LIPID PROFILE** | | | | | |
| CHOLESTEROL, TOTAL | 259 | mg/dl | 12/08/1437 15:12 | H | 50 ___ 200 |
| TRIGLYCERIDES | 378 | mg/dl | 12/08/1437 15:12 | H | 30 ___ 200 |
| HDL CHOLESTEROL | 40 | mg/dl | 12/08/1437 15:12 | | 35 ___ 55 |
| LDL CHOLESTEROL | 143 | mg/dl | 12/08/1437 15:12 | | 0 ___ 150 |

Signature Lamia Hussain Mohammed

Print Location : C7665

*Appendix 12- The Stadiometer and electronic scale*

*Appendix 13- The automated electronic sphygmomanometer*

*Appendix 14- Posters for conferences presentations*

# The association between Vitamin D Deficiency and endothelial function

Wedad Azhar*
Dr. Bartek Buczkowski, Dr. Gladys Onambele-Pearson, Prof. Christopher Smith
Manchester Metropolitan University, Hollings Faculty, Department of Food and Tourism Management
wedad-fouad.o.azhar@stu.mmu.ac.uk

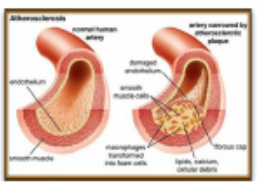
## Introduction

Vitamin D is an essential fat-soluble vitamin required for the maintenance of good health. It is obtained through either exposure to sunlight (ultraviolet B radiation) or diet. Vitamin D has recently been proposed to play a critical role in endothelial cell function, one of the main indicators of atherosclerosis (Jablonski et al., 2011). This study is investigating the correlation between vitamin D status and endothelial function in preventing and treating atherosclerosis especially in country that has ample of sunshine such as Saudi Arabia, Saudis suffer from vitamin D deficiency and insufficiency.

*Studies determining vitamin D status in Saudi Arabia*

| Study | Subjects | Sample size | Vitamin D deficient* |
|---|---|---|---|
| 2012 | Men aged 20-74 | 834 | 51.5 % |
| 2007 | Girls aged 12-15 | 433 | 40% |
| 2009 | Women aged 20-60 | 118 | 78.9% |
| 2003 | People with chronic low back pain aged 15-52 | 360 | 83% |

- *Deficient at 25(OH)D < 20 ng/mL


## Role of vitamin D in atherosclerosis

vitamin D has shown preventive and disruptive effects against the development of atherosclerosis by:

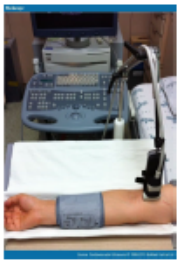
- improving endothelial function.
- regulating vascular smooth muscle cells.
- managing the inflammatory and immune process (Menezes et al., 2014).
- Helping Nitric Oxide synthase.
- Having a role in many atherosclerosis risk factors such as managing body weight, decreasing insulin resistance, improving lipid profiles, and improving blood pressure parameters (Arora and Rehan, 2015).

## Aim of the Study

To determine the best markers of vitamin D deficiency and endothelial function, and any associations between these and the development of CVD risk factors.

## Methods

**Subject**: 30 healthy people, 30 people at risk of devolving atherosclerosis, and 30 atherosclerosis patient.



**Measurement**: blood analysis (25(OH)D3,parathyroid hormone, high sensitivity C-reactive protein, lipids profile, and blood glucose ), FMD test on the brachial artery , 3 days dietary recall, and questionnaire on socioeconomic status, education levels, daily exposure to sunlight, smoking status, and consumption of supplements.
In addition, Body height, mass, waist/hip ratio, body mass index (BMI), and resting blood pressure of subjects will also be noted.

## Conclusion

Vitamin D deficiency or insufficiency is a huge risk factor for many diseases. Preliminary results from previous studies indicate an impact of vitamin D status on endothelial function and atherosclerosis. This may suggest a use of vitamin D in preventing and treating atherosclerosis, the most common type of cardiovascular disease. This study will attempt to demonstrate the link between vitamin D deficiency and atherosclerosis and will suggest options for improving vitamin D status either by more sunlight exposure or by dietary changes and supplementation.

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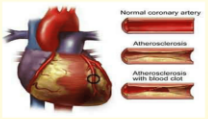
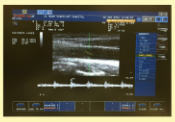
# Measuring the effect of vitamin D status on occurrence of atherosclerosis in patients living in Saudi Arabia

Wedad Azhar*
Dr. Bartek Buczkowski, Dr. Gladys Onambele-Pearson, Prof. Christopher Smith
Manchester Metropolitan University
Faculty of Health, Psychology & Social Care
Department of Food and Tourism Management
wedad_azhar@Hotmail.com




- **Introduction**
- Vitamin D is a fat-soluble vitamin required for the maintenance of bone health, calcium metabolism and other aspects of good health. The sources of vitamin are diet and cutaneous synthesis following exposure to UV radiation. It has been proposed that vitamin D plays a critical role in atherocsclerosis (Menezes, 2014), possibly because of its involvement in vascular smooth muscle cells' health. This study is investigating the impact of vitamin D status on the occurrence of atherosclerosis. Saudi Arabia has ample sunshine but yet vitamin D deficiency and insufficiency are prevalent amongst the Saudis.
- **Methods**
- **Subjects**: 33 healthy people, 30 people at risk of devolving atherosclerosis, and 34 atherosclerosis patients.
- **Measurement**: The following tests were conducted
- Ten hours fasting blood tests for serum 25(OH)D, Thyroid stimulating hormone, high sensitivity C-reactive protein, fasting blood glucose, total cholesterol, triglycerides, high-density lipoprotein, and low-density lipoprotein.
- Carotid artery measurements
- (Intima-media thickness IMT, blood vassal diameter BVD, Resistance index RI, heart rate HR)
- Complior tests (central Blood pressure, pulse wave velocity PWV)


- Resting blood pressure
- A questionnaire (socioeconomic status, education levels, daily exposure to sunlight, smoking status, consumption of supplements, and food frequency questionnaire for food Items that contain vitamin D).
- Measurement of height and weight for Body Mass index (BMI) waist/hip ratio, skin colour was determined using Fitzpatrick skin colour classification.

- **Conclusion**
- Vitamin D deficiency or insufficiency is a huge risk factor for many diseases. It is anticipated that this study will provide an insight into a link between vitamin D status and incident or development of atherosclerosis. This may suggest a use of vitamin D in the preventing and treating atherosclerosis, the most common type of cardiovascular disease.

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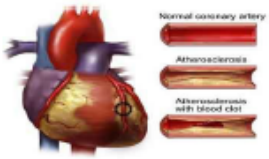
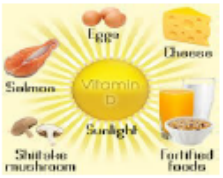
# Any association between vitamin D status and factors contributing to atherosclerosis incidence in Saudi Arabia dwellers

Wedad Azhar*
Dr. Bartek Buczkowski, Dr. Gladys Onambele-Pearson, Prof. Christopher Smith
Manchester Metropolitan University, Faculty of Health Psychology and Social Care Department of Health Professions
wedad-fouad.o.azhar@stu.mmu.ac.uk

## Introduction

Vitamin D is a fat-soluble vitamin required for the maintenance of bone health, calcium metabolism and other aspects of good health. The sources of vitamin D in the human body are diet and cutaneous synthesis following exposure to UV radiation. Whilst, Saudi Arabia may have ample sunshine, vitamin D deficiency and insufficiency are prevalent amongst the Saudis. Many factors have been proposed to play a role in vitamin D status. This study investigated the impact of socioeconomic status, education level, gender, skin tone and smoking status on vitamin D status.




## Methods

97 participant were recruited in this study through a combination of convenience sampling at the local hospital and word of mouth. Vitamin D status was assessed through serum 25(OH)D, using a standard ELISA technique on fasted blood sampled. Potential lifestyle were assessed using a questionnaire.

## Results

Primary results on vitamin D status indicate that 49.5% of the participants were deficient, 28.9% insufficient, whilst the rest had an adequate amount of 25(OH)D. A series of non-parametric pairwise comparisons and/or multiple level difference test however, revealed that none of the tested lifestyle factors stratifications, significantly impacted on 25(OH)D level.

## Conclusion

Our study has uncovered a worrying trend for a high prevalence of vitamin D deficiency in Saudi Arabia. It remains unclear however, what lifestyle factors may affect the presence of this deficiency given that neither family income, education level, gender, skin tone nor smoking status appears to have any bearing on participants' vitamin D status. Future work should look to physiological factors that could be better predictors/correlates of, serum 25(OH)D level.

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