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The effect of vitamin D status on lung function and airway inflammation in adults in Saudi Arabia and the UK

By

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Declaration

I confirm that this is my own work and all materials that used in this work from other sources have been acknowledged

Samih A. Kokandi

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Abstract

Vitamin D deficiency is a worldwide health problem and more extensively present in the gulf countries such as Saudi Arabia and in high latitude countries such as the United Kingdom. Many previous cross-sectional studies have investigated the correlation between vitamin D status and lung function, but the effect of vitamin D status on lung function and airway inflammation is still debatable. This project aimed to investigate the effect of vitamin D intervention through pharmacological supplements or dietary intake, on lung function and airway inflammation, among asthmatic adults in Saudi Arabia and in healthy individuals in the United Kingdom.

The main findings of the study were; vitamin D deficiency is highly prevalent in asthma participants in Saudi Arabia (86%) and in healthy individuals in the UK (81%). Asthma participants with higher serum vitamin D levels had trend of better lung function (FEV1, *P* 0.287; FVC, *P* 0.391), slightly lower fractional exhaled nitric oxide levels (*P* 0.719) and lower blood inflammatory biomarkers, however, did not reach significant levels. Healthy participants with higher vitamin D levels had also better lung function (FEV1, *P* 0.104, FVC, *P* 0.158) but no differences in airway inflammation.

Vitamin D intervention through oral supplements or dietary intervention for three weeks, significantly increased serum vitamin D levels (Supplement, *P* <0.001, dietary, *P* 0.002) and improved lung function, especially the forced vital capacity (*P* 0.031), but did not have effects on airway inflammation. Dietary vitamin D intake was lower than the recommendation in both groups. During the study sun-exposure was at the minimal level due to hot weather in Saudi and cold and cloudy weather in the UK. Vitamin D intervention did not affect weight or percentage of body fat.

Both chemiluminescence immunoassay and liquid chromatography-tandem mass spectrometry method can be used in measuring serum

vitamin D levels but they cannot be used interchangeably due to systematic differences. However, they showed an acceptable agreement levels. Overall, the study found that vitamin D supplement in high dose, low dose or dietary intervention were effective in raising serum vitamin D level and had a trend of positive effect on lung function but not airway inflammation.

Table of abbreviations

Abbreviation	Description
FEV1	Forced expiratory volume in one second
FVC	Forced vital capacity
FeNO	Fractional exhaled nitric oxide
25(OH)D	25-dihydroxy vitamin D
IgE	Immunoglobulin E
IU	International unit
UVB	Ultra violet-B
ng/ml	Nano gram per millilitre
nmol/l	Nano moll per litre
mmol/l	Millie moll per litre
µg	Microgram
BMI	Body mass index
IPAQ	International physical activity questionnaire
SEQ	Sun-exposure questionnaire
FFQ	Food frequency questionnaire
HPLC	High performance liquid chromatography method
ELISA	Enzyme-linked immunosorbent assay
LC-MS/MS	Liquid chromatography tandem mass spectrometry
CLIA	Chemiluminescence immunoassay

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Chapter 1: Literature review

1.1. Introduction

Vitamin D, also called calciferol, is one of the fat soluble vitamins that is essential for biological functions. Vitamin D is mainly obtained from sunlight exposure and only small amounts are present in foods and dietary sources. Vitamin D is also considered a hormone, because it can be produced in the body both in the skin and in other cells such as monocytes and macrophages and not only obtained from dietary sources like other vitamins (Deluca, 2008; Christakos *et al.*, 2010).

Vitamin D has become a topic of interest for researchers worldwide (Spiro and Buttriss, 2014; Cashman, 2012). Many studies have been conducted in different areas concerning vitamin D such as: non-calcaemic functions of vitamin D (Bischoff-Ferrari, 2010), daily recommendation (Cashman, 2015), safe dose of vitamin D supplementation (Glade, 2012), vitamin D deficiency and factors that can affect its levels and the correlation between vitamin D deficiency and diseases (Zhang *et al.,* 2016; Yousef *et al.*, 2013). Although many investigational studies have been published about vitamin D, only few clinical control trials have been conducted to investigate the effect of vitamin D supplementation on disease outcome. The causal correlation between vitamin D and disease is still debatable and more clinical trials are needed (Aspray *et al.,* 2014).

1.2. Vitamin D overview, structure and metabolism

Vitamin D generally consists of two main substances: vitamin D2 (Ergocalciferol) and vitamin D3 (Cholecalciferol). The form of vitamin D present in food sources is generally vitamin D3 or its metabolite 25(OH)D3, except in mushrooms, which contain the vitamin D2 form (Holick, 2007;

Holick *et al.*, 2011). The functional and biological form of vitamin D that acts in the body is 1,25(OH)2D3 (Calcitriol), while the form 25(OH)D (Calcidiol) can reflect the vitamin D status in the body (Holick, 2007).

Structurally, vitamin D is a seco-steroid component and it is stable during heating and storage. The steroid ergosterol is derived from plants and activated to ergocalciferol by irradiation while cholecalciferol is mainly obtained from animal steroids. The ultraviolet irradiation of ergosterol obtained from yeast or fungi can be used to produce vitamin D2 supplements or used in food fortification. Vitamin D2 as a supplement is widely used in the United State while in the UK both vitamin D3 and D2 forms are available as supplements (SACN, 2015; Holick, 2007). The effect on blood vitamin levels (25(OH)D) of supplementation with the D2 or D3 forms will be discussed in more detail in section 1.5.3. Figure 1.1 shows the chemical structure of vitamin D2 and D3.

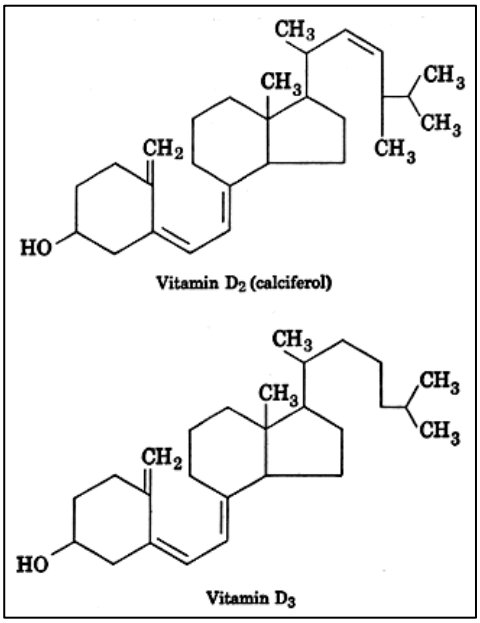

Fig 1.1. Chemical structure of vitamin D2 and D3

http://www.cyberlipid.org/vitd/vitd0001.htm 21-3-13

Cholecalciferol formation is initiated by the component 7-dehydrocholesterol, which is made in the sebaceous glands in the skin,

then absorbed by skin layers and distributed into the epidermis and dermis. The component 7-dehydrocholesterol absorbs the ultraviolet-B photons from the sunlight (wavelength 290-315 nm) to form pre vitamin D3 (Pre cholecalciferol). Rearrangement of the unstable double bonds by thermal isomerization of pre vitamin D3 then occur forming cholecalciferol. Figure 1.2 shows the process of formation of vitamins D2 and D3. Many factors can affect the photosynthesis of vitamin D in the skin, such as skin colour, age, type of clothing, use of sunscreen and geographical factors including latitude and season (Christakos *et al.*, 2010; Holick, 2007). These factors will be discussed in more detail in section 1.6.2.

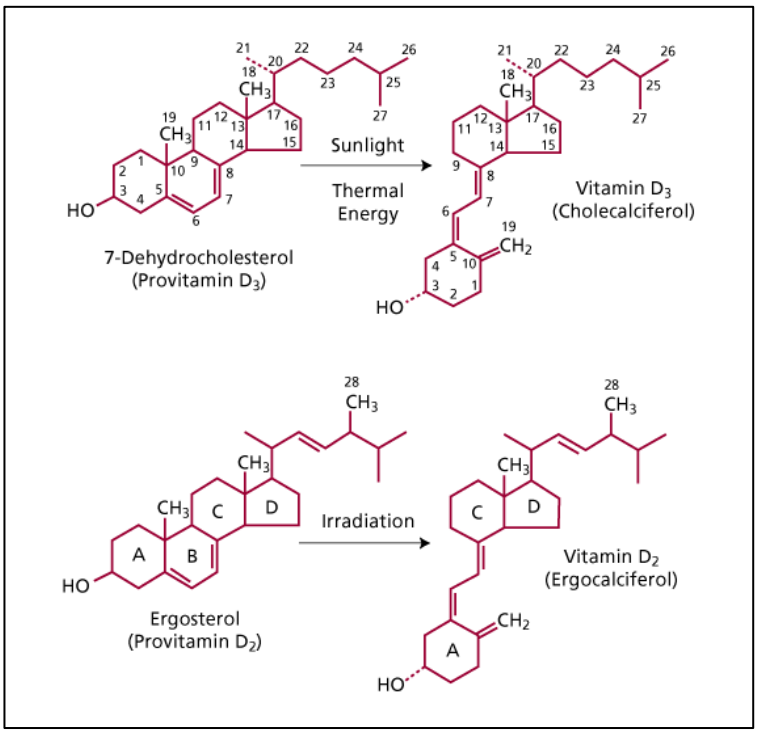

Fig 1.2. Formation of vitamin D2 and D3 (Jovicic *et al.*, 2012)

Cholecalciferol is transported from the skin to the blood by α-2 globulin vitamin D-binding protein (DBP). This binding protein is generated in the liver. Bound vitamin D will mainly be transported to the liver and may also be taken up by other tissues such as muscular or adipose tissues

(Christakos *et al.*, 2010). Vitamin D that comes from food follows a different mechanism. It is absorbed by the intestine from the micelles with help of fats and bile. In the intestine it compounds with chylomicrons that can enter into the lymphatic system and then into the blood flow. Subsequently chylomicrons transport the vitamin to the liver (Searing and Leung, 2010).

In the liver the enzyme 25-hydroxylase hydroxylates the cholecalciferol to 25(OH)D3 (Calcidiol), which is released into the blood and transported by vitamin D binding protein (DBP). The serum concentration of 25(OH)D is the best biomarker to reflect vitamin D status due to its long half-life and because the blood is the storage site of this form of vitamin D (Christakos *et al.*, 2010). Bound 25(OH)D3 is then absorbed by kidney tissues and another hydroxylation occurs by the enzyme 1-hydroxylase to form 1,25(OH)2D3 (Calcitriol) the active form of vitamin D. Once calcitriol is released from the kidneys it is transported to the target tissues and bound to the vitamin D receptors (VDR) which creates the biological effects (Christakos *et al.*, 2010). Figure 1.3.

Vitamin D receptors are present in many tissues including the intestine, bones and kidneys. They are also present in cardiac muscles, the pancreas, the brain, skin and hematopoietic cells. The form 1,25(OH)2D3 is then degraded by the enzyme 24-hydroxylase that converts 1,25(OH)2D3 to calcitroic acid, which can be discharged from the body (Deluca, 2008; Jovicic *et al.*, 2012).

[Click on link to view image]

Figure 1.3. Vitamin D metabolism pathway

http://www.nature.com/nrc/journal/v7/n9/images/nrc2196-f1.jpg

1.3. Vitamin D sources

Dietary vitamin D, sunlight exposure and supplements are the three sources of vitamin D (Matyjaszek-Matuszek *et al*., 2015). Sun exposure supplies the body with the largest amount, about 80% of vitamin D can be obtained from skin production via sun exposure, compared to a low percentage obtained from food (Searing and Leung, 2010). Vitamin D supplementation is also a source and it can be delivered via an oral or in intramuscular route (Pearce and Cheetham, 2010).

1.3.1. Dietary sources of vitamin D

Vitamin D is present in a small amount in a few types of foods. Food sources that provide vitamin D contain vitamin D naturally such as; fatty fish, egg, meat and liver, or be fortified with artificial vitamin D such as; fat spread, cereals, juices, babies' food, milk and milk products (Allen *et al.,* 2014; Buttriss, 2015). Table 1.3 shows the content of vitamin D in some food sources (Spiro and Buttriss, 2014; O´Connor and Benelam, 2011; McCance and Widdowson´s, 2010).

In the UK, the main food sources of vitamin D are, fatty fish such as sardines and salmon, eggs, liver, fortified fat spread and fortified cereals (O´Connor and Benelam, 2011). Milk contains a small amount of vitamin D unless it is fortified with vitamin D (More, 2013). Although meat and meat products contain a small amount of vitamin D, it had been stated that it is the main contributor to vitamin D intake for individuals in the UK (except for children), providing 23-35% of total vitamin D intake, followed by

fortified fat spreads which provide 19-21%, then fish with 17-23% and breakfast cereals with 13-20% of the vitamin D intake (Spiro and Buttriss, 2014; Bates *et al.,* 2014). According to the NDNS, fish was the main source of dietary vitamin D in people who are 65 years and older, providing 31% of vitamin D intake followed by fat spreads with 21% and cereals with 20% of vitamin D intake (Smithers *et al*., 1998). Generally, in the UK, infant formula is fortified with 1-1.25 µg vitamin D/100 kcal formula (Pearce and Cheetham, 2010).

In France and Spain, the main food source of vitamin D is oily fish, while in the US fortified milk is the main source of dietary vitamin D intake (Spiro and Buttriss, 2014). Vitamin D2 (ergocalciferol) and D3 (cholecalciferol) are suitable to be used in food fortification, usually vitamin D2 can be obtained from fungi and yeast, while vitamin D3 can be obtained from sheep´s wool (Lanolin) and also can be extracted from Lichens (Spiro and Buttriss, 2014). Vitamin D2 and D3 that extracted from the Lichens are suitable choice for vegetarians (Spiro and Buttriss, 2014).

Table 1.1. Food sources of vitamin D

Food source	**Vitamin D (µg/100g)**	**Food source**	**Vitamin D (µg/100g)**
Salmon, raw	5.9	Lamb, cocked	0.5
Sardine, fresh	11	Beef, stewed	0.8
Tuna, fresh	7.2	Chicken cooked	0.1
Mackerel, raw	8	Margarine	7.9
Egg	1.75	Butter	0.9
Pork, raw or grilled	0.9	Fat spread	5.8

(Spiro and Buttriss, 2014; O´Connor and Benelam, 2011; McCance and Widdowson´s, 2010).

It has been reported that consuming the daily vitamin D recommendation is not easy even if the individual follows a balanced diet (Spiro and Buttriss, 2014). Dietary supplements may be needed to achieve body requirements especially in winter months in high latitude countries, where food and supplements are the primary sources of vitamin D (Spiro and Buttriss, 2014). Heaney and colleagues (2003) stated that each 1 μg of vitamin D3 consumed can equal approximately 0.7 nmol/l of serum 25(OH)D.

The mean dietary intake of vitamin D in European countries is typically about 4.8 μg per day for males and 3.3 μg per day for females, the lowest intake being reported for Spain (1.1 μg per day) and the highest reported in Norway (23.5 μg per day; Spiro and Buttriss, 2014). These differences could be due to differences in availability of vitamin D rich food sources and population dietary habits. Dietary vitamin D intake in the UK is low according to the (NDNS; Gregorgy *et al*., 2000; Ruston *et al*., 2002). It was reported that the mean vitamin D intake from diet is 3.7 μg per day for adult males and 2.8 μg per day for adult females between 19 and 64 years of age (O´Connor and Benelam, 2011).

1.3.2. Sunlight exposure

Sunlight exposure is a natural way to synthesise vitamin D via the skin and the photoreceptor 7-dehydrocholesterol in the keratinocytes. Production of vitamin D from the sunlight can never lead to excessive synthesis or toxicity, because when large amount of pre cholicalceferol is produced, other products can be also produced such as, lumisterol, eoxisterols and tachysterol that can be then extracted from the body. However, vitamin D supplements very rarely may lead to toxicity if taken in very large doses for long periods because the production of 25(OH)D is not limited (Hart and Gorman, 2013).

Sunlight exposure and ultraviolet radiation (UVR) have many benefits for human health and also have risks at the same time. Ultraviolet B (UVB) with wave length (290-315 nm) is one type of UVR and it is the main source of vitamin D synthesis via the skin. Ultra violet radiation can cause eye problem, immune system problems and also can affect skin health and cause sunburn. Sunburn is a risk factor for skin cancer including malignant melanoma (Seckmeyer *et al*., 2013). Even a sub-erythemal amount of UVB can lead to damage of the DNA in the skin cells. Despite these risks, short exposure to sun light is very important for human health and for obtaining the vitamin D that is required by the body (Rhodes *et al*., 2010).

Many factors affect UVB intensity such as, cloudiness, ozone, latitude, season and diurnal variability (Terenetskaya and Orlova, 2011). For example, it is impossible to synthesis enough vitamin D from sunlight exposure if the UV index is less than 3, because the wavelength between 290-315 which is needed for vitamin D synthesis is only available when the UV index is greater than 3 (Seckmeyer *et al*., 2013; Pearce and Cheetham, 2010). It is believed that 1000 IU of vitamin D3 can be produced in one minute via skin for a pale skinned person with a completely uncovered body in summer with UV index 10 in a place with mid latitude, in a vertical position. This amount will be different with different situations such as body orientation, standing vs lying down, winter months and different UV index (Seckmeyer *et al.,* 2013).

It has been recorded that in the UK in summer, people with fair skin can produced about 2000 IU if they have sunlight exposure for 20-30 minutes per day exposing their face, arms and legs. Two or three times per week of this amount of exposure are enough to achieve and maintain vitamin D levels during summer (Pearce and Cheetham, 2010). A study by Seckmeyer *et al* (2013) in Germany, aimed to compare the time needed to produce 1000 IU in five cases, four of them in Hannover (Latitude 52.39) in different seasons and of different UV indexes. They found a large difference in vitamin D production (Table 1.2).

Table 1.2. Differences in vitamin D production according to season and UV index (Seckmeyer *et al.,* 2013)

Situation	**Case 1**	**Case 2**	**Case 3**	**Case 4**
Place	Hannover	Hannover	Hannover	Hannover
Latitude	52.39	52.39	52.39	52.39
Date	June 21	March 21	December 21	December 21
Cloudiness	None	None	None	Homogeneous
UV index	8	3.3	0.4	0.04
Required exposure time to produce 1000IU with uncovered body (vertical position)	1.1 minute	2.3 minutes	39 minutes	2.1 days

People in high latitude countries >40°N such as the UK are at risk of having low vitamin D in winter months because the UVB radiation is unavailable or insufficient to initiate vitamin D synthesis in the skin, because the angle of the sunlight is skewed and prevents the UVB from passing through the ozone layer (Lanham-New, 2011; Cashman, 2012). However, a sufficient vitamin D level can be achieved in winter because vitamin D can be stored in the body from the intake in the summer (Seckmeyer *et al.,* 2013). Webb and colleagues concluded that having a level of ≥32 ng/ml in late summer can maintain the winter levels at sufficient level ≥20 ng/ml (Webb *et al.,* 2010).

Rhodes and colleagues found that 5% of white Caucasians in Manchester (latitude 53.5 N) in winter have vitamin D deficiency 25(OH)D< 15ng/ml, 62.5% have vitamin D insufficiency 25(OH)D <20 ng/ml and only 2.9% have the optimum level 25(OH)D ≥32 ng/ml. They investigated the effect of summer sun exposure on 25(OH)D levels and found that after sunlight exposure of 13 minutes three times per week for six weeks with 35% of skin surface area exposed, 25(OH)D increased by 10.4 ng/ml. They also stated that UVB was at a maximum at solar noon when the solar radiation

is reaching the earth in the shortest time and the sun is directly overhead (Rhodes *et al*., 2010).

A study carried out in Saudi Arabia between October to December in 2010 among healthy children (n=510), 4-15 years old from Jeddah, aimed to investigate the correlation between serum vitamin D levels and dietary vitamin D intake and sunlight exposure (Mansour and Alhadidi, 2012). The authors found that only 14% had normal vitamin D levels >20 ng/ml, 59% had vitamin D insufficiency 7-20 ng/ml and 27% had severe vitamin D deficiency <7 ng/ml. Mean serum 25(OH)D among these children was 13±8 ng/ml, mean dietary intake was 3.3±1.9 µg/D with 83% of them consuming less than 5 µg/D. The average of daily sunlight exposure was 7.64±7.49 min per day. They stated that this low duration of direct sunlight exposure could be due to the avoidance and reduction of outdoor activity due to the hot and humid weather resulting in children spending more time indoors playing video games or watching television. The authors also found a significant correlation between higher 25(OH)D levels and higher duration of sunlight exposure and also with more BSA (body surface area) exposed to the sunlight and concluded that 20% of the body surface should be exposed to sunlight at least to increase vitamin D concentration. In addition they also found a significant positive correlation between 25(OH)D levels and dietary intake (Mansour and Alhadidi, 2012).

From October to March, people cannot synthesis enough vitamin D from the sunlight exposure in the northern hemisphere such as in the UK and 50% of northern America. People living in these areas are dependent on the vitamin D stored in their body from summer sunlight (Pearce and Cheetham, 2010). Heaney *et al* (2003) concluded that healthy individuals need a minimum of an additional 12.5 µg/day of dietary vitamin D3 to maintain the autumn 25(OH)D concentration throughout the winter. Cashman and colleagues (2008) concluded that healthy adults from 20-40 years old need approximately from 7.2 to 41.1 µg per day vitamin D to maintain serum 25(OH)D above the cut-off value (>25nmol/l) during

winter. This wide range is because of the variety in sunlight exposure (Cashman *et al*., 2008).

However, the current Cancer Research UK´s Sunsmart and NHS (National Health Services) sun exposure recommendations are;

- Spend time in the shade between 11.00 am and 3.00 pm
- Make sure you never burn
- Aim to cover up with a T-shirt, hat and sunglasses
- Remember to take extra care with children
- Then use factor 15+ sunscreen

1.3.3. Vitamin D supplements

Many vitamin D supplements are available to be used in different countries. They are safe and effective to treat vitamin D deficiency and physicians usually use three forms of vitamin D supplements; pills, drops or injections (Pearce and Cheetham, 2010). Each supplement form has different doses, and their use depends on the level of vitamin D deficiency (Lewis *et al*., 2012). Many doses are available in the UK as pills; 400 IU, 10 000 IU, 20 000 IU and 50 000 IU. The intramuscular injection usually has higher doses such as 200 000 IU or 300 000 IU per ampoule and it is used for one single load dose followed by a maintenance dose. High doses of 200 000 IU and 300 000 IU are also available as oral solutions (Aspray *et al*., 2014; Pearce and Cheetham, 2010). Although the prevalence of vitamin D insufficiency is high during winter and spring in the UK, the prevalence of using vitamin D supplements in the UK population is only about 5% of the population (Hypponen and Power, 2007; Glass *et al*., 2009).

Both vitamin D2 and D3 forms of supplement elevate serum 25(OH)D and a number of studies have been conducted to compare the effect of D2 and D3 on serum 25(OH)D concentration. A study by Romagnoli and colleagues (2008), studied the difference between vitamin D3 and D2 supplement

either by an oral or intramuscular route. They found that the D3 form increased the total 25(OH)D significantly more than the D2 form due to faster metabolism. The authors found that oral route increased the level more promptly than the intramuscular injection and stated that the intramuscular route may be not the proper physiological route of administration (Romagnoli *et al.,* 2008). Another study used the dose (4000 IU) of D2 or D3 daily for two weeks in winter among 72 healthy adults and found that 25(OH)D concentration increased after using the supplements in both groups but the increase was greater by 70% in D3 group (Trang*et al.,* 1998). Another study by Glendenning and colleagues used supplement with 1000 IU of D2 or D3 for three months among 95 patients who had hip fracture and they found that 25(OH)D increased by 52% more in D3 group when compared to D2 group (Glendenning *et al*., 2009).

Heaney and colleagues aimed to study the response of oral vitamin D3 supplementation on serum 25(OH)D and they concluded that an additional 1 µg of vitamin D3 was equal to 0.7 nmol/l of serum 25(OH)D, the vitamin D3 required to maintain serum 25(OH)D concentration in winter was 12.5 µg per day and the body´s stores can provided 78 to 82 µg per day. They also concluded that healthy man uses about 3000-5000 IU cholecalciferol per day to meet winter requirements, in addition to the accumulated vitamin D in the body from the summer (Heaney *et al.,* 2003).

Overdose (toxicity) of vitamin D is rare and can increase the absorption of calcium causing hypercalcemia that can lead to renal failure, accumulation of calcium stones, aortic calcification or accumulation in soft tissues. Vomiting, anorexia and headache are symptoms of vitamin D toxicity (Deluca, 2008; Spiro and Buttriss, 2014; Heaney, 2008). Vitamin D toxicity has not been observed with dietary intake or sunlight exposure (Hart and Gorman, 2013); however, very high doses of the pharmacological form of vitamin D over a long period could lead to toxicity. Up to 10 000 IU per day is safe but 20 000 to 50 000 IU per day for a sustained period could cause vitamin D toxicity (Heaney, 2008). In a systematic review and meta-analysis

done in 2016 by Malihi and colleagues including 48 studies and 19833 participants, found that vitamin D supplementation for long period can increase the risk of hypercalciuria or hypercalcemia and this finding is not related to the supplement dose. The National Institute of Health (2008) considered serum 25(OH)D more than 500 nmol/l as vitamin D toxicity. Heaney agreed that the toxicity cannot happen with 25(OH)D of less than 500 nmol/l (Heaney, 2008).

Previous intervention studies have been conducted to investigate the effect of oral vitamin D supplementation on serum 25(OH)D levels and toxicity. Holick *et al.* (2011), stated that 2000 to 4000 IU of vitamin D supplement per day for deficient people is not enough to achieve a normal serum 25(OH)D level, and up to 10 000 IU of vitamin D supplement per day is safe and patients did not show any symptoms of toxicity. However, Ross *et al* (2011) stated that over 10 000 IU of vitamin D supplement per day may cause renal and tissue damage. A study by Ilahi *et al* (2008) found that a single oral dose of 100 000 IU of vitamin D3 is safe and increased serum 25(OH)D by 27 ng/ml in 30 healthy people. In another study in adolescents in Normandy, the authors used a single dose of 200 000 IU of vitamin D3 in winter and measured serum 25(OH)D at baseline, after three weeks and over three months after the supplementation. Their results showed, that serum 25(OH)D increased significantly after three weeks then decreased after three months without any adverse reaction (Mallet *et al.,* 2010).

The UK National Osteoporosis Society (NOS) published vitamin D guidelines in 2014 (Aspray *et al.,* 2014) stated that oral vitamin D3 is the best choice rather than vitamin D2 for treatment and correction of vitamin D deficiency due to the availability of vitamin D3 supplementation in the UK. They stated also that when there is the need for fast or urgent correction of vitamin D levels such as in symptomatic diseases, the treatment strategy should be a high dose of vitamin D3 (300 000 IU) given as separate doses, daily or weekly over a period of 6 to 10 weeks in total. This high load dose

is then followed by a maintenance dose of 800-2000 IU daily. With less urgent cases, an oral maintenance dose can be used without the high load dose. When a high dose is used, calcium levels should be measured and checked after one month of completing the total dose because in rare situation increased calcium levels may be occurred after using a high dose of vitamin D supplementation (Aspray *et al.,* 2014).

1.4. Vitamin D recommendations

Dietary vitamin D recommendations vary by country, health situation, and age. In the UK there was no reference nutrient intake (RNI) for dietary vitamin D for individuals 4 years and above until the new SACN recommendations published in 2016. When the past RNI was established by the Department of Health in the UK, they assumed that healthy adults have sufficient sunlight exposure in the summer, providing them with an adequate amount of vitamin D synthesised by the skin, and that this amount will maintain the serum level of 25(OH)D above 25 nmol/l in the winter months (Buttriss, 2015). However, the current UK RNI for vitamin D is 10 µg per day for people who are 4 years and above, young children and pregnant women, who are at a higher risk of developing vitamin D deficiency (SACN, 2016; Table 1.3). The SACN reviewed many studies in order to set the RNIs for vitamin D and they found that evidence regarding non-musculoskeletal outcomes were insufficient. So the DVRs of vitamin D were set according to the high quality studies of the musculoskeletal health. The available data about non-musculoskeletal outcomes were tend to be observational, cross-sectional studies, case reports, in addition to, high inter-assay and inter-laboratory variations in measuring serum vitamin D . In a meta-analysis by Cashman and colleagues (2011), they reviewed 44 RCTs including studies in adults, adolescents, children and elderly to investigate a model of relationship between vitamin D intake and serum 25(OH)D concentration in order to established the reference intake value

of vitamin D. using this meta analysis and meta regression they concluded that the model to predicted serum 25(OH)D in nmol/l is (0.044 X (total vitamin D intake in IU) + 33.035) and by using this model they found that the predicted RDA of vitamin D at serum 25(OH)D 50 nmol/l is about 359 IU per day (Cashman *et al.,* 2011).

In the light of above, SACN considered the threshold 25(OH)D of 25 nmol/l as desirable levels based on RCTs and meta analysis. According to the threshold, the RNI established to be 10 µg per day vitamin D to achieve serum 25(OH)D ≥ 25 nmol/l in 97.5% of the UK population (SACN, 2016). There is no dietary reference intake of vitamin D in Saudi Arabia.

Table 1.3. The UK Reference Nutrient Intake (RNI) for vitamin D

Age	**Men**	**Women**
0-11 months	8.5-10 µg	8.5-10 µg
1- <4 years	10 µg	10 µg
4 years and above	10 µg	10 µg
Pregnancy	-	10 µg
Lactation		10 µg

Older adults experience a higher rate of muscle pain, loss of bone strength and were more likely to experience bone fracture (Bischoff-Ferrari *et al.,* 2004). Their skin is less effective at producing vitamin D from the sunlight (Spiro and Buttriss, 2014) due to a decrease of VDR (Wicherts *et al*., 2007). They therefore need to ensure they consume sufficient daily vitamin D to meet their needs. Data from the National Diet and Nutrition Survey (NDNS) shows that people who are 65 years and above are more vitamin D deficient compared to younger individuals (Ruston *et al.,* 2002; Finch *et al*., 1998). In addition, they also consume a lower amount of dietary vitamin D because the most common foods eaten by this age group were potatoes, bread, biscuits and milk however; none of these foods are high source of vitamin D (Smithers *et al*., 1998). Loss of mobility, lower sunlight exposure

and lower dietary intake of vitamin D, could be factors leading to vitamin D deficiency in older people (Salamone *et al.,* 1993).

Pregnant women need more calcium to supply the foetus with the calcium needed for bone formation. Vitamin D plays an important role in calcium homeostasis, so maintaining sufficient levels of vitamin D is particularly important during pregnancy (SACN, 2015). In a randomized trial done (Hollis *et al.,* 2011) 350 pregnant women received a 400, 2000 or 4000 IU vitamin D supplement for 4-5 months until they delivered their baby. The authors aimed to achieve the level 80 nmol/l, this level being considered the optimal level for calcium absorption and preventing secondary hyperparathyroidism. They found that the concentration of 25(OH)D increased after the supplementation in all groups. They also found that the concentration of vitamin D in neonates was correlated with 25(OH)D levels of the mothers during the maternal period. They concluded that the level of serum 25(OH)D at 100 nmol/l was needed to optimize the production of the active form 1,25(OH2)D during the period of pregnancy (Hollis *et al.,* 2011). New born infants and young children need calcium and vitamin D to form healthy bones. In addition, vitamin D deficiency can lead to seizures in neonates (Hollis *et al*., 2011). A study by Hazell *et al* (2015) found that children who had serum 25(OH)D of >75 nmol/l had higher bone mineral content compared to children who had levels <75 nmol/l.

In the UK, the cut-off level of vitamin D of 25 nmol/l is considered as vitamin D deficiency. This value was based on the absence of rickets in children or osteoporosis in adults (SACN, 2016). However, for the non-calcaemic effects of vitamin D, higher levels are reported (Lanham-New *et al*., 2011). According to other studies, SACN (Scientific Advisory Committee on Nutrition) in 2016 stated evidence supporting the value of >50 nmol/l as desirable for bone and muscle functions. The US Institute of Medicine (IOM) in 2011, (Ross *et al.,* 2011) concluded that a serum 25(OH)D level <30 nmol/l was considered to be deficiency, 30-50 nmol/l considered to be inadequate and >50 nmol/l was the sufficient level. The level at 50 nmol/l

is agreed also by the World Health Organization, and by the Netherlands recommendation (Spiro and Buttriss, 2014). The Endocrine Society in the USA recommended a level of 25(OH)D at 75 nmol/l (>30ng/ml) to be sufficient and recommended consumption of 1500-2000 IU of vitamin D daily to achieve this level (Holick *et al.,* 2011). Heaney (2005) stated that 4000 IU (100 µg) of vitamin D per day is needed to maintain a serum 25(OH)D of 80 nmol/L.

The first reference value for vitamin D in the USA in 1997 was 200 IU (5 µg) per day for adults >50 years old, 400 IU (10 µg) per day for people from 51 to 70 years old and 600 IU (15 µg) for people above 70 years irrespective of gender (Institute of Medicine, 1997). In 2011 the Institute of Medicine increased the recommendation for vitamin D based on numerous studies about its beneficial effects (Table1.4). They reviewed a large number of studies on the beneficial effects of vitamin D on non-calcaemic functions such as cancers, CVD, diabetes, falls and infection and concluded that only bone health had sufficient evidence to identify an increase in the DRI. Accordingly, people who had serum 25(OH)D <40nmol/l were at high risk to have adverse bone outcomes. It is still debatable as to whether other diseases should be considered in deciding the vitamin D recommended or desirable levels; however, more evidence is needed to investigate the causal relationship with vitamin D (Ross *et al.,* 2011). It has been reported that consuming a daily amount of 10 µg can maintain serum 25(OH)D at the level 40 nmol/l and 15 µg daily can maintain the level at 50 nmol/l (Ross *et al*., 2011).

Table 1.4. The dietary reference intake for vitamin D (IOM, 2011)

Category, age groups	**(EAR)** µg/day	**(RDA)** µg/day	**(UL)** µg/day
Infant 0-6 months	-	-	25
Infants 6-12 months	-	-	37.5
1-3 years old	10	15	62.5

4-8 years old	10	15	75
9-13 years old	10	15	100
14-18 years old	10	15	100
19-30 years old	10	15	100
31-50 years old	10	15	100
51-70 years old males	10	15	100
51-70 years old females	10	15	100
>70 years old	10	15	100
14-18 years, pregnant/lactating	10	15	100
19-50 years, pregnant/lactating	10	15	100

EAR, Estimated Average Requirement.RDA, Recommended Dietary Allowances. UL, Upper level.

The highest recommended dietary intake of vitamin D in European countries is 20 µg per day for adult (in Austria, Germany and Switzerland), while in France, the recommendation for adults is 5 µg per day. However, France has the highest recommendation for children <1 year of age at 20-25 µg per day (Spiro and Buttriss, 2014). Recently, the SACN proposed 10 µg per day vitamin D as the RNI for individuals of 4 years and above in the UK (Buttriss, 2015). There are no recommended reference intakes in Saudi Arabia; here, dietitians and practitioners apply the same recommendations as the USA (The Institute of Medicine, 2011) and vitamin D research studies in Saudi Arabia usually use the US recommendation. For example, in a study done in Saudi Arabia to investigate the prevalence of vitamin D deficiency in children, they used the US recommendation (10 µg per day) when measuring and comparing the dietary vitamin D intake in this group (Mansour and Alhadidi, 2012). Another recent study done in Saudi Arabia among pregnant women also used the US recommendation 15 µg per day to compare and assess the intake of vitamin D in this group (Al-Faris, 2016). Researchers should be aware of the recommendation of vitamin D that used in the study when compare to other studies.

1.5. Vitamin D functions

1.5.1. Vitamin D and calcium homeostasis

The main function of vitamin D in the body is calcium homeostasis and promoting bone health (Jovicic *et al.*, 2012; Weaver, 2007). Calcium homoeostasis refers to ensuring that the calcium concentration is maintained in the desirable range. Calcium homeostasis is essential for the body because calcium is an important mineral that is involved in many functions including: bone calcification, muscle function, muscle contraction and neurological function (Sunyecz, 2008). Calcium regulation depends on calciotropic hormones which are; parathyroid hormone PTH, calcitonin (a hormone secreted by the thyroid gland) and vitamin D. Calcitonin hormone works as inhibitor of calcium absorption and bone re-absorption, its activity being the opposite of that of PTH (Carter and Schipani, 2006). Vitamin D´s role in the process is well known. When calcium intake or calcium level in the blood is low, PTH is released and vitamin D is converted to the active form 1,25(OH)2D in the kidney. The active form of vitamin D then binds to the VDR and stimulates the synthesis of ca-binding protein, increasing calcium re-absorption in the kidney and increasing bone resorption. When the calcium level is high, PTH secretion is suppressed and vitamin D regulates and decreases the absorption of calcium in the kidney and bone resorption; this is a negative feedback process for vitamin D (Weaver, 2007).

Phosphorus is an important element that has many essential functions in the body, it is involved energy release, protein synthesis and important for enzymes function. Phosphorus also is important in bone health because due to its essential role in combine the calcium in the blood to form hydroxyapatite the component needed for bone rigidity. Vitamin D deficiency and low calcitriol levels can decrease phosphorus absorption from the gut leading to hypophosphatemia. In contrast, high level of phosphorus in the blood can affect calcium metabolism leading to hypocalcemia (Ahmed, 2010). Measuring calcium status is difficult as 99% of calcium is located in the bones and because serum total calcium

measurement is also effected by albumin concentrations as half of the calcium in the blood is bound to albumin (Ahmed, 2010).

Low levels of vitamin D and high levels of PTH increase bone remodeling and fractures. A level of 25(OH)D between 75-80 nmol/l is the desirable level for optimum calcium absorption (Weaver, 2007). Calcium homeostasis and a normal range of vitamin D levels are needed to prevent the incidence of rickets and osteoporosis.

Building bone mass in childhood is necessary to prevent osteoporosis in the future (Weaver, 2007). Osteoporosis is a widespread health problem worldwide requiring a large amount of resources and resulting in morbidity and mortality (Sunyecz, 2008). The bone becomes fragile, weak and thin in osteoporosis patients. This loss of strength in bones can increase the fracture rates, especially causing hip fractures (Sunyecz, 2008). In summary, vitamin D maintains calcium and phosphorus concentrations at the optimum range for bone mineralisation. This function of vitamin D is a result of three actions: active transportation of calcium into the enterocyte of the small intestine; active transportation of phosphorus into the enterocyte of the small intestine with help of parathyroid hormone supporting bone resorption when calcium intake is insufficient to avoid hypocalcaemia (Deluca, 2008). (figure 1.4)

[Click on link to view image]

Figure 1.4. Calcium homeostasis

http://accesspharmacy.mhmedical.com/data/Books/gano24/gano24_c021f003.png

1.5.2. Vitamin D and falls, muscle function and bone fractures

Vitamin D can prevent bone fractures and increasing bone density. Vitamin D also has a role in muscle health, it can decrease muscle pain and in addition, vitamin D receptors (VDR) are located in muscles tissues and VDR can activate synthesis of muscle protein. There is also a correlation between vitamin D deficiency and muscle weakness (Bischoff-Ferrari, 2010). Bone fractures can cause pain, loss of mobility, need of long term care and also can lead to psychological problems (Sunyecz, 2008).

Among older adults, muscle weakness and pain, falls, decrease in muscle function and bone fractures are common. In a study by Bischoff-Ferrari and colleagues (2004), they studied the correlation between VDR expression and age. They included 20 women with a mean age of 72 years and investigated a gluteus mediusspecimen taken by biopsy during surgery (total hip arthroplasty). They assessed VDR expression and found that older patients had decreased VDR expression in skeletal muscle.

A large cross-sectional study from Amsterdam on 1234 participants aged 65 years and above found that participants who had serum 25(OH)D of >30 ng/ml (75 nmol/l) had better physical performance compared to participants who had serum 25(OH)D <10 ng/ml (25 nmol/l). Physical performance was assessed as a total score of: walking test, chair stands and tandem stand (Wicherts *et al.,* 2007).

Among older adults hip fracture is costly and nearly all cases involve falls (Bischoff *et al.,* 2003). Vitamin D status and supplementation can benefit muscle function and strength and vitamin D deficiency is a predictor of falls (Bischoff *et al.,* 2003). In a clinical randomized control trial in Switzerland, 122 women who were 63 years and above were enrolled from a geriatric care unit. Sixty-two women were enrolled to receive 1200 mg calcium and 800 IU vitamin D3 supplement, and 60 women were enrolled to receive

1200 mg calcium supplement alone, every day for 12 weeks. They found that the group who received calcium and vitamin D had 49% less falls compared to the calcium only group, these falls were recorded by nurses. The authors also found improvement in musculoskeletal function measured as a sum score of "knee flexor and extensor strength, grip strength and the timed up and go test" (Bischoff *et al.,* 2003).

In a systematic review and meta-analysis (Dao *et al*., 2014), they found that low vitamin D levels was associated with stress fractures, imbalanced bone remodelling and structural bone fatigue.

1.5.3. Vitamin D and other chronic disease

In the past it was believed that vitamin D was important only for calcium homeostasis and bone and muscle health. Recently, many studies have focused on the non-calcaemic effects and functions of vitamin D such as the positive effect on diabetes, cardiovascular disease, cancers, autoimmune disorders and anti-inflammatory effects from epidemiological studies and randomized controlled trials (Bischoff-Ferrari, 2010; Matyjaszek *et al.*, 2015; Wang *et al*., 2008).

1.5.3.1. Cardiovascular disease (CVD)

Different levels of serum vitamin D are associated with positive effects for many diseases. Bischoff-Ferrari (2010), reported that a 25(OH)D levels of 100 nmol/l was associated with a decreased risk of CVD. Vitamin D can play a role in reducing CVD in different ways: vitamin D supplements of 800 IU per day for two months may reduce blood pressure, thus vitamin D deficiency may be associated with the prevalence and incidence of CVD, renin activity in plasma and associated with calcification of the coronary artery (Wang *et al.*, 2008).

Forman *et al* (2007), studied the correlation between serum 25(OH)D and the risk of hypertension from two large cohort studies included 613 males

and 1198 females who did not have hypertension at baseline. They found that a higher level of 25(OH)D was inversely correlated with the incidence of hypertension (Forman *et al*., 2007). Raising renin enzyme can lead to increased blood pressure due to its role in the renin-angiotensin system which results in high blood pressure, after the angiotensin1 is converted to angiotensin2 (Li *et al*., 2002). Increased angiotensin2 constricts the blood vessels and enhances the secretion of the hormone aldosterone from the adrenal gland. Aldosterone hormone stimulates the kidneys to reabsorb water and sodium into the blood, causing high blood pressure (Li *et al*., 2002). In an animal study, Li and colleagues (2002) found that vitamin D receptor-null mice had more plasma renin and angiotensin2, causing higher blood pressure, increased water consumption and cardiac hypertrophy. They also found that 1,25OH2D3 injection suppressed renin expression in these mice.

Moreover, an intervention study in Germany enrolled 148 older females to a calcium 1200 mg supplementation group or a calcium 1200 mg plus 800 IU vitamin D3 supplementation group for eight weeks. They found that the calcium and vitamin D supplementation group increased serum 25(OH)D significantly and decreased the systolic blood pressure by 9.3% compared to the calcium only supplement group (Pfeifer *et al*., 2001).

1.5.3.2. Diabetes mellitus

A review by Mathieu *et al* (2005) reported that vitamin D is linked to both type 1 and type 2 diabetes mellitus. By reviewing previous studies they explained the vitamin D effect on diabetes as follows: firstly, VDR were founded in beta cells of the pancreas. Secondly, low 25(OH)D levels may impair the secretion and synthesis of the insulin hormone in the pancreas and this can play a role in type 2 diabetes. Thirdly, vitamin D supplements may have an effect in decreasing the risk of type 1 diabetes and treating vitamin D deficiency in diabetic patients may improve their glucose

tolerance. In addition to these effects, the active form 1,25(OH)2D3 may protect beta cells and prevent the damage that can be caused by the inflammatory cytokines such as IFN-α. This damage in the beta cells is a step in the development of type 1 diabetes (Mathieu *et al.*, 2005; Bikle, 2009).

In a large cross sectional study which included 960 adult participants from the first national nutritional survey in Kuwait, Zhang and colleagues (2016), aimed to examine the correlation between 25(OH)D levels and the prevalence of diabetes. They found that median serum vitamin D was 13.8 ng/ml and 83% of the participants had vitamin D deficiency or insufficiency. They also found a two-fold increase in diabetes prevalence among vitamin D deficient or insufficient participants compared to participants who had serum 25(OH)D> 20ng/ml (50 nmol/l). Their explanation of these results were similar to Mathieu *et al* (2005) which were that VDR are located in beta cells in the pancreas, vitamin D may regulate insulin secretion, increase insulin sensitivity and decrease insulin resistance and decrease inflammation (Zhang *et al.,* 2016).

In a randomized clinical trial done in India, 137 participants diagnosed with pre-diabetes were enrolled into a vitamin D supplement group (n=69) or placebo (n=68). The supplement was as 60 000 IU once a week for four weeks followed by 60 000 IU per month for total period of 12 months. After 12 months, fasting glucose, 2-hour glucose and HbA1c (Glycated haemoglobin) were significantly lower in the supplementation group than placebo (Kuchay *et al.,* 2015).

In the light of the above, vitamin D can play a role in diabetes in many ways; vitamin D may improve insulin secretion, improve insulin resistance, decrease the inflammation and prevent the cells in the pancreas from the pro inflammatory cytokines.

1.5.3.3. Cancers

Many studies have found that vitamin D is linked to cancer and cancer markers, especially breast, colon and rectal cancers (Feskanich *et al*., 2004; Lappe *et al*., 2007; Yousef *et al.,* 2013). Vitamin D receptors are expressed in many malignant cells and the active form of vitamin D can reduce the proliferation of the malignant cells and at the same time increase the differentiation of the normal cells. It has also been found that high vitamin D intake and higher 25(OH)D levels were associated with lower incidence of colon and rectal cancers (Bischoff-Ferrari, 2010).

A case control study was done by Feskanich *et al* (2004), on 193 women aged 46-78 years with colorectal cancer. They aimed to investigate the association between vitamin D levels and the risk of colorectal cancer and they located these women within a large cohort of data which included 121,700 females, from the Nurses' Health Study. 193 cases of colorectal cancer were recorded and they found that mean 25(OH)D in cases 27 ng/ml, was significantly lower than matched control women 30.3 ng/ml (*P* 0.03). They also found that higher level of 25(OH)D was negatively correlated with the risk of colorectal cancer (Feskanich *et al.,* 2004).

A study in Saudi Arabia on 120 women with breast cancer found that patients with breast cancer had significantly lower serum 25(OH)D than control subjects, despite all Saudi women being at high risk of vitamin D deficiency due to low sunlight exposure and clothing style. The author explained the role of vitamin D in breast cancer by the anti-proliferation and pro-differentiation action of vitamin D on cancer cells (Yousef *et al.*, 2013).

In a 4-year randomized control clinical trial done in Nebraska, Lappe and colleagues (2007) recruited 1179 women of 55 years and above to investigate the effect of 1500 mg of calcium supplement, calcium with 1100 IU of vitamin D supplement or placebo, on the risk of cancer. They found that both intervention groups reduced the risk of all type of cancers; however the calcium and vitamin D group had the better effect with significantly lower incidence of cancer compared to the placebo group. Participants' baseline vitamin D was 72±20 nmol/l and the dose of 1100 IU per day increased the level by 24±19 nmol/l. The authors concluded that increasing serum 25(OH)D by 25 nmol/l may reduce the risk of cancers by 35%. They explained this benefit by the role of vitamin D on genes including the regulation function of cell proliferation, differentiation and cell apoptosis and they stated that if the vitamin D level was low these functions will be defective (Lappe *et al.,* 2007).

1.5.3.4. Asthma

Many studies have focused on the effect of vitamin D on immunity and the relationship between vitamin D deficiency and diseases involving immune dysfunction, including asthma. It has been found that vitamin D has effects on different immune cells such as macrophages, dendritic cells, T lymphocytes and B lymphocytes (Szczawinska-Poptonyk and Breborowicz, 2012). Recent studies have found a positive correlation between higher 25(OH)D levels and asthma outcomes such as better asthma control, better lung function, lower inflammation and better response to corticosteroid (Raissy and Blake, 2015).

Asthma is a chronic inflammatory disease and it is a potentially fatal condition. Wheezing, cough, shortness of breath, lung obstruction and chest tightness are the most common symptoms of asthma. These symptoms can range from occasional mild symptoms to life threatening attacks (Al-Zahrani*et al*., 2015). The pathophysiology of asthma starts with

airway inflammation followed by airway hyper responsiveness and if chronic inflammation is not treated then airway remodelling can occur. Airway remodelling is a late stage of asthma, causing change and damage of the epithelial cells in lungs (Al-Moamary *et al*., 2012). According to the global initiative for asthma (FitzGerald et al., 2012) family history of asthma, allergy, environmental allergens exposure, exercise, smoking, respiratory infection, genetics and some medications such as aspirin are risk factors that can both increase the chances of developing asthma or increase the symptoms and exacerbation periods (FitzGerald *et al*., 2012).

A diagnosis of asthma should be made based on patient medical history, physical examination, blood analysis such as eosinophil counts and total Immunoglobulin E (IgE) and clinical assessment such as spirometry to measure lung function (Al-Moamary *et al*., 2012). The most important spirometer measurements are: forced expiratory volume in one second (FEV1), which is "the volume of air that the patient is able to exhale in the first second of forced expiration". The second measurement is forced vital capacity (FVC), which is "the total volume of air that the patient can forcibly exhale in one breath" and the FEV1/FVC ratio, which is "the ratio of FEV1 to FVC expressed as a percentage" (Bellamy *et al.,* 2005).

Asthma outcomes or asthma biomarkers are classified as core or required, supplemental and emerging (Szefler *et al*., 2012). These biomarkers can be used in studies to investigate treatments or measure the improvement and monitoring asthma. The only core outcome that is required in every study is total IgE to determine if the patient has atopy or allergy. Eosinophil count in blood or sputum, fractional exhaled nitric oxide, leukotrienes in urine and specific IgE are supplemental outcomes that can be measured depending on the study aims. Airway imaging, skin prick test and genetic analysis are emerging outcomes that need more research (Szefler *et al*., 2012). Asthma control is the physician's goal and consists of treating asthma, as well as giving safe treatment with minimum adverse effects of

medication and with a supplemental aim of cost effectiveness. Asthma control means minimal symptoms, normal physical activity, no night-waking due to symptoms, minimum use of quick relief medication, no exacerbation and normal lung functions (FitzGerald *et al*., 2012).

Asthma can be correlated with other allergic diseases such as food allergy, eczema, conjunctivitis and rhinitis (Pawankar, 2014). The prevalence of allergic diseases including asthma has increased recently (Anandan *et al*., 2010). The World Health Organization (2011) stated that 235 million people have asthma. In 2014, the worldwide prevalence of asthma was 300 million (Pawankar, 2014). The prevalence of asthma in the United Kingdom is 5.4 million, 8% of the total population (Asthma UK, 2012). In Saudi Arabia, the Saudi Health Interview Survey found that prevalence of asthma among adults is 4% and authors stated that although the prevalence is low, asthma in these patients is not well controlled and patients are not taking the proper medical care (Moradi-Lakeh *et al.,* 2015).

Asthma prevalence is estimated to be 20% in adolescents from 16 to 18 years and from 8% to 25% in children depending on a number of factors such as the environment and weather (coastal, humid, dry), presence of sand storms, dust, allergens and locations such as urban or rural dwelling (Al-Moamaray *et al*., 2012). A study undertaken by Hijazi *et al* (1998) found that the prevalence of asthma in Yunbu (urban) is 14% and in Al-Furash and Al-Gafure villages (rural) is 8%. Animal dander, smoking, dust, pollen, dust mites and emotional stress can be triggers of asthma however, in addition to these triggers, a high rate of asthma in Saudi Arabia can be due to changes in climate (sand storms), poor knowledge about the disease as well as patient fear of using new medications, and an unhealthy lifestyle. Physicians also have lack of awareness regarding the effect of controlling asthma symptoms can also increase the burden of the disease (Al-Moamary *et al.*, 2012).

In recent years there has been an emergence of epidemiological data, supported by animal and in vitro studies, which suggest that low vitamin D status may be an underlying cause of the increase in the prevalence of asthma and its symptoms, both in the UK (SACN, 2007) and in Middle-Eastern countries (Ardawi *et al*., 2012). Vitamin D deficiency is also correlated with higher prevalence of asthma among children in Saudi Arabia (Aldubi *et al*., 2015).

A cross sectional study of 100 children, 50 asthmatics and 50 non-asthmatics, by Alyasin *et al* (2011) found that serum 25(OH)D was significantly lower in the asthmatic children than in non-asthmatic children. They found also that lower 25(OH)D was significantly correlated with lower FEV1 in asthmatic children (Alyasin *et al*., 2011). Similar findings have been found in adults with asthma. According to the Third National Health and Nutrition Examination Survey (a cross-sectional study on 14 091 individuals), there was a significant correlation between serum 25(OH)D and improved lung function including FEV1 and FVC (Black and Scragg, 2005). Another large cross-sectional study in Canada on 3359 adults found that vitamin D less than 50 nmol/l was associated with reduced FEV1 and FVC, especially in obese and overweight participants (Khan *et al*., 2013). In China, in a cross-sectional study on 435 adults with asthma, Li and colleagues found that 89% of the patients had vitamin D deficiency (<50 nmol/l) and they also found that higher serum 25(OH)D concentration had a positive correlation with better FEV1 and FVC (Li *et al*., 2011).

In adults with asthma, vitamin D deficiency was also correlated with poorer asthma control, higher severity and higher level of exhaled nitric oxide (Korn *et al*., 2013) and reduced response to corticosteroid medication (Korn *et al*., 2013; Sutherland *et al*., 2010).

Previous studies have highlighted the inflammation suppression effect of vitamin D. Vitamin D in its active form 1,25(OH)2D3 is known to suppress the synthesis of inflammatory cytokines in the lung. It also regulates the

activity of other immune cells involved in the pathophysiology of asthma, such as mast cells (Chang, Chung and Dong, 2010; Sandhu and Casale, 2010). Histamine can stimulate bronchial hyper-responsiveness (Bradding, 2007) and that can lead to worsening asthma symptoms. Levels of mast cells which are responsible for releasing histamine in airways of patients with asthma are higher than in normal healthy people. Vitamin D may suppress the activity of mast cells (Yip *et al*., 2014).

Vitamin D regulates the levels of inflammatory cells by suppressing their maturation in the bone marrow and increasing the likelihood of apoptosis (Baroni *et al.,* 2007). Vitamin D supplementation has been shown to reduce the maturation and activation of dendritic cells in patients with the autoimmune disease systemic lupus erythematosus (Ben-Zvi *et al*., 2010). It can also regulate the differentiation of the immune cells and cell proliferation by stimulating the innate immunity and cellular differentiation and suppressing the adaptive immunity and cell proliferation (Di Rosa *et al*., 2011). While these studies provide preliminary evidence that vitamin D deficiency may increase the risk of asthma and its severity, thus far there have been no clinical trials to determine the efficacy of vitamin D supplementation in improving outcomes in patients with asthma in Saudi Arabia.

1.6. Measuring vitamin D status

Three methods can be used to estimate vitamin D status in humans. The first method is estimating vitamin D level from sunlight exposure by direct methods such as a special watch worn around the wrist. These ambulatory watches can calculate the absorbed UVB and can be used to estimate vitamin D synthesised via the skin (Hoon Lee *et al.,* 2012). Film badge dosimeters can also be used to calculate the UVB absorbed by the skin (Webb *et al.,* 2010) and a specific formula used to calculate and estimate 25(OH)D (Seckmeyer *et al*., 2013; Rhodes *et al.,* 2010). However, these

methods are not very easy to use and do not give a strong correlation between the sunlight exposure and the level of serum 25(OH)D, this could be due to different age groups, variability in skin pigmentation and differences in high vitamin D food consumption (McCarty,2008). Sunlight exposure can also be estimated indirectly using a sunlight exposure questionnaire (Kimlin *et al.,* 2007; Hoon Lee *et al.,* 2012). Another way to estimate vitamin D status is using dietary intake assessment methods such as food frequency questionnaires and dietary records followed by analysis of the food consumed through the dietary analysis data base (Macdonald *et al.,* 2008). However, according to the National Osteoporosis Society Vitamin D guidelines, the best way to estimate 25(OH)D level in humans is by measuring total 25(OH)D concentration in serum (Aspray *et al*., 2014).

Measuring total serum 25(OH)D is the best biomarker of vitamin D status in the body due to its long half-life, from two to three weeks, and because the concentration of total 25(OH)D is an indicator for both forms of vitamin D both from skin and from foods (Deluca, 2008). The levels also can be replenished by vitamin D stored in the fat stores, although the active form of vitamin D (1,25(OH)2D3) is not the best biomarker to measure to reflect vitamin D status in the body due to its very short half-life (about four-six hours) and the fact it is linked to PTH levels (Pearce and Cheetham, 2010).

A number of methods are available to assess and measure serum 25(OH)D levels. These methods are valid and are prepared to give an accurate quantifying 25(OH)D. Examples of these methods that can be used to assess circulating 25(OH)D are; competitive protein blinding assay (CPBA), radio immunoassay (RIA), automated direct chemiluminescent assay (CLIA), high performance liquid chromatography (HPLC), enzyme-linked immunosorbent assay (ELISA), and liquid chromatography-tandem mass spectrometry (LC-MS/MS) method (Hollis, 2008; Gerbe and Singh, 2011).

The chemiluminscent assay is widely used in many countries and it is suitable to be used in large laboratory and clinical laboratories that need

high throughput; however, this method is not able to detect vitamin D2, it can only measure 25(OH)D3, this will not affect countries that use only vitamin D3 form such as the UK, however, this method cannot be used in countries that use vitamin D2 supplementation such as the United States because it will underestimate the 25(OH)D levels (Hollis, 2008; Wallace *et al.,* 2010).

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been considered as a gold standard to measure 25(OH)D concentration (Lai *et al*., 2011). This method offers specific analysis for low molecules weight components when compared to the immunoassays or HPLC. LC-MS/MS method developed and spread through the clinical laboratories and it is able to do hundreds of variable tests. It is easier to be used and have higher throughput when compared to HPLC method. The test reagents are variable in costs but in general its cost is lower in total when compared to other techniques for large laboratories (Grebe and Singh, 2011).

There is large variability in results measured and reported when chemiluminscent assay and LC-MS/MS method were used to measure 25(OH)D concentration in the same samples (Grebe and Singh, 2011). Thus, standardization of methods is urgently needed. The National Institute of Standards and Technology developed a quality control solution to be used in LC-MS/MS method containing D2 and D3 in known different concentrations by using human serum (SRM 972). LC-MS/MS then have the accurate results and the required sensitivity and specificity. However, the method still has some limitations such as it requires high manual workflow, the operation and maintenance are complex, the cost is high initially to set the method in the laboratory, and the throughput is still limited when compared to immunoassays (Grebe and Singh, 2011). In addition, the UK Standard Agency recommend using LC-MS/MS method and using the control standard solution (SRM 972) that was developed by the National Institute of Standard and Technology in the US, to measure vitamin D levels in the National Diet and Nutrition Survey of the UK (Lai *et al*., 2011).

1.6.1 Vitamin D deficiency and vitamin D status

Globally, vitamin D deficiency is widely spread. Holick and Chen (2008) estimated that over one billion individuals have vitamin D deficiency worldwide. Generally, it had been estimated that 2% to 30% of adults in Europe are vitamin D deficient (Lips, 2007). In the United Kingdom, 24% of adult males and 28% of adult females have serum 25(OH)D of less than 25 nmol/l (Ruston *et al*., 2002). These percentages are higher in older people, 36% of males and 38% of females who are 65 years old and above have vitamin D lower than 25nmol/l (Finch *et al*., 1998).

Saudi Arabia is one of the countries that has the lowest level of vitamin D in the world (Hussain *et al.,* 2014). Vitamin D deficiency is a major problem in Saudi Arabia although it is a sunny country, it is estimated that 85% of the population are vitamin D deficient (Mansour and Alhadidi, 2012). Alarge study done by Ardawi and colleagues (2012) on 834 adult males estimated that 88% had vitamin D deficiency and 49% had severe deficiency with serum 25(OH)D less than 25 nmol/l. Eighty percent of Saudi adult females had vitamin D deficiency and 56% had severe deficiency with serum 25(OH)D less than 25 nmol/l according to a study done on 1172 females (Ardawi *et al*., 2011; Soliman *et al*., 2014). In a recent large retrospective observational study done in Saudi Arabia among 10,709 participants, they found that mean vitamin D level was 44.6±34.8 nmol/l, over all 83.6% of the subjects had serum 25(OH)D<75 nmol/l, 31.9% of them had severe vitamin D deficiency <25 nmol/l, 32% had levels at <50 nmol/l and 19.7% had levels at <75 nmol/l. They also found that vitamin D deficiency was higher among females than males (Hussain *et al.,* 2014). They explained the lower levels of vitamin D among females by low sunlight exposure, covered traditional clothing style, low dietary vitamin D due to few natural sources of vitamin D and few fortified foods being available in Saudi Arabia.

Additionally females could be pregnant or lactating and also females are more likely to be overweight or obese, which can lead to decreased vitamin D levels (Hussain *et al.,* 2014). Vitamin D may store in the body fat in obese individuals decreasing the vitamin D bioavailability in the body (Lagunova *et al*., 2009). In a cross-sectional study of 331 Saudi children and adolescents from 6-17 years old who were recruited into the study during the summer, authors found that all participants (100%) had vitamin D deficiency, with serum 25(OH)D of <30 nmol/l (Al-Othman *et al*., 2012).

Vitamin D deficiency in children can lead to low calcium levels in the blood, causing seizures or tetany (Pearce and Cheetman, 2010). Vitamin D deficiency also can cause rickets, bow legs, soft and deformable skulls and defective growth especially height. More frequent infections and respiratory symptoms can also be associated with vitamin D deficiency in children (Pearce and Cheetman, 2010). Among adults, painful and weak muscles, fibromyalgia, low density of bones and osteopenia can be caused by vitamin D deficiency (Pearce and Cheetman, 2010). Vitamin D deficiency is also correlated with the incidence and severity of a number of diseases. Vitamin D deficiency seen as low concentration of 25(OH)D can lead to decreased bone mass, causing rickets in children and osteoporosis in adults. Low concentration of 25(OH)D is found more often in postmenopausal women which is linked to a higher risk of developing osteoporosis (Wozniak-Holecka and Sobczyk, 2014; Matyjaszek-Matuszek *et al*., 2015). In a recent study by Kanel *et al* (2015), they found that vitamin D deficiency of 25(OH)D less than 50 nmol/l was associated with depression and low mood in adult patients. According to many studies and clinical trials, vitamin D deficiency is correlated to an increase in the risk of cardiovascular disorders, both types of diabetes, obesity, cancer and autoimmune diseases (Matyjaszek-Matuszek *et al*, 2015). More details about vitamin D and diseases are discussed in section 1.3.

A number of studies found that vitamin D deficiency is also correlated with asthma and exacerbated asthma symptoms in both adults and children. This relationship can be explained by the role of vitamin D in growth and maturation of foetal lungs, the effect of decreasing wheezing in children, the immune modulatory effects of vitamin D and a reduction in steroid resistance when used to treat asthma (Litonjua, 2009; Litonjua and Weiss, 2007; Raissy and Black, 2015).

1.6.2. Factors affecting vitamin D status

Many factors can affect vitamin D status in humans such as: season and latitude, sunlight exposure pattern and physical activity, skin colour, ethnicity, clothing style, dietary intake and body weight. Latitude, sunlight exposure and dietary intake were discussed previously in section 1.5.1 and section 1.5.2.

In general, Asian people have lower 25(OH)D levels than Caucasian people. In a study in the UK done by Smith (2010) among 200 healthy individuals Caucasian and Asian they found that during summer and winter, Asian males and females had significantly lower 25(OH)D levels than Caucasians. During winter 64% of Asian have 25(OH)D levels <10 ng/ml and 53% in summer while for Caucasians the percentage are 7.7% in winter and 3.5% in summer, Smith (2010) concluded that these different levels could be due to skin pigmentation and darker skin colour, lower dietary vitamin D intake and a covered clothing style. Glass *et al* (2009) stated that Asian and African American individuals have lower 25(OH)D levels and this difference between ethnicities could be related to genetic variation in vitamin D metabolism, different vitamin D dietary intake and dietary habits, darker skin colour and cultural differences (Glass *et al.,* 2009; Mansour and Alhadidi, 2012).

Previous studies have investigated the relationship between 25(OH)D status and skin colour in different ethnic groups. Generally, individuals with dark skin had lower serum 25(OH)D than individuals with fair skin and need more time exposed to sunlight to synthesis the same amount of 25(OH)D. Melanin pigmentation can reduce and limit 25(OH)D synthesis as it limits the penetration of UVR. However, a large study done in the UK investigated the relationship between 25(OH)D level and skin type using the Fitzpatrick skin type scale in the Caucasian population and found that females with fairer skin (type 1 and type 2) had lower vitamin D than females with darker skin. This finding could be due to avoidance of sunlight as a result of the primary care recommendations for individuals with light skin due to the risk of skin cancer (Glass *et al*., 2009).

Despite Saudi Arabia being a sunny country, vitamin D status is markedly low in the Saudi population in all age groups. A review by Christie and Mason (2011) reported the most important factors that may lead to low 25(OH)D in the Saudi population, especially in women. These factors are poor knowledge about vitamin D, low sunlight exposure because of the extreme hot weather, covering style clothing and low dietary vitamin D intake (Christie and Mason, 2011). Another study on adults in Saudi Arabia reported that a vitamin D intervention, including advice to eat more food sources of vitamin D and to increase exposure to sunlight before 10 am or after 3 pm (the time between 10 am to 3 pm is reported to be carcinogenic in Saudi Arabia) twice a week for 5 to 30 minutes led to a significant increase in serum 25(OH)D (Al-Daghri *et al*., 2012). However, it is estimated that exposing face and arms to sunlight between 10 am and 3 pm in the UK in summer, spring and autumn for 5 to 15 minutes can lead to production of an additional 1000 IU of vitamin D through skin (Glass *et al*., 2009).

Being overweight or obese is correlated with lower serum 25(OH)D. In a large study included 2126 adult participants in Norway, the authors found that half of males and one third of females who had BMI of 40 or more were vitamin D deficient (Lagunova *et al*., 2009). It is also reported that UV

radiation and oral supplementation interventions have less effect on obese individuals when compared to normal weight individuals (Wortsman *et al*., 2000). In a recent systematic review and meta-analysis including 15 trials, authors found that serum vitamin D was increased with weight loss (Mallard *et al.,* 2016).

Possible explanations of the negative correlation between obesity and vitamin D levels could be because vitamin D can be stored in body fat and that leads to reduced vitamin D bioavailability in the body. Secondary hyperparathyroidism is also common in obese individuals and this can lead to lower vitamin D in addition to the slower release of vitamin D from fat to blood flow (Lagunova *et al*., 2009). Limited outdoor activity and lower mobility of obese people can also decrease vitamin D levels. Obese individuals tend to wear more clothes and cover a larger part of their body in the summer this is also can lead to a decrease in sunlight exposure (Lagunova *et al*., 2009; Wortsman *et al*., 2000).

From a large cohort study in 2007 undertaken by Forman, which included 613 males and 1198 females, they found that higher BMI and lower physical activity were correlated with lower 25(OH)D levels (Forman *et al.,* 2007). In a large cross-sectional study in Kuwait, it was found that among 960 adults, 83% of them were vitamin D deficient or insufficient with 25(OH)D<20 ng/ml. They also found that people with low 25(OH)D had significantly higher BMI than people with adequate 25(OH)D level. However, their physical activity did not differ significantly (Zhang *et al.,* 2016).

In a large study in the US, Wanner *et al* (2015) collected the data from NHANES 2003-2006 included 6370 adults. They aimed to investigate if there was a correlation between physical activity assessed by accelerometers and by self-reported physical activity and vitamin D levels. They found that a 10 minute increase in physical activity measured by the accelerometer was associated with increased 25(OH)D by 0.32ng/ml, and

by 0.18 ng/ml for self-reported moderate or vigorous physical activity (Wanner *et al*., 2015). Another study from Saudi Arabia among 331 children and adolescents aimed to investigate the correlation between physical activity and 25(OH)D levels. Physical activity and sunlight exposure were measured by a questionnaire which found that children and adolescents who were moderately active or very active had higher serum 25(OH)D when compared to inactive participants (17.7±1.6 nmol/l vs 22.7±1.5 nmol/l). They also found that BMI was higher in children who were inactive. The authors concluded that outdoor exercise or physical activity during the daytime can increase sunlight exposure, which may result in increasing vitamin D synthesis and physical activity also can decrease calcium excretion and increase its absorption. Finally, exercise and physical activity can lead to weight loss and reduce adiposity resulting in increased vitamin D levels (Al-Othman *et al.,* 2012).

1.7. Rationale and aims of study

This project is important because it is one of the few studies investigating the outcomes and characteristics of adult asthma patients in Saudi Arabia. A number of studies have previously been undertaken but these have been carried out in Saudi asthmatic children. Therefore, this current study will provide baseline characteristics of adult asthma outcomes in Saudi Arabia. To the best of our knowledge, this is the first study to investigate the prevalence of vitamin D deficiency among adult asthma patients in Saudi Arabia. In addition, it is also the first intervention study using two doses of oral vitamin D3 supplement in Saudi asthma patients.

The second study is also an intervention study to investigate the effect vitamin D intake via diet on serum 25(OH)D levels and on lung function after the winter months in healthy adults living in the UK. This is of importance as there is currently no reference nutrient intake (RNI) for vitamin D for healthy adults aged 18 to 65 years in the UK as it is assumed that this age group is able to have normal vitamin D levels due to a

balanced diet and proper sun exposure. However, people in the UK do not have sufficient sunlight exposure during the winter months and during cloudy weather which can be up to half of the year. The study will investigate the effect of consuming 15µg per day of dietary vitamin D for three weeks on the total 25(OH)D, lung function and airway inflammation, adding the novelty of studying the effect of dietary intervention on vitamin D levels and the feasibility of consuming this amount via diet only. This study is also novel in investigating the role of dietary vitamin D intervention and its correlation with lung function in healthy individuals.

1.7.1 Main objective

The main objective of this project is to investigate the effect of vitamin intervention by oral supplementation or by a diet rich in vitamin D on lung function and airway inflammation among asthma patients and healthy individuals.

1.7.2 Specific objectives

- The specific objectives for the study were; to investigate the prevalence of vitamin D deficiency in adult asthma patients in Saudi Arabia and to investigate if there any correlation between vitamin D levels and asthma outcomes in adults with asthma in Saudi Arabia.
- To compare the effect of oral vitamin D supplements on 25(OH)D using different doses.
- To investigate the prevalence of vitamin D deficiency in healthy adult living in the UK after the winter months.
- To investigate the effect of a daily intake of 15 µg dietary vitamin D for three weeks on 25(OH)D levels after the winter months.
- To investigate if there any difference between chemiluminescence immunoassay, the method widely used to measure vitamin D levels, and the LC-MS/MS method, the gold standard method to measure vitamin D status.

Chapter 2: Methods

2.1. Introduction

A cross-sectional study, two randomized interventional studies, followed by a vitamin D measurement study were conducted to answer the following research question "To what extent can vitamin D affect lung function and airway inflammation?".

In this chapter, the study design, participants, instruments used in measurements, variables and outcomes assessment methods for each study will be discussed. Figure 2.1 shows the studies in the order that they were conducted.

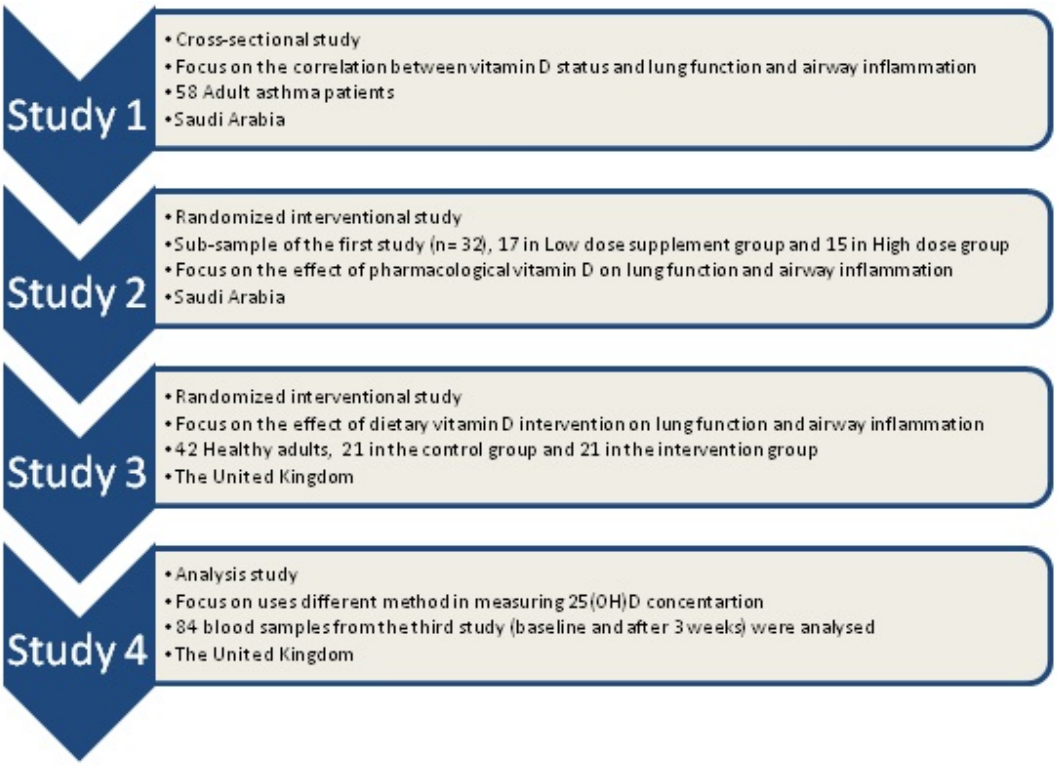

Figure 2.1. Flow chart shows the four studies conducted.

2.2. STUDY 1: The correlation between vitamin D level and asthma outcomes in Saudi Arabia

2.2.1. Study design and participants

A cross-sectional study was conducted between December 2013 and June 2014, in Saudi Arabia (KSA). Male and female adult asthma patients aged between 18–60 years were recruited from the out-patient allergy and asthma clinic in King Abdul Aziz University Hospital (KAUH). The eligibility criteria were: physician diagnosed with asthma, normal calcium level, low or normal serum 25(OH)D level. Any patient with chronic illnesses such as cancer, tuberculosis, cardiac problems, hepatic or renal diseases were excluded. For this study, 58-66 participants were aimed to be enrolled based on study sample calculation and study power of 80%. However, 58 participants took part in the study.

The study methods were approved by the University Research Ethics Committee (UREC) of Oxford Brookes University (OBU) in the UK and also by the ethics committee of King Abdul Aziz University in KSA (Appendix 1,2). Each participant signed a consent form before taking part in the study (Appendix 3).

2.2.2. Study protocol

Participants were invited to take part in the KAUH study after checking the patient list of the allergy and asthma clinics and sending the information sheet to eligible participants via email (Appendix 4), before their routine clinical appointment, to allow at least 24 hours to decide if they wanted to take part. The investigator informed them that the clinical services would not be affected if they decided not to participate. Once informed consent

had been obtained, initial baseline data were collected including: age, gender, ethnicity, smoking habits, medical history, and use of medications or any dietary supplements (Appendix 5).

Following this, four short questionnaires were completed by the participants with the help of the investigator at their visit time in the clinic. These questionnaires were:

- An asthma control test ACT (Appendix 6)
- A self-designed sun exposure questionnaire SEQ (Appendix 7)
- The international physical activity questionnaire IPAQ (Appendix 8)
- A self-designed food frequency questionnaire FFQ to estimate vitamin D dietary intake (Appendix 9)

A non-fasting venous blood sample was collected by a nurse or other qualified clinician at the hospital, and used to screen vitamin D level by measurement of serum 25(OH)D as well as serum calcium. Those who did not meet the inclusion criteria were excluded from participating in the study and reverted to routine clinical care.

Inclusion criteria

- Adults with asthma with physician diagnoses, according to global initiative asthma GINA guidelines (FitzGerald *et al.,* 2012)
- Vitamin D as 25(OH)D < 75nmol/l
- Serum calcium within the normal range (2.2-2.6 mmol/l)
- No severe asthma exacerbation in last 4 weeks

Exclusion criteria

- Any serious medical illnesses such as cancer, renal or hepatic or gastrointestinal diseases, tuberculosis and chronic obstructive pulmonary disease.
- Currently taking vitamin D supplement
- Abnormal serum calcium

- Taking medications that can interact with vitamin D supplement. These medications are; Atorvastatin (Lipitor), Calcipotrine (Dovonex), Calcium channel blokers (Niedipine, Procardia) (Verapamil, Calan) (Nicardipine, Cardene) (Diltiazem, Cardizem, Dilacor) (Amlodipine, Norvasc), Digoxin (Lanoxin), Anti acids, Anti seizures, Bile acid sequestrants, Rifampin, Orlistat (Xenical), Omalizumab, Mepolizumab.

Anthropometric measurements (see section 2.2.5) were then taken by the investigator. After that, lung function (see section 2.2.4) was assessed by portable spirometer (Appendix 10) including forced vital capacity (FVC) and forced expiratory volume in one second (FEV1). Fractional exhaled nitric oxide (FeNO) was also measured using a portable device.

2.2.3. Blood tests

Venous blood samples were taken by venepuncture by a trained person. Six ml of whole blood sample were collected in a serum tube (BD vacationer® serum tubes, UK) at the clinic in KAUH to measure 25(OH)D and calcium levels. Samples were then centrifuged at 4000 rpm for five minutes and collected in 1.5 ml aliquots. Aliquots were then kept in a freezer at –40°C until the time of analysis. Measurements of serum 25(OH)D, calcium, eosinophil and total IgE were conducted in the central laboratory in KAUH. Eosinophil cationic protein was measured in the King Fahad Research Centre.

Serum 25(OH)D concentrations were measured using electrochemiluminescence immunoassay by (Cobas® e 601 analyser, Roche Diagnostic, USA). The detection limits were 7.5-175 nmol/l and with CV% for inter-assay analysis 18.4% at a serum 25(OH)D level of 39.5 nmol/l and 11.7% at 12-125 nmol/l (Snellman *et al.,* 2010). The levels and status classified as deficiency at<30 nmol/l, insufficiency 30-50nmol/l and

sufficiency at >50nmol/l (The US Institute of Medicine, 2011). Total serum calcium levels were measured using the spectrophotometry method by (Dimension Vista®, Siemens, USA). The detection range was 1.25–3.75 mmol/l. Normal range is 2.2-2.6 mmol/l. Total calcium was measured at the baseline to identify the eligible participants who could participate in the study.

Total immunoglobulin E (IgE) was measured using fluoroenzyme immunoassay (ImmunoCAP 250, Phadia, Sweden). Total IgE is a core measurement in asthma patient to identify allergic patients (Szefler *et al*., 2012). Normal range of IgE is <75kU/L (NHS Foundation Trust), however it can be different according to the laboratory ranges (Phillips, 2009). According to Laurent *et al* (1985) the accepted normal level for total IgE is 150-300 Ku/L. In the present study, this was dependent on the KAUH laboratory, normal range which was <195 kU/L.

Eosinophils were measured as part of the complete blood count test (CBC) using stain with flouresinse by (Sysmex XE-2100-1, Japan). High eosinophils in blood can be an indicator of an airway inflammation (Reddel *et al.,* 2009). The normal level of eosinophils percentage in the blood sample is 1%-6% (Curry, 2015). However, according to KAUH the normal range is less than 3%. Eosinophilic cationic protein (ECP) was measured by enzyme-linked immunosorbent assay ELISA (Cusabio, China). Eosinophil cationic protein is a granular protein released from activated eosinophils during inflammation in asthma patients. ECP has a pro-inflammatory role and increase airway narrowing. It is correlated with severity of asthma, allergy and inflammation (Blay *et al.,* 1998). ECP is recommended to be measured in chronic asthma patients due its sensitivity to airway inflammation and airway obstruction, giving the chance to prevent the exacerbation symptoms (Wever *et al.,* 1994). Serum range of ECP in healthy adults is 3.5 to 19 µg/l (Kroegel *et al*., 1998).

2.2.4. Lung function and fractional exhaled nitric oxide (FeNO)

Spirometer and lung function is an essential measurement at baseline in the intervention studies (Tepper *et al.,* 2012). Spirometer is a standard test and it is used to measure the lung function and airway limitation. Bronchoconstriction, structural pulmonary change and airway inflammation can cause airway limitation and reduce spirometer readings. Spirometer is a simple, safe and repeatable valid test to be used in asthma patients including children above 5 years, adolescents, adults and older adults. The change or improvement of spirometer readings can be detected in weeks or months responding to anti-inflammatory treatment and rapid change can be happening within minutes with bronchodilators (Tepper *et al.,* 2012). The minimal clinically important difference in FEV1 and FVC is 100-200 ml, or about 10% (Reddel *et al*., 2009).

Forced expiratory volume in one second (FEV1) and forced vital capacity (FVC) were assessed using a portable spirometer (Micro I diagnostic spirometer, CareFusion, UK). The values and predicted percentages presented in the screen of the spirometer after each exhalation were recorded. These percentages are dependent on age and height of the participants according to the ATS/ERS 2005 guidelines (The American Thoracic Society/ European Respiratory Society). The researcher first asked the participants some question before use the spirometer (appendix 10). Participants were asked to wear comfortable loose clothing and were seated in a comfortable position with a straight back resting on the chair. Participants first inhaled deeply and then exhaled through the disposable mouthpiece attached to the spirometer. Initially the participant completely exhaled in a relaxed way and the reading was recorded, then second and third readings were taken for faster and stronger exhalation. The differences between each measurement could be not more than 5% (Reddel *et al.,* 2009); if the differences were more than 5%, up to five further manoeuvers can be completed if the participant agreed.

Measuring fractional exhaled nitric oxide is a simple, valid, repeatable and non-invasive way to assess the airway inflammation (Dillon *et al.,* 2014). Three devices for measuring FeNO were assessed by the National Institute for Health and Care Excellence (NICE). They recommended FeNO measurement in asthma patients and agreed that all three devices (NIOX MINO aerocrine, NIOX VERO, NO breath) are diagnostic and monitoring methods that are valid to be used in asthma patients (Dillon *et al.,* 2014). Fractional exhaled nitric oxide was measured in this study using the NIOX MINO®(Aerocrine, Sweden).The time for change in FeNO readings with inhaled corticosteroid is about 3 days to 8 weeks (Raddel *et al.,* 2009).

The participant first exhaled the air then deeply inhaled from the disposable mouthpiece attached to the machine, then exhaled again through the mouthpiece for 10 seconds. Supportive visual symbols were available on the laptop in front of participant. Then the result was shown immediately on the screen of the set. The results 20-25 part per billion (ppb) was considered as normal, the level 25-50 ppb indicated an airway inflammation and >50 ppb was significantly high and indicated airway inflammation according to American Thoracic Society (ATS) guidelines (Dweik *et al.,* 2011; Berry *et al.,* 2005).

2.2.5. Anthropometric measurements

Body weight and height were measured using digital scale (Detecto scale, Weight-Height 750, USA). Height was taken for each participant with his or her heels touching the wall, back against the height scale. Participants were asked to remove their shoes and the height recorded to the nearest 0.5 cm. Weight was taken with minimal clothing and without shoes and recorded to the nearest 0.1 kg. Body mass index (BMI) was calculated as weight in kg divided by height in m^2. BMI status was classified as normal (BMI 18.5-24.9 kg/m^2), overweight (25-29.9 kg/m^2), or obese (≥30 kg/m^2) (WHO, 2006).

2.2.6. Food frequency questionnaire

Many FFQs are valid and available to use to estimate all nutrient intakes or a single nutrient in special groups. They are adapted for different countries, to match the dietary habits of the population being studied (Thompson and Subar, 2013). No valid FFQ is available to assess vitamin D intake in Saudi Arabia. A self-designed new FFQ was therefore developed to estimate dietary vitamin D intake in the Saudi population based on the study by Taylor *et al* (2009). It was designed to estimate vitamin D intake over the last year. The developed FFQ included five food groups and 60 food items. Food groups included: milk and milk products, fish, meats (e.g. camel meat and camel liver) and other foods such as cornflakes, eggs, mushrooms and butter. Camel milk, meat and liver are tradition foods in Saudi Arabia. Each liter of camel milk contains 640 IU of vitamin D (Zhang *et al.*, 2005). Most milk and milk products in Saudi Arabia are fortified with vitamin D (400 IU/l). Specific brand names of milk products were used in the developed FFQ due to differences in vitamin D content. The choice (per Ramadan month) was added to the Arabic version that was used in this study. Although Ramadan accounts for 1/12 of the year, many families only consume some special foods that can affect the vitamin D intake during this period such as yogurt and Laban. The average of daily dietary vitamin D intake was estimated in µg per day using the UK food composition tables to calculate the content of vitamin D in foods (McCance and Widdowsons food composition tables, 2010).

2.2.7. Questionnaires and forms

2.2.7.1. Development of the sun exposure questionnaire (SEQ)

The developed SEQ included eight questions. The first two questions were asking about the period of time the participant was exposed to sunlight and the duration in minutes at week days and weekends. Other questions asked about which part of the body was exposed to sunlight, the type of clothes usually worn in daytime, skin colour type, use of sunscreen and

frequency of getting a tan. This questionnaire was developed to match the present study participants based on the study undertaken by McCarty (2008). The FitzPatrick skin scale (Sachedeva, 2008) was used in the questionnaire allowing the investigator to classify the skin colour to one of the six types from fair skin to darker skin, (Figure 2.2). The Fitzpatrick skin scale is a valid scale used by dermatologist and used within research to help identify the skin colour (Sachedeva, 2008).

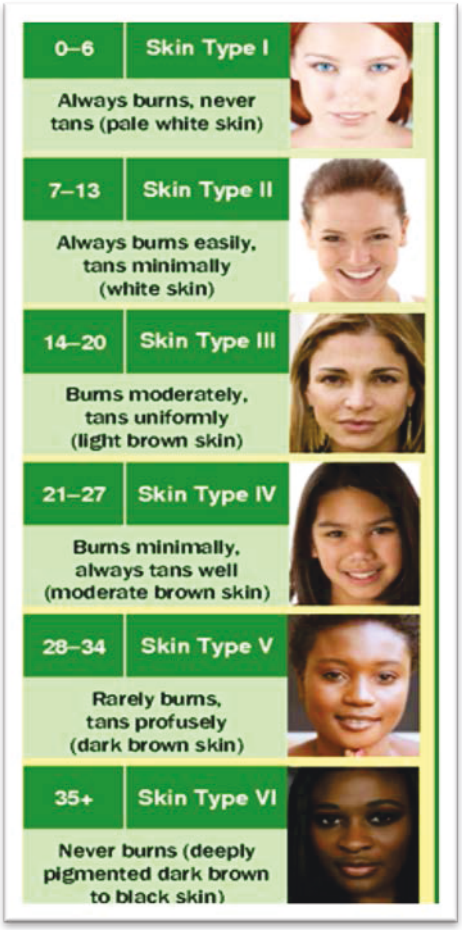

Figure 2.2. FitzPatrick skin colour scale (Sachedeva, 2008).

2.2.7.2. Asthma control test (ACT)

The asthma control test (ACT) is a questionnaire recognized by the National Institute of Health. It is a valid method used to measure the control level of asthma. The asthma control test is simple, short, reliable, valid and free

cost method (Schartz *et al.,* 2006). The questionnaire is previously translated into 34 languages, Arabic is one of them (FitzGerald *et al*., 2012). The Arabic version was used in study 1. The asthma control test consists of five simple questions that the patients can answer by themselves usually taking no more than five minutes. Every question has five choices to answer and each answer have a score. Then by calculating the score patients can be divided into three categories; well controlled asthma if the score ≥20, not well controlled asthma if the score 16-19 and very poor controlled asthma when the score ≤15 (Al-Moamary *et al.,* 2012).

2.2.7.3. International physical activity questionnaire (IPAQ)

Physical activity was measured using the short form of the IPAQ. The questionnaires in Arabic and in English were used in studies 1 and 3. Participants answered questions about the type of activity, intensity and duration of the physical activity during the last week. Participant´s activity was then classified as; low level of activity, moderate level of activity and high level of activity depending on the calculated score of the questionnaire. The IPAQ test is a valid and reliable instrument to assess a person's physical activity levels. This IPAQ test has been translated into many languages and consists of a long and a short version of which the short version was used in the studies (Craig *et al.,* 2003).

2.3. STUDTY 2: The effect of different doses of oral vitamin D supplements on asthma outcomes

2.3.1. Study design and participants

A randomized intervention study was conducted using a sub-sample of participants from study1.For the intervention studies 2 and 3, the sample size estimated to be 30 to 35 in each group to detect the minimal clinical important difference for FEV1 and FVC, which is 10%. Considering the confidence level as 95%, and error of 0.05 and a standard deviation taken from previous studies of 15-17% for the FEV1% and power of study at 80%.

Baseline data, post 3 weeks of the intervention and post 6 weeks of the intervention data were measured to investigate the effect of different doses of oral vitamin D3 supplementation on lung function among adult asthma patients. Eligible patients were randomly allocated to a low dose vitamin D3 supplementation group (LDG) or to a single high dose vitamin D3 supplementation group (HDG).

2.3.2. Intervention and study protocol

Participants in the HDG were provided with oral 200,000 IU of vitamin D3 (cholecalciferol D3 ampoule, Devarol-S by Memphis, Egypt) as a single high dose. Participants in the LDG were provided with oral vitamin D3 drops (cholecalciferol D3 drops Novartis, Switzerland) 800IU per day for three weeks, 16 800 IU in total over three weeks. The 800 IU per day is the lower maintenance dose of vitamin D supplement and the 200 000 IU is a safe load dose, which has been used in previous studies without any adverse effects (Groningen *et al.,* 2010; Aspray *et al*., 2014; Pearce and Cheetham,

2010). The difference between the two doses allowed an excellent separation of vitamin D status between the two groups, enabling the effect of vitamin D status on lung function and airway inflammation to be observed. It was considered unethical to give nothing to any participants as serum 25(OH)D measured and participants with levels <75nmol/l were only included in the study.

Participants who were allocated in the study 2 then attend a second appointment. In the second visit, participants attended the clinic again and start their supplementation. Participants in the HDG provided the supplement at the clinic and participants in the LDG provided with the first dose of supplement and given the instruction to use the supplement every day until the next visit. The third appointment was arranged after 3 weeks of the second visit.

The third visit was three weeks after the start of the intervention and the following were carried-out:

- A blood sample was taken to measure 25(OH)D and calcium (see section 2.2.3)
- Lung function was measured using the spirometer (see section 2.2.4)
- FeNO level was measured (see section 2.2.4)
- Advice on how to take the supplement were given to participants in LDG
- Last visit appointment was arranged for 3 weeks time

In the last visit:

- A blood sample was taken to measure 25(OH)D and calcium (see section 2.2.3)
- Lung function was measured using the spirometer (see section 2.2.4)

- FeNO level was measured (see section 2.2.4)

Three weeks was the duration between the start time of taking the supplement, the middle visit and the last visit, as the half-life of 25(OH)D concentration in the blood is 2-3 weeks and3 weeks can show potential change in 25(OH)D levels in blood (Wootton, 2005; Holick, 2004). A summary of the recruitment and protocol study 2 is shown in Figure 2.3.

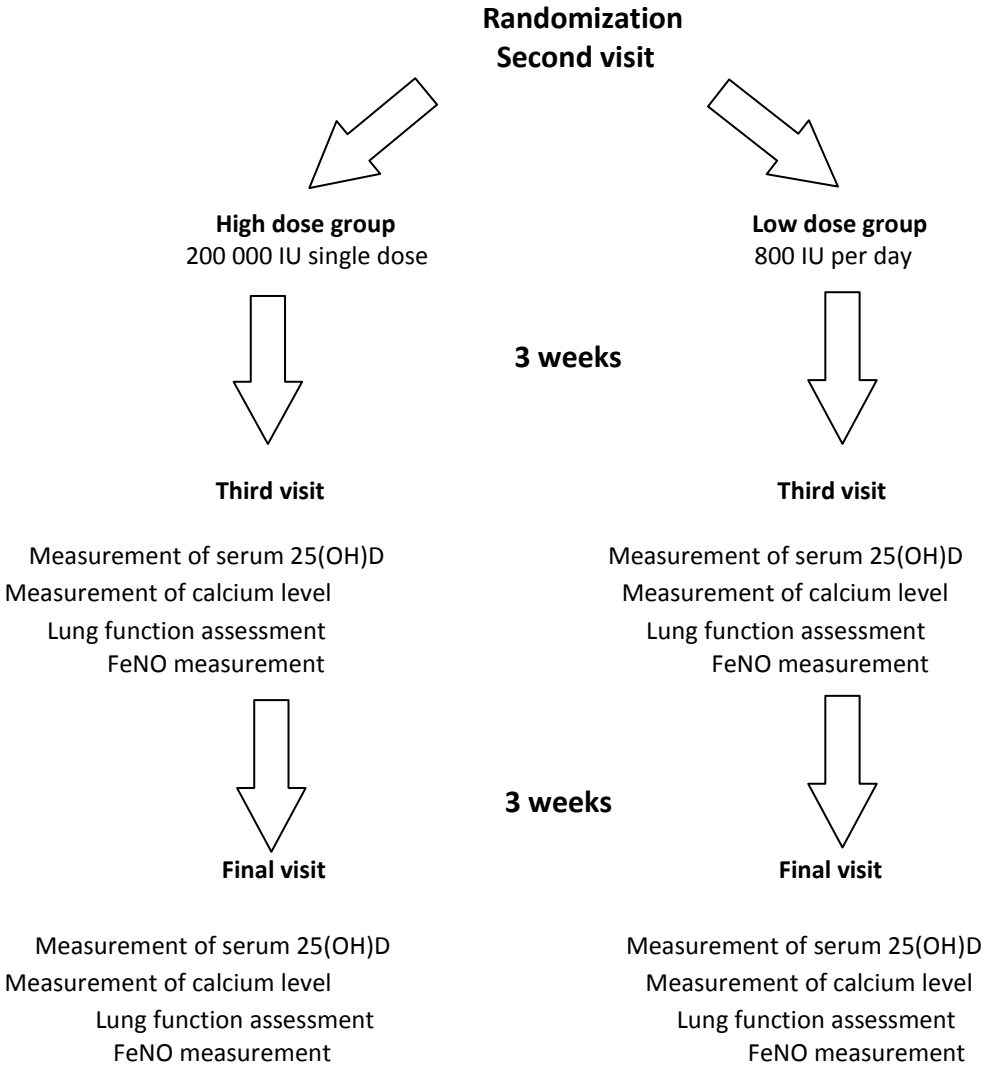

Figure 2.3. Recruitment and study protocol for study 2

2.4. STUDY 3: Dietary intervention and lung function among healthy adults in the UK

2.4.1. Study design and participants

A randomized intervention study was conducted between February 2015 and May 2015 in the United Kingdom (UK). Advertisements containing the contact details of the researchers were attached to the University advertisement boards, in each department. A detailed participant's information sheet (Appendix 11) and consent form (Appendix 12) were sent to any participant responding to the advertisement. Eligible participants were randomly assigned to a control group (CG) or to a dietary intervention group (IG). Baseline data and post intervention data were measured to investigate the effect of a dietary vitamin D intervention on lung function and airway inflammation in healthy adults. This study was approved by the University Research Ethics Committee (UREC) of Oxford Brookes University (OBU) (Appendix 13).

2.4.2. Study protocol

Healthy male and female adults aged between 18–60 years were recruited from OBU. Eligible participants, not taking any vitamin D supplements were recruited via advertisement displayed in the University advertisement boards.

Inclusion criteria

- Healthy adults who are not having any chronic diseases or on any medications
- Body mass index ≤30 kg/m²

Exclusion criteria

- Any serious medical condition such as cancer, renal failure, hepatic failure and cardiac problems
- Taking vitamin D supplement
- Known current or past hypercalcemia
- Food allergies

The study included three visits: the first visit was the screening visit to assess the eligibility of the participant. The participants were given time to read and understand the participant information sheet (Appendix 11) and had the chance to ask any questions. Then each participant signed a consent form (Appendix 12). Baseline data were collected including age, gender, medical conditions, use of any medications or dietary supplements and food allergies (Appendix 14). Anthropometric measurements were taken in the nutrition department laboratory at OBU.

Eligible participants were randomly assigned to either a control group (CG) or to a diet intervention group (IG). Participants who were assigned to the control group were asked to consume their normal diet. Participants assigned to the intervention group were asked to choose their preferred food items from a food list, to be provided in a weekly basis (Appendix 15). The dietary intervention was consumption of 15 µg vitamin D via diet only. There is no dietary recommendation fot vitamin D for adults in the UK, so the US recommended dietary allowance of vitamin D for adults (15 µg) per day was used in the dietary intervention (O´Connor and Benelam, 2011). Measured and selected food items were provided to participants to be consumed within their normal diet for three weeks. All foods provided were containing vitamin D in the form D3 due to its greater effect in raising serum vitamin D. A four-day diet record was also provided for all participants at the end of the first visit to be taken away, filled in and returned for the next visit (Appendix 16) to estimate the dietary intake of vitamin D. A sheet of the available food items containing vitamin D, were shown to the participants to choose their preferred items (Table 2.1). For

each participant, the researcher then drew up a weekly plan to ensure that each day include food choices that totalled 15 µg vitamin D.

At the second visit, after at least four days, a sample of blood was collected to measure baseline vitamin D as 25(OH)D before the dietary intervention commenced.

Table 2.1 Food items that provided to the participants and their content of vitamin D (µg)

Product	Quantity	Vitamin D content
Actimel Danone flavoured 0% fat, 100ml	2 bottles	1.5
Petitsfilous flavoured yogurt, 47 g	4 pots	2.4
Nestle Munch Bunch flavoured yogurt 100g	2 pots	1.5
Tesco dried skimmed milk powder	100g	1.5
Nestle evaporated milk	170g	2.6
Tesco UHT unsweetened soya milk	250ml	2
Flora light low fat spread	30g	2.25
Cheese fingers	2 fingers	2.5
Egg	2 medium eggs	1.8
Vitamin water (flavoured water)	500 ml	1.25
Mango and passion fruit drink (vitamin drink)	Bottle (500ml)	10
Sardines in sunflower oil	Can (84g)	4.2
Tuna canned in oil	Can (120g)	3.6
Kellogg's Corn Flakes	50 g	2.2
Kellogg's Coco Pops	50 g	2.2
Kellogg's Rice Krispie	50 g	2.2
Kellogg's Special K	50 g	4.2
Nesquik Chocolate cereals	50 g	1.5
Cheerios' cereals	50 g	1.5
Alibi juice pomegranate or blue berry or citrus	Can (330ml)	5

Lung function was assessed using a spirometer and FeNO was measured (see section 2.2.4). The participants completed three questionnaires with the investigator´s assistance, SEQ, IPAQ and a self-designed FFQ (Appendix 17). In addition, a four-day diet record was collected and a daily sun exposure form was given to the participants to be completed over the next

three weeks (Appendix 18). At the third visit after three weeks of the second visit, a blood sample was collected to measure 25(OH)D, lung function (FVC and FEV1) was assessed, FeNO was measured and the daily sun exposure form was collected. A summary of the study protocol is shown in Figure 2.4

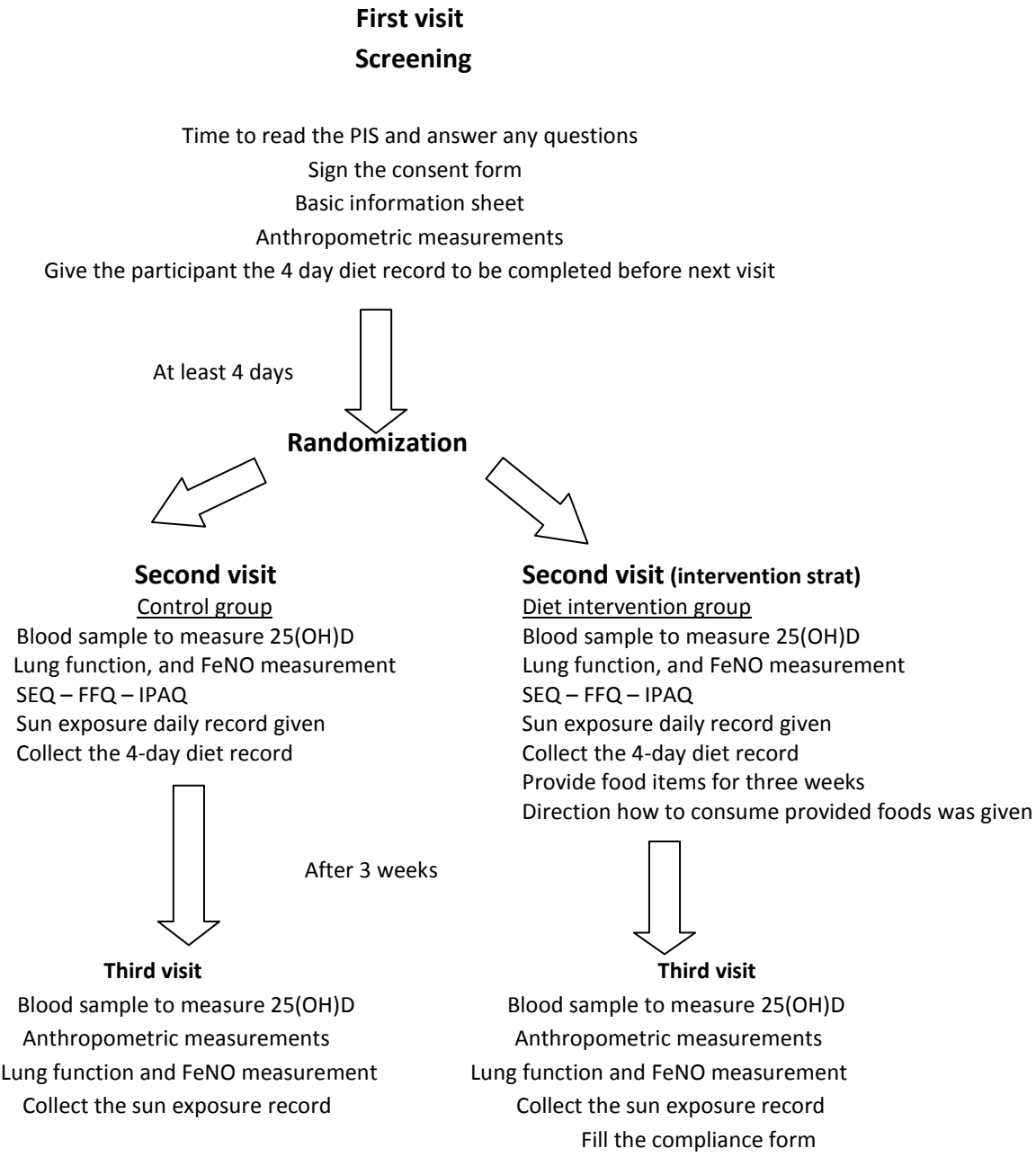

Figure 2.4 Protocol for study 3

2.4.3. Blood tests

Venous blood samples were taken by venepuncture by a trained person. Four ml were collected in a serum tube (BD vacationer® serum tubes, UK) to measure vitamin D concentration. Samples were then centrifuged at 4000 rpm for five minutes and collected in 1.5 ml aliquots.

Aliquots were then kept in a freezer at –40°C until analysis time. Serum 25(OH)D was measured at the Functional Food Centre laboratory at OBU, using the electrochemiluminescence immunoassay (Cobas® e 411 analyser, Roche Diagnostic, USA).The detection limits were 3.00-70.0 ng/ml and CV% of 43.5 (37.7-48.9) with 95% confident levels (Snellman *et al.,* 2010).

2.4.2. Lung function and fractional exhaled nitric oxide (FeNO)

Lung function assessment and FeNO were measured as described in section 2.2.4.

2.4.3. Anthropometric measurements

Body weight, body fat percent and fat-free mass were measured using the (Tanita body composition analyser BC-418 MA, UK). Height was taken using a somatometre (SECA, Germany). Waist and hip circumferences were taken using an inelastic non-stretchable tape. Participants were asked to remove any outer clothing, light clothing remained on and waist circumference was taken at the midpoint between the hip bone and the bottom rib. Hip circumference was taken around the widest circumference of the hip and results recorded to the nearest 0.5 cm.

2.4.4. Dietary assessment

Dietary assessment was estimated using two nutritional tools: a four-day dietary record and a food frequency questionnaire (FFQ). The developed

FFQ used in study 1 (see section 2.2.6) translated to English and few amendments were made in the English form. Some food items were removed such as camel meat and camel milk and few food items were added to the English FFQ such as soya milk, pork and fat spreads. The average of daily dietary vitamin D intake was estimated in µg per day. McCance and Widdowsons UK food composition table (2010), were used to calculate the content of vitamin D in food items.

A four-day diet record was used to investigate food intake and to estimate vitamin D intake of all participants. Instructions to use and fill the record were given. Participants were asked to report all foods and beverages consumed for four days with details such as type of food, cooking method, serving size and any leftover. Serving size was estimated by household measurements such as; cups, table spoon, tea spoon, or by grams if known, then converted to a serving size or grams to estimate vitamin D content in each food item.

2.4.5. Questionnaires and forms

Basic information screening sheets were designed including; age, gender, ethnicity, smoking habits, medical history, medications, food allergies and if the participant was taken any vitamin D supplements (Appendix 14). Sun exposure questionnaire (SEQ) used in study 1 (see section 2.2.7.1) and the international physical activity questionnaire IPAQ (see section 2.2.7.3) used in study 1 were used in this study.

Daily sunlight exposure record was used in study 3 to investigate the trend of sun-light exposure during the study period. Seasonal variation in Saudi Arabia may not play an important role in vitamin D level. The form included; the day and date, time of exposure to the sunlight, duration in minutes, parts of body exposed, use of sunscreen and weather conditions (sunny, cloudy, and partially cloudy) (Appendix 18) participants asked to fill out the questionnaire over three weeks between the second and third visit.

2.5. STUDY 4: Comparison of two methods in measuring serum 25(OH)D

2.5.1. Serum samples

During study 3, an additional serum sample of was obtained during each participant´s visits. All samples were centrifuged and separated in aliquots, then stored at –40°C until analysis. Each aliquot had a number and the same serum sample for each participant was analysed by two methods: electrochemiluminescence assay and liquid chromatography-tandem mass spectrometry (LC-MS/MS).

2.5.2 Electrochemiluminescence assay (CLIA)

Total serum 25(OH)D concentration was assessed by electrochemiluminescence immunoassay using (Cobas® e 411 analyser, Roche Diagnostic, USA) at Oxford Brookes University. Electrochemiluminescence by Roche Diagnostic is a competitive protein binding assay. This assay is able to quantify 25(OH)D in serum or plasma. The method uses vitamin D binding protein (DBP) as a capture to bind 25(OH)D2 and 25(OH)D3. The assay includes three incubations. Firstly, the sample is incubated with the pre-treatment reagent, then bounded 25(OH)D released from the DBP. Secondly, the sample is incubated again with a ruthenium labeled DBP making a complex component consist of 25(OH)D and ruthenylated DBP. Then a third incubation occurs with added micro particles coated with streptavidin and 25(OH)D with biotin. The component then occupies the free sites of the ruthenium DBP creating a complex of rutheniumed DBP and biotinylated vitamin D (Abdel-Wareth *et al.,* 2013). After the incubation steps, a washing step removes the unbound material and the reagent is added again. After that, the flash chemiluminescent reaction starts. A photomultiplier then measures the

light signals giving a proportional concentration of serum 25(OH)D (Snellman *et al*., 2010).

2.5.3. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay

LC-MS/MS method is widely used among laboratories and research centres (Grebe and Singh, 2011). The mass spectrometer is a tool assessing the mass-charge ratio in the charged particles. In clinical practice usually the mass spectrometer is either single or combined with tandem mass filter and can be fronted by LC. The LC-MS/MS consists of; atmospheric pressure ionization source attached to an ion-inlet component, two mass-filtered devices, collision chamber and an ion-impact detector. The sample first becomes ionized in the atmospheric pressure ionization source, then passes to the first mass-filter. After that the sample passes to the collision chamber, and then again through the second mass-filter. Finally to the detector that is quantifying the ions. An auxiliary component is attached to the MS which is the device for data reduction of detector signals attached usually to the computer software that quantify the detection component (Grebe and Singh, 2011).

In this study, a water LC-MS/MS system was used to quantify 25(OH)D concentration in serum samples. The column used was water Acquity UPLC BEH pheny1.17µm,201X50 mm. This analysis was conducted in the clinical biochemistry laboratory (City Hospital), Sandwell and West Birmingham Hospitals. Samples were sent to Birmingham Hospital laboratory to be analysed as LC-MS/MS method was not available at OBU.

2.6. Statistical analysis

Data were analysed using the Statistical Package for the Social Sciences (SPSS software version 21). Descriptive statistics with calculated means (M) and standard deviations (SD) were used to describe the baseline characteristics of the participants and to describe the baseline variables. The Shapro-Wilks test was used to assess the normality of the data.

The independent t-test and Mann-Whitney U-test were used to compare parametric and non-parametric data between groups (control sv interventions). To compare the variables within the group (baseline vs after intervention) the paired sample t-test or Wilcoxon signed rank test were used for parametric and non-parametric data, respectively. The Freidman test was used to investigate differences between data at baseline, after three weeks and after six weeks of the supplement in Study 2. To investigate correlations and associations between variables, Pearson and Spearman rank test were used for parametric and non-parametric data, respectively. Statistical significance was set at $P < 0.05$.

MedCalc software (version 16.4.3, Ostend, Belgium) was used to do Bland Altman test, Passing-Bablok regression test and Kappa analysis to investigate the agreement between the two methods used to measure 25(OH)D in Study 4.

Chapter 3

The correlation between vitamin D levels and asthma outcomes in Saudi Arabia

3.1. Justification and aims

Vitamin D deficiency is a major public health problem in Saudi Arabia despite the almost unlimited availability of sunlight (Alharbi *et al*., 2013). It is estimated that vitamin D deficiency affects approximately 85% of the Saudi population (Mansour and Alhadidi, 2012) and may modulate the risk of type 2 diabetes, cardiovascular disease and allergic disorders such as asthma (Al-Daghri *et al.,* 2012; Maki *et al*., 2011; and Bozzetto *et al*., 2012). The prevalence of asthma has risen globally over recent decades (Pawanker, 2014) and in Saudi Arabia in particular (Al-Moamary *et al*., 2012).

Vitamin D is an important element correlated with asthma and previous research has found that vitamin D has a positive impact on many functions involved in asthma such as immunity, improving lung function, lung capacity and reduction in airway hyper-responsiveness (Chinellato *et al.,* 2011; Zosky *et al.*, 2011; Black and Scragg, 2005). Most of these previous studies have been undertaken in different populations in different countries. To date, only one recent study (Aldubi *et al.,* 2015) has been carried out in Saudi Arabia investigating the correlation between serum 25(OH)D levels and asthma severity biomarkers but this was in children. This study showed that vitamin D deficiency was more prevalent in asthmatic children than in healthy children, and those

children with vitamin D deficiency had increased asthma symptoms and allergy markers (Aldubi *et al.,* 2015). To the best of our knowledge, the current study is the first study to investigate the prevalence of vitamin D deficiency and the correlation between vitamin D level and asthma outcomes in adult patients in Saudi Arabia.

Aims and objectives

1. To determine serum 25(OH)D levels among adult asthma participants in Saudi Arabia
2. To estimate dietary intake of vitamin D among adult asthma participants in Saudi Arabia
3. To evaluate the sun exposure patterns among adult asthma participants in Saudi Arabia
4. To investigate the correlation between 25(OH)D levels and lung function, airway inflammation, control of symptoms and other inflammatory biomarkers

3.2. Subject characteristics

3.2.1. Demographic information

Sixty-two asthma participants were initially recruited to the study and signed the consent form. Fifty-eight participants (mean age 36±12 years) attend the appointment and took part in the study. Personal information and medical history were taken by a questionnaire, 74% of the participants did not have any medical conditions except asthma. Other medical conditions such as diabetes, hypertension and allergic rhinitis were recorded for some participants. Only five participants were smokers and seven participants were exposed to passive smoking. The usage of inhaled corticosteroids, anti-histamine and rapid acting bronchodilators were recorded. Forty-nine percent of participants were using one medication to treat or control their symptoms, 45% were taking two medications and 6%

were taking three types of medications to control their symptoms. Fifty-two percent of participants were using inhaled or oral corticosteroid and only 7% were using oral corticosteroid only.

For any participant who was recently prescribed medication, two weeks were given before starting the study to decrease any effect of the medication on the study outcomes (only two participants were using medication for the first time and they were given two weeks before they took part in the study). Detailed baseline characteristic of the participants including, gender, smoking status, educational level, medical history and physical activity level are shown in, Table 3.1.

Serum 25(OH)D levels were lower in smokers and passive smokers than non-smokers (28.6±20.5, 22.7±8.2, 34.2±20 nmol/l respectively); however, these were not significant (*P* 0.284). In addition, no significant differences were seen in serum 25(OH)D according to different educational levels (*P* 0.695) or different activity levels (*P* 0.158). Moreover, serum 25(OH)D was similar in participants who took one type of medication to control their symptoms or two types of medication or three types of medication (*P* 0.687).

Table 3.1. Baseline characteristics of the participants

Variables	Number of participants	Percentage %
Gender		
Males	14	24%
Females	44	76%
Smoking		
Smoker	5	9%
None smoker	46	79%
Passive smoker*	7	12%
Education		
Secondary education	1	2%
High school	7	12%
University level	42	72%
Postgraduate	8	14%
Medical history		
Medically free except asthma	43	74%
Diabetes	2	3%
Hypertension	3	5%
Diabetes and hypertension	2	3%
Allergic rhinitis	6	10%
Physical activity*		
Inactive	32	65%
Minimally active	8	16%
Vigorous-intensity active	9	18%

*****Passive smoker defined as exposure to the exhaled smoke and side stream smoke (WHO, 2003)
*****Physical activity classified as the International Physical Activity Questionnaire scores (Craig *et al.,* 2003).

3.2.2. Body weight status

The mean body weight of the participants was 74.8±18.7 kg and the mean body weight for males and females were 80.4±17.8 kg and 73±18.8 kg respectively. The classification of body weight according to the BMI is underweight BMI <18.5 kg/cm², normal body weight BMI is between 18.5 and 24.9 kg/cm², overweight BMI is between 25 and 29.9 kg/cm² and obese BMI ≥30 kg/cm² (WHO, 2006). Sixty-four percent of the participants

were found to be overweight or obese. No significant differences were found in serum 25(OH)D in obese, overweight and normal weight participants (*P* 0.717). Table 3.2 shows the percentages of body weight classifications according to the BMI among males and females participants.

Table 3.2.Body weight classification according to the BMI among males and females participants

Body weight status	Number of participants	Percentage %
Total subject n=56*		
Underweight BMI <18.5	1	2%
Normal BMI 18.5-24.9	19	34%
Overweight BMI 25-29.9	11	20%
Obese BMI ≥30	25	44%
Males n=13		
Underweight BMI <18.5	0	0
Normal BMI 18.5-24.9	4	31%
Overweight BMI 25-29.9	3	23%
Obese BMI ≥30	6	46%
Females n=43		
Underweight BMI <18.5	1	2%
Normal BMI 18.5-24.9	15	35%
Overweight BMI 25-29.9	8	19%
Obese BMI ≥30	19	44%

*Two participants had missing data for weight and height

3.2.3. Sunlight exposure patterns of asthmatic participants

Participants´ most common skin colour was classified as type 3 or type 4 according to the FitzPatrick skin colour scale (Sachdeva, 2008). Serum 25(OH)D was higher in fairer skin type such as type 2 and type 3 compared to darker skin colour such as 4, 5 and 6; however, these differences were not significant (*P* 0.178). Spearman correlation showed a weak negative correlation between 25(OH)D and skin type colour (*r* -0.275, *P* 0.044; Table 3.3).

Table 3.3. Serum 25(OH)D according to participants´ skin type (Means±SD)

Skin colour type (FitzPatrick)	Serum 25(OH)D (Mean±SD)	Number of participants
Type 2	34±16 nmol/l	7
Type 3	39.4±26 nmol/l	11
Type 4	31±18.4 nmol/l	31
Type 5	18.3±2.3 nmol/l	4
Type 6	14 nmol/l	1

Eighty-two percent of the participants were exposed to the sunlight less than 10 minutes per day and 86% of females exposed a very small part of their body to the sunlight, face or face and hands only and 27% of them were using sunscreen most days or every day. All female participants were wearing a hijab or a niqab (hijab is a religious head cover used by Muslim females and niqab is covering the face) and 4% of males were wearing a ghotra (ghotra is a traditional head cover used by males in Saudi Arabia) when they were outside; Table 3.4 shows the sunlight pattern using the self-developed SEQ (see section 2.2.7.1).

Table 3.4. Sunlight exposure pattern of asthmatic patients

Sunlight exposure patterns	Total subject n=54*	Males n=13	Females n=41
Time exposing to the sunlight per day (by minutes)			
Less than 10 minutes	44 (82%)	8 (62%)	36 (88%)
11-20 minutes	6 (11%)	2 (15%)	4 (10%)
21-30 minutes	1 (2%)	0 (0%)	1 (2%)
30-45 minutes	0 (0%)	0 (0%)	0 (0%)
46-60 minutes	1 (2%)	1 (8%)	0 (0%)
60-120 minutes	2 (4%)	2 (15%)	0 (0%)
Parts of body exposed to the sunlight usually			
Face only	1 (2%)	0	1 (2%)
Hands only	20 (37%)	0	20 (49%)
Face and hands	20 (37%)	5 (39%)	15 (37%)
Face, neck and hands	6 (11%)	3 (23%)	3 (7%)
Face, neck and arms	6 (11%)	4 (31%)	2 (5%)
Face, neck, arms and mid legs	1 (2%)	1 (8%)	0 (0%)
How often do you apply sunscreen?			
Never	37 (69%)	11 (85%)	26 (63%)
Occasionally	6 (11%)	2 (15%)	4 (10%)
Most days	3 (6%)	0 (0%)	3 (7%)
Every day	8 (15%)	0 (0%)	8 (20%)
Spend time in the sunlight for getting tan			
Yes, once a year	2 (4%)	0 (0%)	2 (5%)
Yes, sometimes	2 (4%)	0 (0%)	2 (5%)
No	50 (93%)	13 (100%)	37 (90%)

*Four participants had missing data for SEQ

3.3. Vitamin D and calcium levels among asthmatic participants

Mean total serum 25(OH)D was 32.3±19 nmol/l for all participants (n=58). Vitamin D deficiency and insufficiency (<50nmol/l) was found in 86% of participants. Vitamin D levels were slightly higher among females 35±20.7 nmol/l compared to males 24.2±9.8 nmol/l (*P* 0.081). All males and 82% of females had vitamin D deficiency or insufficiency (Table 3.5).

Table 3.5. Vitamin D status according to serum 25(OH)D among participants

Serum vitamin D status nmol/l	**All patients** n=58 (100%)	**Males** n=14 (24%)	**Females** n=44 (76%)
Deficiency , 25(OH)D < 30	32 (55%)	11 (79%)	21 (48%)
Insufficiency , 25(OH)D between 30-50	18 (31%)	3 (21%)	15 (34%)
Sufficiency , 25(OH)D >50	8 (14%)	0 (0%)	8 (18%)

Mean calcium level was 2.24±1.3 mmol/l. No abnormal calcium level was found in any participant. Similar results of calcium levels were found among males and females, 2.23±0.14 and 2.24±0.13 mmol/l respectively (*P* 0.381).

Mean estimated dietary vitamin D intake measured by self-developed FFQ (see section 2.2.6) was 3.7±2.5 µg per day. Females had slightly higher dietary vitamin D intake than males at 4.0±2.7 µg/d vs 2.7±1.5 µg/d (*P* 0.201). No correlation was found between dietary vitamin D intake and serum 25(OH)D (*P* 0.548). Participants with vitamin D deficiency had slightly lower dietary vitamin D intake compared to participants who were vitamin D insufficient or sufficient; however, no significant difference was found (*P* 0.691; Table 3.6).

Table 3.6. Dietary vitamin D intake in participants with different serum vitamin D status (Mean±SD)

Participants	Vitamin D Deficiency	Vitamin D Insufficiency	Vitamin D Sufficiency
All participants (n=46)	n=27	n=13	n=6
Dietary vitamin D intake (µg/D)	3.2±2.0 µg/D	4.7±3.5 µg/D	3.9±2.3 µg/D
(minimum – maximum) µg/D	(0.34 - 9.1) µg/D	(1.5 - 11.3) µg/D	(1.1 - 7.1) µg/D
Males (n=11)	n=9	n=2	n=0
Dietary vitamin D intake (µg/D)	2.6±1.7	2.8±1	----
(minimum – maximum) µg/D	(0.4 – 5.1)	(2.1 – 3.5)	----
Females (n=35)	n=18	n=11	n=6
Dietary vitamin D intake (µg/D)	3.5±2.2	5±3.6	3.9±2.3 µg/D
(minimum – maximum) µg/D	(0.35 – 9.1)	(1.5 – 11.3)	(1.1 - 7.1) µg/D

3.4. Asthma outcomes

A lung function test was conducted using the portable spirometer and fractional exhaled nitric oxide was measured using the NIOX MINO device, to determine if any participants had airway inflammation (see section 2.2.4). Other inflammatory biomarkers such as eosinophil%, eosinophilic cationic protein and total IgE were also measured for some participants (see section 2.2.3).

3.4.1. Lung function

Mean percentage of predicted forced expiratory volume in one second (FEV1%) for all participants was 79±17% and mean percent of predicted forced vital capacity (FVC%) was 81±16%. Females had significantly better lung function than males (Table 3.7). Although lung function followed the same trend as serum 25(OH)D levels, there were no significant differences in means of FEV1 and FVC among different serum 25(OH)D levels groups (Table 3.8).

Table 3.7. Lung function and FeNO among males and females participants (Means±SD)

Variables	**Males,** n=14	**Females,** n=44	***P* value**
FEV1%	71.1±17.2	81.4±17.0	0.038
FVC%	75.1±16.4	83.4±16.2	0.040
FeNO (ppb)	28.5±19.5	34.1±33.8	0.719

Table 3.8. Lung function and FeNO according to serum vitamin D status (Means±SD)

Variables	**Vitamin D deficiency** n= 32	**Vitamin D insufficiency** n= 16	**Vitamin D sufficiency** n= 7	***P* value**
FEV1%	77.3±18.0	78.4±19.3	88.1±11.0	0.287
FVC%	79.4±18.3	82.3±14.0	87.0±14.1	0.391
FeNO (ppb)	37.2±36.2	27.3±23.4	28.1±20.4	0.502

3.4.2. Asthma control scores

According to the asthma control test (ACT), 22 participants (44%) had controlled asthma, twelve participants (24%) had partially controlled asthma and 16 (32%) had very poorly controlled asthma. Controlled asthma participants had slightly better lung function and slightly lower total IgE than participants who were partially controlled or poorly controlled, although there were no significant differences between controlled asthma participants and non-controlled participants, (Table 3.9).

Table 3.9. Vitamin D (mean±SD) and asthma outcomes in participants with different asthma control levels

Variables	**Controlled** n=22	**Partially controlled** n=12	**Poorly controlled** n=16	***P* value**
25(OH)D (nmol/l)	31.1±22.2	28.4±17.4	38.7±18.5	0.177
Dietary vitamin D (IU/d)	153.0±103.3	157.0±94.0	157.3±114.1	0.964
FEV1%	84.0±13.0	81.1±12.4	69.3±24.1	0.126
FVC%	86.1±14.1	82.2±15.0	76.0±21.4	0.198
FeNO (ppb)	35.3±38.4	41.1±30.0	19.4±13.1	0.059
IgE (KU/L)*	269.0±220.4	602.0±978.3	620.0±783.0	0.953

*IgE could not be measured in all participants due to financial constraints, 7 participants in controlled group, 5 in partially controlled and 8 in poorly controlled asthma were measured.

3.4.3. Inflammatory biomarkers

The mean fractional exhaled nitric oxide (FeNO), the airway inflammatory biomarker taken for all participants was 33±31 ppb. Forty-one percent of the participants had FeNO higher than the normal range (normal range <25ppb; Dweik *et al.,* 2011). Other inflammatory biomarkers; total IgE, eosinophil and ECP were not measured for all participants due to financial constraints.

Mean total serum IgE, measured for 25 participants (6 males and 19 females), was 493.4±647.0 KU/L. Fifteen participants (60%) had higher IgE level than the normal range which according to the KAUH laboratory is <195 KU/L (see section 2.2.3). Eosinophils (%) levels were taken for 34 participants (6 males and 28 females). Eosinophil (%) normal range is <3% according to the KAUH laboratory. Eosinophil (%) was higher than 3% in 53% of participants; Mean ECP (eosinophil cationic protein) was 19.5±11.0 ng/ml in 19 participants (6 males and 13 females). Total IgE, eosinophil cationic protein and eosinophil percentage were higher in males compared to females (Table 3.10).

Table 3.10. Inflammatory biomarkers among males and females participants (mean±SD)

Variables	Males	Females	*P* value
IgE (KU/L)	1049.0±753.0	318.0±515.1	0.03*
Eosinophil (%)	6.4±4.2	3.3±2.8	0.008*
ECP (ng/ml)	23.0±8.3	18.0±12.5	0.416

*Significant differences, Mann Whitney U test

3.5. Correlation between vitamin D level and asthma outcomes

Participants with lower serum 25(OH)D also had poorer FEV1% and FVC. Moreover, males who had lower 25(OH)D also had lower FEV1%, FVC% and higher inflammatory biomarkers: IgE, ECP and eosinophil. However, a Spearman rank test showed that there was no correlation between serum 25(OH)D and FEV1% or FVC% (*r* 0.115, *P* 0.399 and *r* 0.2, *P* 0.140, respectively). Serum 25(OH)D was not correlated to the inflammatory biomarkers: IgE (*r* 0.038 *P* 0.858), Eosinophil % (*r* -0.036 *P* 0.833) or ECP (*r* -0.235 *P* 0.332). There was a weak trend showing that participants with higher 25(OH)D had lower FeNO levels (*r* -0.236, *P* 0.08).

Patients who had higher IgE also had poorer lung function (FEV1% *r* -0.423, *P* 0.044) and (FVC% *r* -0.373, *P* 0.079; Figures 3.1 and 3.2). A positive weak correlation was found between eosinophil% and FeNO levels (*r* 0.388, *P* 0.023; Figure 3.3). Better asthma control score measured by ACT was correlated with improved FEV1% and FVC% although this was a weak correlation (*r* 0.293, *P* 0.039 and *r* 0.306, *P* 0.031 respectively).

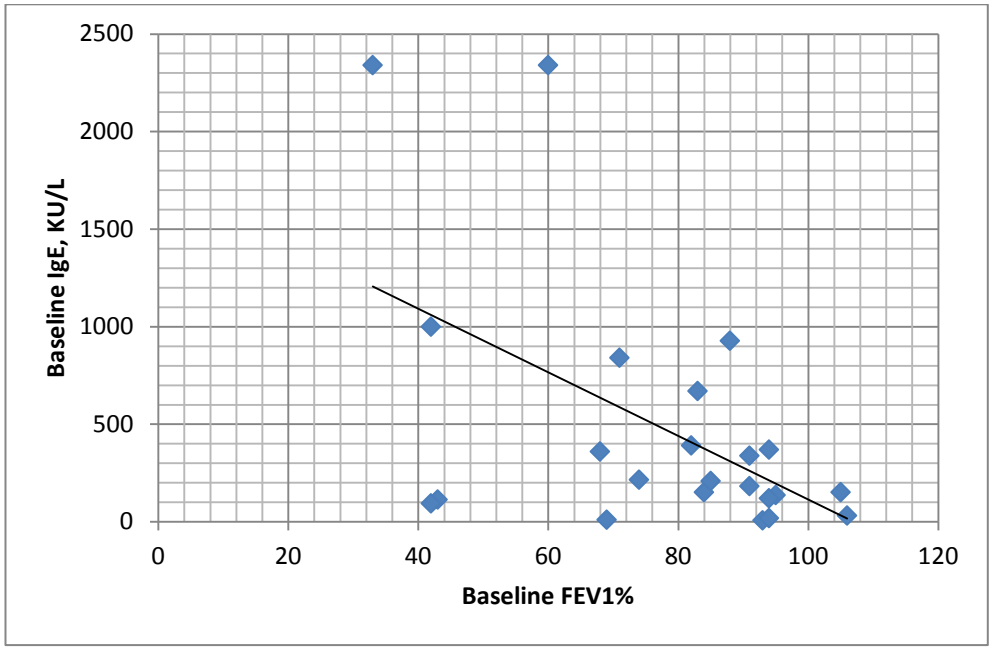

Figure 3.1. The correlation between total IgE and FEV1% (n=25)

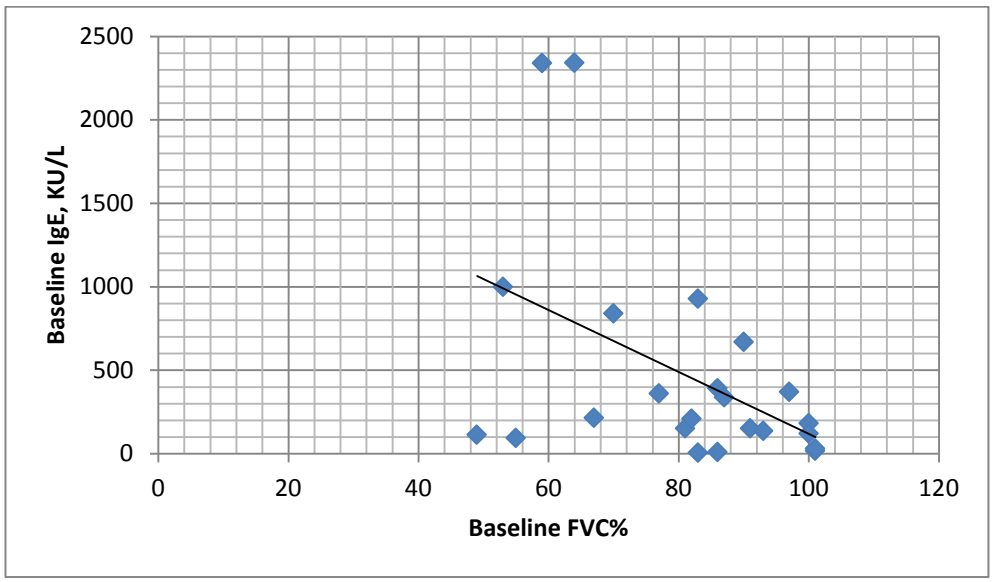

Figure 3.2. The correlation between total IgE and FVC% (n=25)

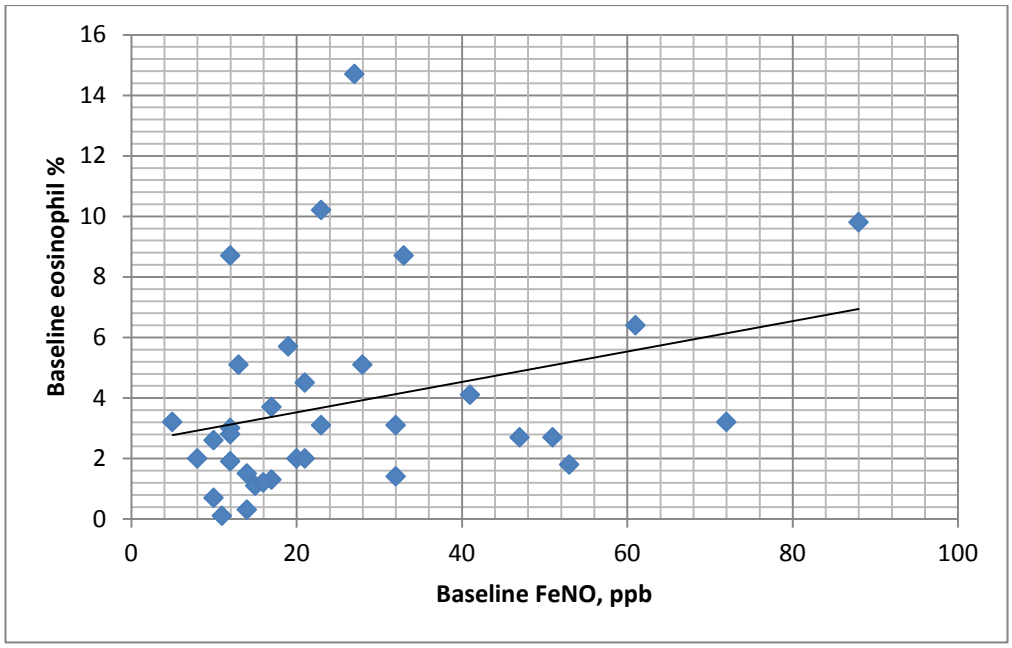

Figure 3.3. The correlation between eosinophil and FeNO (n=34)

3.6. Discussion

Vitamin D deficiency is a worldwide health problem and it is more serious in high latitude countries and Middle Eastern countries (Cashman, 2012; Thomas *et al*., 2010; Alharbi *et al*., 2013). Asthma is a chronic inflammatory disorder, the prevalence of which is increasing globally (Pawanker, 2014). Previous cross-sectional studies and reviews have reported a positive correlation between higher vitamin D levels and better asthma outcomes (Alyasin *et al*., 2011; Black and Scragg, 2005; Khan *et al*., 2013; Li *et al*., 2010). However, there have been no studies in Saudi Arabia investigating vitamin D levels or their correlation with asthma outcomes in adult asthma patients. In the light of the above, the aim of this cross-sectional study was to investigate vitamin D levels among adult asthma patients, to assess baseline asthma outcomes in a sample of Saudi adult asthma patients and to investigate the correlation between vitamin D levels and asthma outcomes. To the best of our knowledge, this is the first study to investigate this in Saudi adult asthma patients.

Previous studies in different countries found a higher incidence of vitamin D deficiency in asthma patients compared to healthy individuals (Samrah *et al.,* 2014; Niruban *et al.,* 2015; Hatami *et al.,* 2014). In the present study, 86% of asthma participants were found to be vitamin D deficient or insufficient. The level of vitamin D deficiency in adult asthmatics in the present study is similar to the levels among healthy adults in Saudi Arabia. Previous studies have shown that the prevalence of vitamin D deficiency and insufficiency in Saudi Arabia ranges from 80% to 88% among adults and adolescents (Ardawi *et al*., 2011, Ardawi *et al*., 2012, Mansour and Al hadidi, 2012). Moreover, a study undertaken in asthmatic children in Saudi Arabia found that all participants were vitamin D deficient (Aldubi *et al*., 2015).This high rate of vitamin D deficiency in Saudi Arabia among healthy people or asthmatic patients can be explained by a number of factors such as very hot weather and low level of outdoor physical activity that lead to low sunlight exposure and outdoor activity usually is after sunset in malls or covered buildings. Also the type of clothing worn, along with poor dietary vitamin D intake and poor knowledge about vitamin D sources and vitamin D function. In addition they are only a few fortified foods available on the market (Hussain *et al*., 2014; Mansour and Alhadidi, 2012).

In the present study, females had a higher level of serum 25(OH)D compared to males. This is in contrast to previous studies in Saudi Arabia, which have found that females have a lower level of serum 25(OH)D for a number of reasons. A study by Elshafie and colleagues (2012) investigated vitamin D levels among Saudi married couples and found that males had a higher level of serum 25(OH)D than females and this was explained by increased sunlight exposure in males because of traditional habits such as females spending most of the time at homes and covered clothes style. Another study (Kensara and Azzeh, 2012) found that female Saudi children had a significantly higher incidence of vitamin D deficiency compared to male children. This was attributed to the fact that boys could play out-door

more than girls, the girl's school buildings are covered, and unlike girls, boys sometimes used bicycles outside (Kensara and Azzeh, 2012).

Muslim females also usually wear fully-covered clothes when they are outside, and this covering style may limit vitamin D synthesis via the skin (Christie and Masaon, 2011). The higher level of serum 25(OH)D in females in our study could be explained by higher dietary vitamin D intake in females compared to males.

Dietary vitamin D intake in the participants in this study was 3.7 µg/d, females consumed more dietary sources of vitamin D than males. In Saudi Arabia, dietary vitamin D intake in general is low. It was found the mean dietary vitamin D intake among children aged from 4 to 15 years was 133±77 IU/d (3.3±1.9 µg/d) in Saudi Arabia (Mansour and Al hadidi, 2012). Another study (Al-Musharaf *et al*., 2012) in Saudi Arabia found a similar result; they found that the mean intake of vitamin D in children and adolescents was 3.45 µg/d. Among Saudi postmenopausal women the average intake is even lower, 0.172 µg/d (Alissa *et al*., 2014).

In this study, more than half of the participants were physically inactive (65%) and 16% were minimally active. This result is similar to the results in a recent large study done in Canada, where the authors found that 57% of adult asthma patients were inactive or had less than 3 hours activity per week (Doggett and Dogra, 2015). Forty-four percent of the participants in the present study were found to be obese and 20% were overweight. The percentage of obesity among asthma participants in the present study is higher than the percentage of obesity in non-asthmatic individuals in Saudi Arabia, which was found to be 29% (Memish *et al.,* 2014). Our findings are similar to those of a study done in Iran by Alipour and colleagues (2015) who found that 38% and 24% of adult asthma patients were overweight and obese respectively. In the present findings the high rate of obesity among asthmatic participants could be because they may be more likely to

have lower physical activity and limited outdoor activity as 81% of participants were as found to be inactive or minimally active.

Asthmatic patients may reduce their physical activity because they are afraid to trigger or increase their symptoms such as shortness of breath during the exercise. However, a programmed physical activity for asthma patients can improve and control asthma, respiratory function and quality of life (Emtner and Hedin, 2005). Starting a balanced planned physical activity program for asthma patients will not lead to exacerbate symptoms. In addition, increasing their physical activity will increase their control of asthma symptoms, improve cardiac health, improve mental health, help in maintain healthy weight and it can also help improve lung function (Mancuso *et al.,* 2013).

The explanation of the correlation between obesity and asthma is controversial. Previous studies state that the increased prevalence of asthma among obese patients could be explained by accumulated abdominal fat, which can limit the movement of the respiratory system and lungs. Also, the fact that obese individuals secrete more inflammatory hormones from adipose tissue, such as leptin and resistin, which also can affect asthma symptoms and increase inflammation (Alipour *et al*., 2015; Sood, 2010). At the same time asthma patients who are obese or overweight and doing few physical activity, have lower serum 25(OH)D than non-asthmatic individuals (Forman *et al.,* 2007). Decreasing physical activity results in lower sunlight exposure and this could lead to lower serum 25(OH)D concentration (Al-Othman *et al.,* 2012).

In the present study, 82% of participants were exposed to the direct sunlight in daylight hours (6 am to 5 pm) for 10 minutes or less per day. Furthermore, only very small parts of the body were exposed such as the face, hands or arms. There was no difference between males and females in exposure to sunlight patterns. The exact amount of 25(OH)D that

synthesized by sunlight exposure was not measured in the present study; however, no correlation was found between sunlight exposure measured by minutes and serum 25(OH)D levels. According to the Lund and Browder chart (Mimianas, 2007) of body surface area (BSA%), the front of the head and one side of each hand is equal to 6% of the body. This small BSA% combined with a very short period of sunlight exposure may lead to low serum 25(OH)D levels due to low level of skin vitamin D synthesis and could be also a reason of not detecting any correlation between sunlight exposure and serum levels. It has been reported in a previous study that, individuals who expose 5% of body surface area to the sunlight will not have more than 35 nmol/l of serum 25(OH)D (Davie *et al.,* 1982). Moreover, skin synthesis of vitamin D can also be affected by many factors such as the period of time exposed to direct sunlight, whether the weather is clear or overcast, skin colour, sunscreen usage and clothing style (Christie and Mason, 2011; Glass *et al.,* 2009; Smith, 2010). In addition, calculation and estimation of serum 25(OH)D synthesized by the skin from sunlight is complex and not easily measured (McCarty, 2008; Buttriss, 2015). In the present study, an overall trend of low sunlight exposure was found in general.

The results from this study showed that, more than half of the asthma participants (58%) were not controlled asthmatics. This high percent of non-controlled asthma symptoms is in agreement with a study by Al-Jahdali and colleagues (2008), which found that in Saudi adult asthma patients, 64% were not controlled. This could be explained by low level of awareness about asthma, poor knowledge of asthma and how to use medications and inhalers correctly, physician under-diagnosis of asthma as well as under-treatment (Al-Moamary *et al.,* 2012).

In the present study, participants with vitamin D deficiency 25(OH)D<30 nmol/l had poorer FEV1% and FVC% compared to participants who had insufficient or sufficient levels of serum 25(OH)D. In addition, male patients

who had lower vitamin D also had significantly poorer lung function when compared to females, including FEV1% and FVC%. In spite of these findings we did not find a significant correlation between serum 25(OH)D levels and FEV1% or FVC%.

The correlation between serum 25(OH)D levels and lung function and other asthma biomarkers is still debatable. Many studies have stated that there is a positive correlation between vitamin D level and better inflammatory biomarkers; however, many studies have also reported no correlation. A large study (THANES) found a positive correlation between vitamin D level and lung function (Black and Scragg. 2005). Another study on children from Costa Rica found that 25(OH)D was positively correlated to lung function and inversely correlated with IgE and eosinophil (Brehm *et al.,* 2009). It has been reported that vitamin D deficiency can affect the immune cells, decrease the pro-inflammatory cytokines (Sutherland *et al.,* 2010) and reduce the smooth muscle function, which can lead to poorer lung function and airway remodelling (Damera *et al.,* 2009). Li *et al.,* in 2011 found that 89% of adult asthmatic patients in China had vitamin D deficiency. They also found that higher serum 25(OH)D levels was associated with better lung function. A study by Chinellato of Italian children with asthma found that serum 25(OH)D was positively correlated with FEV1 and FVC (Chinellato *et al.,* 2011). Tanaka *et al* (2014) in Japan found that higher IgE was associated with poorer asthma control and lower FEV1. However, a study in 2013, stated that serum 25(OH)D was not correlated with asthma prevalence, severity or response to treatment (Gergen *et al.,* 2013). The study by Li and colleagues found no significant correlation between serum 25(OH)D and total IgE levels in adult asthma (Li *et al.,* 2011).

In our study, there was an inverse trend between serum 25(OH)D and fractional exhaled nitric oxide, however this was not significant. The correlation between FeNO level and serum 25(OH)D is controversial. A study by Checkley and colleagues did not find a correlation between serum

25(OH)D and FeNO in asthmatic children (Checkley *et al*., 2014). However, Korn *et al.,* (2013) found an inverse correlation between serum vitamin D and FeNO levels in adult asthma patients. Airway inflammation is one of the most common features in asthma patients as asthma is defined as a chronic inflammatory disease (Al-Zahrani *et al.,* 2015). Previous animal and *in-vitro* studies by Berry and colleagues (2005) showed that, vitamin D has an anti-inflammatory effect by reducing the production of IL-17, which is a cytokine related to chronic inflammatory diseases. This cytokine can stimulate IL-6 expression and enhance nitric oxide production that could result in increasing FeNO level. Other animal studies support the anti-inflammatory effect of vitamin D supplementation in rats and murine models by inhibition of the pro-inflammatory cytokines (Liu *et al.,* 2009; Agrawal *et al*., 2013).

The findings of our study showed that there was no correlation between serum 25(OH)D and total IgE, ECP or eosinophil. However, it was found that controlled asthma patients had lower IgE than patients who are partially controlled or poorly controlled. IgE plays an important role in developing hyper-responsiveness in asthma and it was reported that vitamin D deficiency correlated with higher IgE levels (Paul *et al.,* 2012) and has anti-inflammatory effects (Lange *at el.,* 2009). Eosinophilic cationic protein (ECP) is one of the granular proteins that is released in asthmatic patients and can reflect the severity of asthma and inflammatory activity, and also predict the response to treatment with corticosteroid. In the present study there was no correlation between ECP and lung function, asthma control or eosinophil count. Conversely, a number of studies have stated a correlation between inflammatory biomarkers and lung function. Zimmerman (1993) in Canada found that a higher ECP level was negatively correlated with FEV1 in children with asthma. Another study by Sorkness *et al.,* (2002) found a strong correlation between ECP and eosinophil count; they also found a high level of ECP in adult asthma patients with exacerbated symptoms.

Our negative findings could be explained by small sample size, different population from previous studies. Moreover, IgE levels and eosinophil could be affected by the taken medication such as anti-histamine and anti-inflammatory medication (Wilson, 2002; Banderia-Melo *et al*., 1997; Jayaram *et al.,* 2005). In this study we did not control for the medications that participants therefore took different medications were used to control asthma symptoms.

To the best of our knowledge, this study is the first to investigate the prevalence of vitamin D deficiency among adult asthma patients in Saudi Arabia. In addition, it is also the first study investigating the baseline characteristics of adult asthma patients in Saudi Arabia and investigating the correlation between vitamin D levels and asthma outcomes including inflammatory biomarkers and fractional exhaled nitric oxide which can reflect airway inflammation. The main findings of our study were;

1. Vitamin D deficiency prevalence is high in adult asthma participants in Saudi Arabia
2. Low serum 25(OH)D in asthma individuals could be due to low sunlight exposure in addition to low dietary intake of vitamin D.
3. Overweight and obesity is high as well as low physical activity in adult asthma individuals in Saudi Arabia.
4. Participants with vitamin D deficiency had slightly reduced lung function compared to those with sufficient levels of serum 25(OH)D.
5. A trend of a negative correlation between FeNO level and vitamin D was observed.
6. No correlation was found between serum 25(OH)D levels and IgE, ECP and eosinophil.

The findings from this study have important implications and applications. Measuring serum 25(OH)D could be essential for people in Saudi Arabia as

they may be at high risk for vitamin D deficiency. Increasing sunlight exposure may not be the best solution to prevent and treat vitamin D deficiency in the Saudi population due to very hot and humid weather during daylight hours; traditional clothes type for males and females; very high UV index that could damage the skin if exposed for a long time; and traditional habits and lifestyle especially for women who spend most hours of the day at home or under covered buildings. Therefore, using over the counter vitamin D supplementation on daily basis for individuals who are not severely deficient could be an effective way to maintain serum 25(OH)D levels at the desirable range. Individuals who are severely deficient or display vitamin D deficiency symptoms may be encouraged to take higher prescribed doses of vitamin D supplements from their physician. Encouraging people to consume more dietary sources of vitamin D is also important and could be effective to increase serum 25(OH)D levels. Although dietary vitamin D sources are limited, most milk brands and milk products are fortified with vitamin D in Saudi Arabia; cornflakes are also fortified in Saudi market and eggs and salmon are available to be consumed. Dieticians, Nutritionist and Health Care Providers should increase their clients' awareness of vitamin D and how to consume a balanced diet to achieve daily recommendations. Finally, it is also necessary that the food production market and policy makers within the Saudi Ministry of Health investigate ways to increase the fortification of foods with vitamin D.

The main limitation of this study was that non-asthmatic participants were not included. The addition of a healthy control group may provide appropriate comparable data between asthma participants and healthy normal participants. Time restrictions and financial constraints were the main reasons for this. In addition, the blood inflammatory biomarkers were not available for all patients due to financial constraints.

Future research need to investigate the correlation between vitamin D with lung function and other inflammatory biomarkers among asthmatic and non-asthma participants to provide more conclusive evidence. Increase sample size for the correlation of serum 25(OH)D and other blood inflammatory biomarkers such as IgE, ECP and eosinophil in asthma patients can provide more informative data. It is also important to study the validity of the developed FFQ to measure vitamin D as there is no valid FFQ to measure the dietary intake of vitamin D in the Saudi population. Finally, randomized control trials and interventional studies are necessary to study the correlation and effect of vitamin D supplementation on asthma outcomes to define the causation relationship between vitamin D and asthma outcomes.

Abstract and poster presented in the Annual meeting of the British Society of Allergy and Clinical Immunology (BSACI), Telford, UK (2014)

Vitamin D deficiency and asthma outcomes in adult asthmatics from Saudi Arabia

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Background: vitamin D is an important element for normal lung health. Airway disease such as bronchial asthma is characterised by an immunological mediated inflammation. Few recent reports suggest the potential link of vitamin D deficiency and an increased airway inflammation which can leads to poor asthma control.

Objectives: this study is aimed to investigate the prevalence of vitamin D deficiency in asthmatics and the correlation with asthma outcomes.

Methods: asthmatic participants were recruited from the allergy clinic in King Abdul Aziz University Hospital at Jeddah, Saudi Arabia. Lung function tests (FEV1, FVC), fractional exhaled nitric oxide (FeNO) and asthma control test (ACT) were assessed. Serum vitamin D 25(OH)D was measured by CLIA method.

Results: forty-nine adult asthma patients were included. Their age ranged 18-60 years (mean 35±12) years. 76% of them were females. Levels of asthma control were: 39.5% well controlled, 14% partially controlled and 46.5% poorly controlled. Mean serum 25(OH)D was 32.6±19 nmol/l. vitamin D levels were 50 nmol/l (normal) in 14%, 30-50 nmol/l (insufficiency) in 31% and <30 nmol/l (deficiency) in 55%. Asthmatics who had vitamin D deficiency had lower mean predicted FEV1 (76% vs. 82%), and higher FeNO levels (34 ppb vs. 27 ppb) when compared to ≥ 30 nmol/l. patients who had FeNO levels ≥ 26 ppb had lower vitamin D level (29.6 vs. 34.6) nmol/l, lower predicted FEV1 (76% vs. 81%) and lower predicted FVC (84 vs. 86). None of these differences reached statistical significance.

Conclusion: the study explored that low serum vitamin D is a very common findings in asthmatics (86%). More than half of asthmatic participants were not controlled. There was a trend of association between low vitamin D and poor asthma outcomes.

Chapter 4

The effect of different doses of oral vitamin D supplements on asthma outcomes

4.1. Justification and aims

The prevalence of vitamin D deficiency in Saudi Arabia is markedly higher than in Europe (Ardawi *et al*., 2011; Ardawi *et al.,* 2012; Ruston *et al*., 2002). The hot climate encourages people to stay indoors during daylight hours so very little vitamin D is ever produced in the skin. Those who do go outside tend to wear clothing covering most of the body and there is little vitamin D in the Saudi diet to compensate for lack of continuous synthesis (Hussain *et al.,* 2014) (see section 1.6.1).

Vitamin D deficiency has been associated with greater risk of asthma (Aldubi *et al*., 2015; Alyasin *et al*., 2011; Black and Scragg, 2005). The combination of high rates of both asthma and vitamin D deficiency in the Saudi population provides an excellent opportunity to determine whether vitamin D supplementation in asthmatic individuals can improve asthma control and decreases airway inflammation.

Studies that have explored the relationship between vitamin D status and asthma severity or lung function have tended thus far to be observational (Ji *et al.*, 2010; Devereux and Wagner, 2011; Bozzetto *et al.*, 2012). Associations in such studies may be highly susceptible to confounding bias or even reverse causation and therefore, an experimental study design is warranted. For example, severe asthma may lead to reduced time spent outdoors leading to poorer vitamin D status, or being asthmatic may lead to reduced levels of exercise, leading to greater adiposity and lower levels of serum 25(OH)D. However, the hypothesis that vitamin D deficiency leads directly to increased levels of inflammation and an exacerbation of

asthma symptoms is both biologically plausible and supported by data from *in vitro* and animal studies (Agrawal *et al.*, 2013; Zosky *et al.*, 2011).

Asthma is a chronic inflammatory disease characterised by breathlessness, airway hyper-responsiveness, broncho-constriction and airway remodelling. Wheezing and cough are the most common symptoms and range from occasional, mild symptoms to acute, life threatening episodes (Al-Zahrani *et al*., 2015). Vitamin D deficiency may exacerbate the symptoms of asthma. The active form of vitamin D, 1,25(OH)2D has receptors in many different types of lung cells. Emerging evidence suggests that adequate vitamin D status may prevent asthma through a combination of promoting lung immunity, reducing airway inflammation, slowing cell cycling, reducing hyperplasia and promoting the effects of exogenous steroids (Iqbal and Freishtat, 2011). However, no study to date has investigated the potential remedial effects of vitamin D in improving lung function and airway inflammation in vitamin D deficient Saudi adult asthmatic individuals. Therefore, this study aims to determine whether vitamin D supplementation for six weeks lead to significant improvements in asthmatic patients living in Jeddah, Saudi Arabia.

Aims and objectives:

1. To investigate the effect of two different doses (single high dose and continuous low dose) of oral vitamin D on serum 25(OH)D in adult asthma participants after three weeks and after six weeks.
2. To determine whether increasing vitamin D status in adult asthma participants results in improving lung function.
3. To determine whether increasing vitamin D status in adult asthma participants results in lower levels of airway inflammation.

4.2. Participants recruitment

All participants who took part in the first study (n=62) were invited to participate in this intervention study. Thirty-seven adults with asthma were eligible to participate in this study (see section 2.2.2). However, 32 participants completed the study for three weeks and only 16 completed the study for six weeks (Figure 4.1).

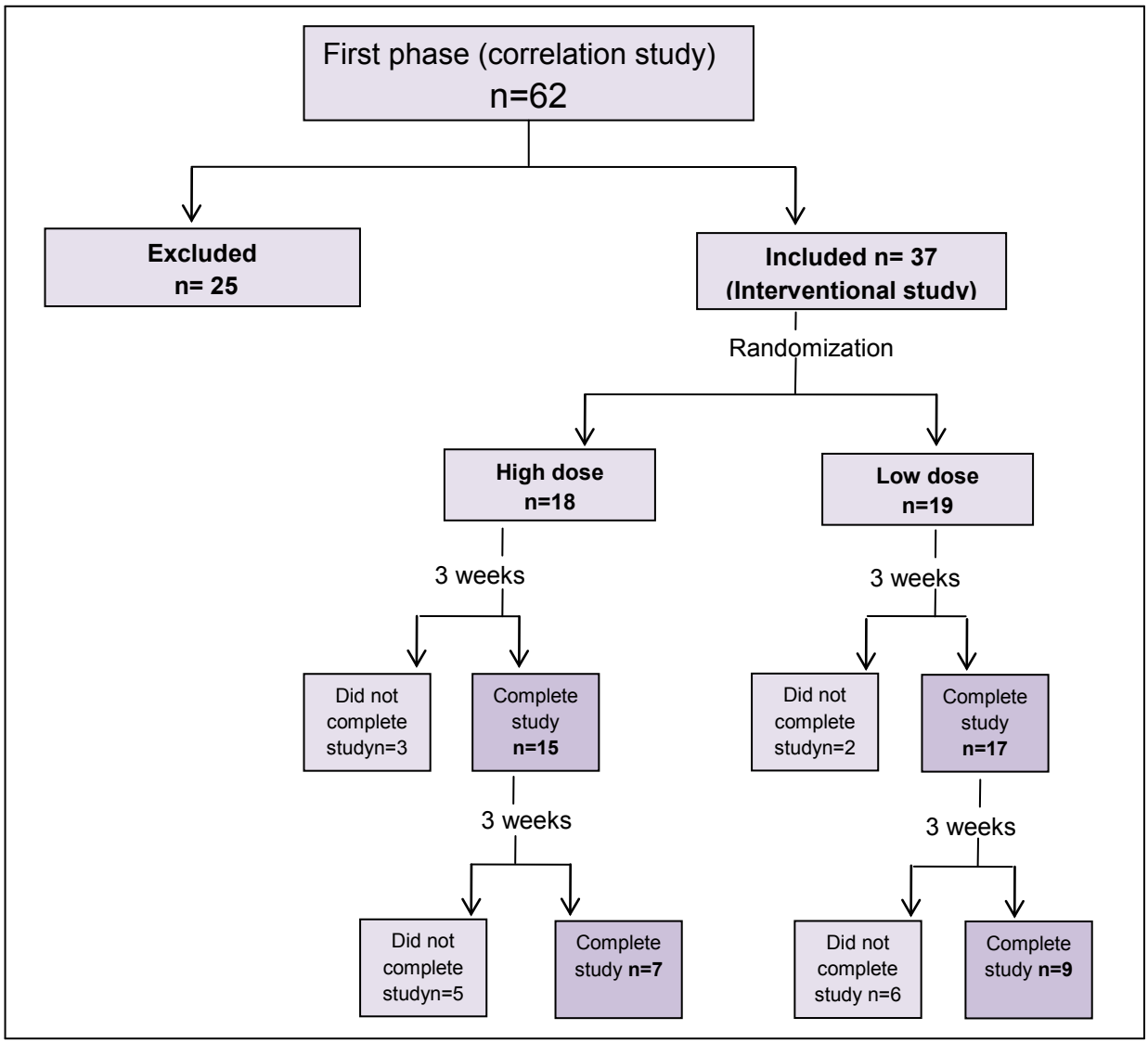

Figure 4.1. Flowchart of participant recruitment and completion

Twenty-five participants were excluded from the study (Figure 4.1) of the participants who were excluded:

- One participant had tuberculosis
- One participant was taking anti IgE medication
- One participant refused to do the spirometer test and FeNO test
- One participant was not able to provide a blood sample
- Two participants had baseline serum 25(OH)D>75nmol/l
- One participant had a high baseline calcium level
- Three participants had transport difficulties
- Four participants were not interested to continue the study after they came for first visit
- Eleven participants were too anxious to attend their appointments due to a new virus appearing in the Middle East (Coronavirus) with transmission that is hospital-related

The large drop-out of participant's number in the sixth week was also because of the virus. Of the 37 participants who were eligible to participate (see section 2.2.2), 18 were randomly assigned to the single high dose group (HDG), 15 completed the three week visit and 7 completed the six week visit. Nineteen participants were randomly assigned to the continuous low dose group (LDG), 17 completed the three week visit and 9 completed the six week visit (Figure 4.1). The intervention study protocol is described previously in section (2.3.2).

4.3. Participant baseline characteristics

Baseline characteristics of participants of both groups are shown in (Table 4.1). The mean age of the participants was 33±12 years. There was no difference in age of participants between the high dose group and the low dose group (32.8±9 and 34.8±14 years respectively; *P* 0.637). The majority

of participants were not smokers and tends to be educated. Most of participants were inactive.

Table 4.1. Baseline characteristics of participants in HDG and LDG

Variables	HDG,(n=18) n (%)	LDG,(n=19) n (%)
Gender		
Males	3 (17%)	7 (37%)
Females	15 (83%)	12 (63%)
Smoking		
Smoker	1 (6%)	3 (16%)
None smoker	14 (78%)	14 (74%)
Passive smoker*	3 (17%)	2 (11%)
Education		
High school	-	3 (16%)
University level	14 (78%)	13 (68%)
Postgraduate	4 (22%)	3 (16%)
Medical history		
Medically free except asthma	14 (78%)	15 (79%)
Diabetes	1 (6%)	-
Hypertension	1 (6%)	1 (5%)
Diabetes and hypertension	-	1 (5%)
Irritable Bowel syndrome	1 (6%)	1 (5%)
Allergic rhinitis	1 (6%)	1 (5%)
Physical activity*		
Inactive	11 (61%)	12 (63%)
Minimally active	1 (6%)	5 (26%)
Vigorous-intensity active	5 (28%)	1 (5%)
Missing	1 (6%)	1 (5%)

*Passive smoker defined as exposure to the exhaled smoke and side stream smoke (WHO, 2003)
*Physical activity classified as the International Physical Activity Questionnaire scores (IPAQ)

The mean weight, height and body mass index of participants in the HDG and the LDG are shown in (Table 4.2). No significant differences were found in weight, height and body mass index between the two groups.

Table 4.2: Height, weight and BMI in the HDG and the LDG (means±SD)

	HDG	LDG	*P* value*
Weight (kg)	77.5±17	69.6±22.2	0.232
Height (cm)	161.6±6.3	160±8.3	0.510
Body mass index (kg/m²)	29.9±7.5	27±7.8	0.261

*Independent *t*-test

Baseline mean serum 25(OH)D, dietary intake of vitamin D estimated by FFQ, serum calcium levels and lung function are described in (Table 4.3) for participants in the HDG and LDG. No significant differences were observed between groups at baseline.

Table 4.3. Vitamin D and asthma outcomes in the HDG and LDG (means±SD)

Variables	HDG	LDG	*P* value*
25(OH)D (nmol/l)	27.2±22.3	34.6±20.7	0.159
Dietary vitamin D (IU per day)	167±110	129±69	0.613
Calcium (mmol/l)	2.23±0.06	2.22±0.1	0.621
FEV1 (%)	83±18.1	79.7±15.7	0.343
FVC (%)	87±16.8	85.6±16.8	0.443
FeNO (PPB*)	23.4±17.6	39.6±43	0.150
ACT	19.5±5.9	17.7±5.5	0.245

*Mann-Whitney U test
*PPB, part per billion

4.4. The effect of vitamin D supplementation on asthma outcomes

To investigate the effect of a single high dose (200 000 IU) of oral vitamin D3 or a continuous low dose (800 IU per day) on serum concentration of 25(OH)D and asthma outcomes, measurements were taken at baseline, after three weeks of the supplementation and after six weeks. However, few participants were able to complete the three visits.

4.4.1. Effect of single high dose vitamin D supplement on serum 25(OH)D concentration and asthma outcomes

Only seven participants who were in the HDG completed all three visits. Serum 25(OH)D concentration was significantly increased after the single high dose vitamin D supplementation (*P* 0.002, Table 4.4). Post-hoc analysis with the Wilcoxon singed rank test with a significant level at <0.017 was conducted. The results showed that there were differences between 25(OH)D level at baseline and after three weeks of the supplementation (*P* 0.018), and between baseline and after 6 weeks after supplementation (*P* 0.018) but did not reach the significant level which was <0.017. However, there was no significant difference between serum 25(OH)D levels at three weeks and after six weeks (*P* 0.043). Calcium level did not change significantly after the supplementation. Although EFV1% and FVC% slightly improved after the supplement, no significant differences were observed between the three visits, FeNO was significantly increased after supplementation (Table 4.4).

Table 4.4. Effect of a single high dose vitamin D supplement on serum 25(OH)D and asthma outcomes after three and six weeks (means±SD), n=7

Variables	**Baseline** n=7	**After 3 weeks** n=7	**After 6 weeks** n=7	***P* value**
25(OH)D (nmol/l)	17.4±7.8	45.7±14.8	38.5±11.7	0.002*
Calcium (mmol/l)	2.22±0.06	2.23±0.13	2.22±0.05	0.651
FEV1 (%)	72.7±22.7	85.7±11.0	80.4±18.8	0.104
FVC (%)	77.1±19.2	90.7±14.0	92.6±15.8	0.158
FeNO (ppb)	25.4±14	34.1±19.3	33.7±18.0	0.034*

*Significant change, Friedman test

Due to the small number of participants who complete both the three week visit and the six week visit, analysis was undertaken to assess the effect of supplementation within participants who completed the three

weeks visit only (n=15; Table 4.5). Serum 25(OH)D increased significantly (*P* 0.001) after three weeks of the single high dose of oral vitamin D3. FEV1% was improved after three weeks, although this was not significant; moreover, FVC% was significantly improved (*P* 0.023) after three weeks. FeNO levels were also significantly increased (*P* 0.004).

Table 4.5. Effect of a single high dose of vitamin D supplement on serum 25(OH)D and asthma outcomes after three weeks (means±SD), n=15

Variables	**Baseline** n=15	**After 3 weeks** n=15	***P* value***
25(OH)D (nmol/l)	21.9±12.0	52.1±16.0	0.001*
Calcium (mmol/l)	2.23±0.07	2.25±0.11	0.683
FEV1 (%)	82.4±19.0	86.0±11.0	0.637
FVC (%)	87.8±18.0	98.0±14.0	0.023*
FeNO (ppb)	25.7±18.0	37.0±21.0	0.004*

* Significant differences, Wilcoxon signed rank test

4.4.2. Effect of a continuous low dose of vitamin D supplement on serum 25(OH)D concentration and asthma outcomes

Nine participants completed the study at three weeks and six weeks in the low dose group. Serum 25(OH)D concentration increased significantly after supplementation (*P* 0.002; Table 4.6). Post-hoc analysis with Wilcoxon singed rank test conducted with a significant level at <0.017 showed that the increase was significant only between the baseline and after six weeks of supplementation (*P* 0.008). Calcium levels did not change significantly after the supplementation. In addition, there was no significant change in lung function (Table 4.6).

Table 4.6. Effect of a continuous low vitamin D supplement dose on serum 25(OH)D and asthma outcomes after three and six weeks (means±SD), n=9

Variables	Baseline n=9	After 3 weeks n=9	After 6 weeks n=9	*P* value
25(OH)D (nmol/l)	31.2±17.2	38.7±13.0	46.9±17.7	0.002*
Calcium (mmol/l)	2.22±0.11	2.20±0.06	2.21±0.05	0.347
FEV1 (%)	72.3±15.5	71.8±17.0	71.0±17.5	0.459
FVC (%)	79.0±13.0	80.1±13.5	83.2±19.0	0.368
FeNO (ppb)	49.8±58.5	57.4±56.5	52.2±45.0	0.490

*Significant change, Friedman test

Due to the small number of participants who completed the three week visit and the six week visit, analysis was undertaken to assess the effect of supplementation within participants who completed the three weeks visit only (n=17; Table 4.7). Serum 25(OH)D increased after three weeks of the continuous low dose of oral vitamin D3, although the different was not significant (*P* 0.076). Lung function improved after three weeks, although this was not significant.

Table 4.7. Effect of a continuous low vitamin D supplement dose on serum 25(OH)D and asthma outcomes after three weeks (means±SD), n=17

Variables	Baseline n=17	After 3 weeks n=17	*P* value*
25(OH)D (nmol/l)	36.0±21.5	40.6±15.3	0.076
Calcium (mmol/l)	2.22±0.1	2.22±0.07	0.448
FEV1 (%)	77.3±14.5	79.1±15.7	0.139
FVC (%)	82.0±12.0	86.5±13.0	0.064
FeNO (ppb)	42.8±44.4	45.8±43.5	0.794

*Wilcoxon singed rank test

The change in serum 25(OH)D concentration between the baseline and three weeks in HDG was 28.3±11.6 nmol/l. However after six weeks the concentration decreased by 7.2±8 nmol/l from the three weeks. The change in serum 25(OH)D concentration between baseline and three weeks in LDG was 7.5±7.8 nmol/l and increased from three weeks to six weeks by 8.1±13.6 nmol/l. The change in 25(OH)D was significantly higher in HDG than LDG after three weeks (*P* 0.001). However, the change was higher in LDG than HDG after six weeks (*P* 0.003).

Comparing the change in serum 25(OH)D from baseline and six weeks, it was found that no significant difference (*P* 0.174) between HDG and LDG (21.1±11.3 and 15.6±18.6) nmol/l respectively.

The change in FVC% between baseline and three weeks in HDG was +13±20.5 % and the level slightly increased from three weeks to six weeks by 2.4±16 %. The change in LDG was 1.1±10 % after three weeks and increased from three weeks to six weeks by 3.1±11.9 %. No significant differences were found between the change in FVC% from baseline and after three weeks in both groups (*P* 0.351), or between three weeks and six weeks (*P* 0.536). The change in FVC% between baseline and six weeks is higher in HDG (15.4±12.8) % than in LDG (4.2±17.0) %, although this difference was not significant (*P* 0.174).

4.5. The correlation between serum 25(OH)D and lung function after supplementation

There were no significant correlation between serum 25(OH)D and lung function after vitamin D supplementation in both groups; HDG and LDG. However, a positive correlation was found between serum 25(OH)D and FVC after three weeks in LDG but this did not reach the significance level (*r* .619 *P* 0.075)

4.6. Discussion

The present study aimed to investigate the effect of oral vitamin D3 supplements, in a continuous low dose or in a single high dose on serum 25(OH)D concentration and lung function (including FEV1 and FVC and airway inflammation), among adult asthma participants in Saudi Arabia. It was aimed to recruit at least 17 participants in each supplementation group. However, 32 participants in total from the 62 participants who participate in the first study agreed and completed the intervention study, moreover, only 16 participants in total completed the six weeks. This large drop-out percentage can be explained by the new virus (Coronavirus) that was spreading among health care providers and hospitals in Saudi Arabia in the same period as the study. Thus a large number of participants were anxious to come to the clinic for the study visits as a result.

Present findings shows that after six weeks of the oral vitamin D3 supplements given either as a single high dose or continuous low dose significantly increased 25(OH)D levels. In the present study both supplements were in D3 form. Vitamin D3 supplement is more effective and has more stability than D2 form. Houghton and Vieth (2006), gave possible mechanisms that could explain the greater effect of D3 than D2 on raising serum 25(OH)D concentration. Firstly, the component 25(OH)D2 has less affinity to the DBP, resulting in a shorter half-life when compared to 25(OH)D3 and the enzyme 25-hydroxlase from the liver has higher affinity to the D3 form than vitamin D2. Also vitamin D3 and its metabolites have more affinity to the VDR. In addition, 25(OH)D2 metabolism is faster than 25(OH)D3 to produce the component 24(OH)D.

The single dose group had an average increase of 28 nmol/l at three weeks, and then the level decreased from three weeks by 7nmol/l after an additional three weeks. Similarly, Mallet and colleagues (2010) found that an average increase in 25(OH)D concentration in adolescents was 32nmol/l after two weeks of administrating an oral dose of 200 000 IU vitamin D3

supplements but the 25(OH)D concentration then decreased by 39 nmol/l after three months of supplementation. In addition, Stern *et al,* (1981) showed that adults receiving a 400 000 IU vitamin D supplement had increased serum 25(OH)D by 53 nmol/l after four days only, suggesting that the rapid increase after a high dose can be detected within days. The continuous low dose vitamin D3 supplement (800 IU/D) increased 25(OH)D concentration by only 7.5 nmol/l at three weeks and remained increased by 16 nmol/l after six weeks. A similar study found that a daily dose of 800 IU oral vitamin D3 supplement increased 25(OH)D by 12 nmol/l after four weeks (Freaney *et al.,* 1993).

The rapid increase in serum 25(OH)D after the high dose at week three and the following decrease at week six in the present study, can be explained by the half-life of 25(OH)D which has been shown to be between two weeks to one month (Holick, 2004; Clements *et al*., 1992; Vieth, 1999). In the first few days of giving an oral vitamin D supplement, the vitamin D3 converts rapidly to the form 25(OH)D. The following decreased is due to the half-life of 25(OH)D, subsequently, 25(OH)D is metabolised and converted by the liver to other metabolites such as 24(OH)D and then discharged from the body (Romagnoli *et al.,* 2008; Vieth, 1999; Jovicic *et al.*, 2012).

No adverse reactions were observed in any patients in the high dose group. Calcium levels were always within the normal range. In very rare cases hypercalcemia can occur after a large dose of vitamin D supplement, and renal damage, calcium stones, headache and vomiting can results from hypercalcemia (Spiro and Buttriss, 2014; Heaney, 2008; see section 1.5.3). However, Ilahi *et al,* (2008) reported that a single dose of oral vitamin D containing 100 000 IU is safe and did not lead to toxicity symptoms. Vitamin D toxicity is a rare condition because of the homeostasis control system which means that serum levels can be maintained within the range due to the livers ability to catabolism 25(OH)D and produce other products that secreted into the bile (Vieth, 1999).

In our study, a trend for improvement in lung function was observed including FEV1 and FVC after three weeks of the oral vitamin D supplement in both high dose group and low dose group. However, the FVC% increased significantly in the HDG after three weeks and was slightly improved in the LDG after three weeks. Similarly, a study in Italy found that a higher vitamin D level was associated with higher FVC% and FEV1% but was not statistically significant (Chinellato *et al.,* 2011). Another study found similar results; higher vitamin D concentration was significantly correlated with improved FVC (Hagman *et al.,* 2011).

The positive effects of vitamin D supplementation on the lung function could be explained by a number of different mechanisms. Firstly; the change in the histological and pathological structure of the lungs is supported by animal studies. Zosky and colleagues (2011) measured the lung function of mice with vitamin D deficiency and made a histological assessment of lung structure and found a decrease in lung volume and lung size. Another animal study on rats in China found that vitamin D supplements improved pathological changes in the lungs caused by vitamin D deficiency (Liu *et al.,* 2009). Secondly, the effect of vitamin D on the respiratory infection and reduced airway inflammation can lead to better lung function (Iqbal and Freishtat, 2011; Sandhu and Casale, 2010). Thirdly, reducing airway hyper-responsiveness by decreasing the histamine levels can lead to better lung function (Bradding, 2007; Sears, 2007). Finally, the effect of vitamin D on the smooth muscle function and airway remodelling by reducing smooth muscle cells proliferation (Li *et al.,* 2010).

In the present study, no significant correlations were found between serum 25(OH)D and lung function after supplementation in either groups and this could be due to small sample size in both groups. However, a positive correlation was found between serum 25(OH)D and FVC% after three weeks of a continuous low dose vitamin D supplement but did not reach significance. The correlation between vitamin D levels and lung function

and the effect of vitamin D supplementation on lung function are still debatable.

Many studies have reported positive correlation between higher vitamin D status and improved lung function. Data from the Third National Health and Nutrition Examination Survey (NHANES) found a strong significant correlation between serum 25(OH)D and better FEV1 and FVC (Black and Scragg, 2005). Li *et al,* (2011) found a significant weak positive correlation between total 25(OH)D and better FEV1 % in Chinese adults with asthma. Another study found an association between higher vitamin D levels and FEV1 % in 54 adult patients with asthma (Sutherland *et al.,* 2010). However, the effect of vitamin D supplementation on lung function is still debatable. Vitamin D supplementation of 100 000 IU per month for a period of one year resulted in no significant improvement in FEV1 compared to a placebo group in 182 COPD patients (Lehouk *et al.,* 2012). In a large randomized control trial (VIDA) by Castro *et al,* (2014), they found that vitamin D supplementation of 100 000 IU as a single dose followed by a daily dose of 4000 IU for 28 weeks among adult asthma patients did not significantly improve lung function or asthma control scores. These differences in findings could be due to differences in the baseline characteristics of the participants such as different baseline vitamin D status, different ethnicity, different duration of supplement and final vitamin D levels.

The increase in FeNO levels after the supplement in our study could be un-related to the supplementation and could be explained by the three sandstorms that occurred in the same period of the study in Jeddah. During the period between Februarys to July, sandstorms are very common in Saudi Arabia, and two to three sandstorms can happen during this period of time per month. Sandstorms in general can cause coughing, wheezing, headache, eye irritation and acute asthma attacks in healthy individuals who are exposed to the sandstorm for about 30 minutes (Meo *et al.,* 2013). During the sandstorm, the air is high in bacteria, fungi, microorganisms and

aeroallergens such as Acacia, Alternaria and cat dander. It also can carry many other components such as, silica, iron, aluminium and nitrogen dioxide NO2 (harmful gas), these components can affect health and cause many problems especially to the respiratory system. Asthma symptoms and episodes of exacerbation can be triggered by dust and sandstorms (Kwaasi *et al.,* 1998; Alangari *et al.,* 2015). Nitrogen dioxide (NO2) can increase the level of FeNO and can also convert to Nitric oxide by combining with oxygen in the air. Nitric oxide is a free radical and atmospheric pollutant (Fedoseev *et al.,* 2015; Grutta *et al.,* 2012).

The main limitations of this study were: small sample size, few patients completed the whole six week intervention, three sandstorms which happened at the period of the study that can affect the airway inflammation biomarkers of the participants. Obesity can affect vitamin D utilization in the body due to storing vitamin D in fat tissues. Compliance was not assessed in the low dose group. In addition, the inflammatory biomarkers such as eosinophil, IgE and ECP that were measured in study 1, were not available for patients after supplementation due to a limited budget.

In a conclusion, oral vitamin D3 supplementation as a single high dose or a continuous low dose increased serum 25(OH)D. However, the single high dose increased serum 25(OH)D significantly after three weeks. In addition, no adverse reactions, such as high calcium levels were observed after the single high dose. Increasing levels of 25(OH)D may improve lung function. The single high dose improved FVC% significantly but did not affect airway inflammation. Sandstorms could be a strong factor contributing to this result. Up to this date, this is the first study done to investigate the effect of oral vitamin D supplement in two doses on asthma outcomes of Saudi adult asthma patients.

As a practical implication of the study, High dose vitamin D3 could be better use to increase serum 25(OH)D levels as it increased serum 25(OH)D

significantly as well as, improving FVC in asthma participants. In addition to, there is an advantage of better compliance to the supplement (Romagnoli *et al*., 2008). However, further research needs to be completed with a larger sample. Investigating the effect of high dose vitamin D supplement on a larger sample size of asthmatic participants while monitoring calcium levels and investigating the changes in serum 25(OH)D as well as the lung function is needed.

Treating vitamin D insufficiency by giving a continuous low dose is also effective in raising serum 25(OH)D levels, although a high dose followed by a maintenance dose could be more effective in severely deficient patients. A trend towards lung function improvement with the continuous low dose of vitamin D supplement was found in the present study, and a future study with a longer supplementation period until reaching the desirable level of 25(OH)D could be helpful to investigate any positive effect on lung function in adult asthma patients.

Encouraging people in Saudi Arabia to consume more dietary sources of vitamin D is also necessary. As well as, increase the awareness of vitamin D function and beneficial effects in the body, along with, advising people who are in a risk of vitamin D deficiency to measure the 25(OH)D concentration and to reach the desirable level.

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High Dose Oral Vitamin D Supplement Improves Lung Functions in Asthmatics

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Abstract

Background: Globally and locally, vitamin D deficiency is a common health issue. Few studies have been done to investigate the effect of vitamin D supplementation on asthma. The aim of this study was to investigate the effect of different doses of oral vitamin D supplements on lung function and airway inflammation in asthmatics.

Methods: Adult asthma patients were recruited from an allergy and asthma clinic in King Abdul Aziz University Hospital in Jeddah. Sixty two patients were screened and 32 were randomly enrolled to receive either a single high dose (200 000 IU, n=15) or low continues dose (800 IU per day, n=17) of vitamin D3 supplement. Serum 25(OH)D, serum calcium, fractional exhaled nitric oxide (FeNO), forced expiratory volume in one second (FEV1) and forced vital capacity (FVC) were assessed at the baseline and after three weeks.

Results: Serum vitamin D increased significantly by 30.21 nmol (from 21.88±11.85 to 52.10±16.46) after three weeks of the high dose supplement ($p<0.001$) and increased by only 4.61 nmol (from 36.01±21.52 to 40.62±15.28) after continuous low dose ($p=0.071$). Predicted FVC improved significantly from 88.14% to 98.07% ($p=0.025$) in the high dose group and from 81.94% to 86.47% in the low dose group ($p=0.06$). Predicted FEV1 increased from 81.64% to 86.07% and from 77.29% to 79.12% respectively. Calcium level did not differ in both groups. FeNO level (inflammatory biomarker) increased significantly from 26.29 ppb to 37ppb ($p=0.016$) after the high dose supplement and increased from 42.82 ppb to 45.82 ppb ($p=0.607$) after the low dose.

Conclusion: Vitamin D deficiency is common in asthmatics from Saudi Arabia. Oral high dose vitamin D intake is safe and well tolerated in deficient patients and could be helpful in improving lung functions but not inflammatory marker.

Chapter 5

Dietary intervention and lung function among healthy adults in the UK

5.1. Justification and aims

Very small quantities of vitamin D are present in food, the main food sources being oily fish such as salmon and, sardines, and to a much lesser extent, egg yolk and fortified foods. Vitamin D is mainly obtained through exposure of the skin to ultraviolet-B (UVB) radiation. Vitamin D is essential for optimal bone mineralization but recently it has also been found to play an important role in lung function and development and have a positive effect on immune functions (Hewison, 2012). Vitamin D deficiency is a global issue, especially during the winter in regions with high latitude such as the United Kingdom. In the UK, continuous synthesis of vitamin D from the UVB is not sufficient for approximately half of the year (Macdonald *et al.,* 2008). Thus, in the winter months, foods rich in vitamin D become the only natural source of this micronutrient.

Lung function is not only affected by asthma or other lung disease, it can also be affected by age in healthy individuals. FEV1 can be standardized in healthy people at age 25 years and then it could start to be declined (Calveley, 2015). The decline in lung function could be observed due to respiratory system anatomical, physiological and immunological changes associated with age (Goodwin, 2006). Previous studies estimated the decline in lung function in healthy individuals by 22 ml per year compared to 38 ml per year among people with asthma (Sears, 2007). In other study carried out in the USA among individuals from 18-30 years and followed up for 10 years, they found a decline in FEV1 by 8.5% in non-smokers healthy people compared to 10.1% in asthmatic people who are non-smokers (Apostol *et al*., 2002). On the light of above, we hypothesised that raising

vitamin D concentration could also lead to improve lung function in healthy individuals due to its effect on reducing lung remodelling.

Studies that have explored the relationship between vitamin D status and lung function have tended thus far to be observational (Ji *et al.,* 2010; Devereux and Wagner, 2011; Bozzetto *et al.,* 2012). Associations in such studies may be highly susceptible to confounding bias or even reverse causation, therefore an experimental study design is warranted. In our previous study on asthma participants in Saudi Arabia (study 2), we examined the effects of an oral vitamin D supplement with different doses on serum 25(OH)D levels, lung function and airway inflammation among adults with asthma. We found that continuous low dose or single high dose of vitamin D increased 25(OH)D levels and increasing 25(OH)D improved FVC but not airway inflammation.

A healthy diet has become a point of interest for individuals and people may prefer to obtain their vitamin and mineral needs from a healthy diet instead of using pharmacological supplementation. In this proposed study, we aimed to investigate the effect of consuming 15µg of vitamin D from dietary sources for three weeks on serum 25(OH)D level after the winter months and lung function including FEV1, FVC and FeNO. The specific objectives of this study were:

1- To estimate the dietary vitamin D intake in healthy adults living in the UK

2- To investigate the feasibility of consuming 15 µg of vitamin D daily for three weeks from food sources only

3- To examine the effect of consumption of 15 µg of vitamin D from diet for three weeks on serum 25(OH)D levels

4- To examine the effect of consumption of 15 µg of vitamin D from dietary sources for three weeks on lung function and airway inflammation

5.2. Participants baseline characteristics

Forty-seven participants responded to the study advertisement and each participant signed an informed consent form before taking part in the study. Forty-two participants completed the study. Five were excluded from the study for a number of reasons:

- One participant had a BMI greater than 30 kg/m².
- One participant did not attend their second visit due to University exams
- One participant failed to complete the dietary intervention
- One participant had difficulties with transport.
- One participant had an extremely high level of FeNO and was advised to visit their GP, they was subsequently diagnosed with asthma

Of the 42 participants who completed the study visits, 21 were randomized to the control group (5 males, 16 females) and 21 were randomized to the dietary intervention group (11 males, 10 females).

The mean age of the participants was 29±6.6 years. Sixteen participants (38%) were males and 62% were females. All of participants were healthy and were not taking any medication or dietary supplements. The World Health Organization (1946) defines health as “a state of complete physical, social and mental well-being, and not merely the absence of disease or infirmity”. In our study we asked participants if they suffer from any acute or chronic diseases, or had any diagnosed psychological disorder or if they were on any medication. If they did not have any diseases and were not on any medication, they were considered healthy and included in the study.

Using the IPAQ tool to assess the physical activity of the participants, it was found that the majority of the participants were moderately active (58%) followed by very active (22%) and (19%) were inactive.

5.2.1. Baseline body weight status

At the baseline, 60% of the participants had normal BMI and 40% were classified as overweight. Significant differences were found in weight, BMI, waist and hip circumference between participants in the (control group) CG and the (intervention group) IG (Table 5.1). However, these differences were only found among female participants (Table 5.2). No association was found between baseline serum 25(OH)D levels and weight (*P* 0.941), BMI (*P* 0.292), IPAQ score (*P* 0.288) or fat percentage (*P* 0.352).

Table 5.1. Physical characteristics of the participants in the control (CG) and intervention (IG) groups at baseline (means±SD)

Variables	**CG,(n=21)**	**IG,(n=21)**	***P* value**
Weight (kg)	63.0±10.0	71.0±8.0	0.004*
Height (cm)	165.0±8.0	168.0±8.6	0.246
BMI (kg/m²)	23.1±3.0	25.4±2.5	0.010*
Fat percentage (%)	25.4±7.3	23.7±11.0	0.552
Waist circumference (cm)	74.3±8.0	79.4±6.0	0.032*
Hip circumference (cm)	99.5±7.0	104.4±6.3	0.029*
Waist/Hip (ratio)	0.74±0.1	0.76±0.1	0.380

* Significant difference, Independent t-test used to compare the variables between control group and intervention group

Table 5.2. Physical characteristics of males and females in the control group (CG) and intervention group (IG) at baseline (means±SD)

Variables	**Males**			**Females**		
	CG,(n=5)	IG,(n=11)	*P* value	CG,(n=16)	IG,(n=10)	*P* value
Weight (kg)	73.4±9.6	73.5±8.0	0.993	59.5±7.5	67.0±7.0	0.004*
Height (cm)	175.0±3.3	173.5±7.0	0.551	161.6±5.7	161.5±5.7	0.979
BMI (kg/m²)	24.0±3.0	24.4±2.4	0.780	22.8±3.0	26.4±5.3	0.002*
Fat percentage (%)	19.0±6.0	15.0±5.3	0.196	27.5±6.6	34.0±5.3	0.013*
Waist circumference (cm)	83.0±7.4	82.3±5.0	0.854	71.0±6.0	76.2±6.2	0.064
Hip circumference (cm)	102.6±8.0	102.8±7.0	0.964	98.3±6.4	106.0±5.5	0.006*
Waist/Hip (ratio)	0.80±0.2	0.80±0.1	0.734	0.72±0.02	0.72±0.06	0.744

* Significant difference, Independent t-test used to compare the variables between males and females in both groups

5.2.2. Sunlight exposure pattern

Using the Fitzpatrick skin type scale, most participants were classified as type 2 or type 3 skin colour. Twelve percent of the participants were having less than 10 minutes per day exposure to sunlight and 45% were exposing only their face and hands to sunlight (in the winter months) and 36% of participants were typically wearing a hat or hijab. Forty-seven percent reported having painful sunburn 1-2 times during the previous summer. Females in general applied sunscreen more often than males (Table 5.3)

Table 5.3. Sunlight exposure pattern of the participants in the control (CG) and intervention (IG) groups

Sunlight exposure patterns	All participants = 42 n (%)	CG = 21 n (%)	IG = 21 n (%)
Fitzpatrick skin colour types			
Type 1	4 (10%)	4(19%)	0
Type 2	15 (36%)	6(29%)	9 (43%)
Type 3	17 (41%)	8(38%)	9 (43%)
Type 4	4 (10%)	3(14%)	1 (5%)
Type 5	1 (2%)	0	1 (5%)
Type 6	1 (2%)	0	1 (5%)
Time exposing to the sunlight per day (by minutes)			
Less than 10 minutes	5 (12%)	3 (14%)	2 (10%)
11-20 minutes	7 (17%)	4 (19%)	3 (14%)
21-30 minutes	4 (10%)	2 (10%)	2 (10%)
30-45 minutes	12 (29%)	6 (29%)	6 (29%)
46-60 minutes	3 (7%)	1 (5%)	2 (10%)
60-120 minutes	6 (14%)	3 (14%)	3 (14%)
More than 120 minutes	5 (12%)	2 (10%)	3 (14%)
Parts of body usually exposed to the sunlight			
Face only	4 (10%)	3 (14%)	1 (5%)
Face and hands	19 (45%)	9 (43%)	10 (48%)
Face, neck and hands	9 (21%)	5 (24%)	4 (19%)
Face, neck and arms	8 (19%)	3 (14%)	5 (24%)
Face, neck, arms and mid legs	2 (5%)	1 (5%)	1 (5%)
How often do you apply sunscreen?			
Never	15 (36%)	5 (24%)	10 (48%)
Occasionally	16 (38%)	7 (33%)	9 (43%)
Most days	5 (12%)	4 (19%)	1 (5%)
Every day	6 (14%)	5 (24%)	1 (5%)
Spend time in the sunlight for getting a tan			
Never	27 (64%)	10 (48%)	17 (81%)
Rarely	5 (12%)	3 (14%)	2 (10%)
Sometimes	5 (12%)	4 (19%)	1 (5%)
Often	5 (12%)	4 (19%)	1 (5%)

In the present study, daily sun exposure during the period between the second visit and the third visit were also estimated for each participant. Most of the days in the period of the study were cloudy and rainy.

However, the average minutes reported during the three weeks between the visits were calculated only if the weather was sunny. There was no difference in sunlight exposure between participants in the control group and in the intervention group (221±193 minutes and 387±331 minutes, respectively; *P* 0.175). Conducting this dietary intervention study in the winter months was to minimize the effect of sunlight on vitamin D status. No association was found between baseline 25(OH)D levels and sun-exposure in minutes (*r* .060 *P* 0.706) or skin colour type (*r* -.070 *P* 0.661).

5.2.3. Baseline lung function

The mean forced expiratory volume in one second (FEV1) for the total sample at the baseline was 3.0±0.4 L (89.3±17 %) and the mean forced vital capacity (FVC) was 3.7±0.9L (92.5±21.7 %). The normal average of FEV1 % and FVC% is 80 % or above (Bellamy *et al.,* 2005). The mean fractional exhaled nitric oxide level was 20.3±11 ppb. No significant differences were observed between the CG and IG (Table 5.4).

Table 5.4. Lung function and FeNO levels among CG and IG at the baseline (means±SD)

Variables	CG,(n=21)	IG,(n=21)	*P* value
FEV1 (L)	3.0±0.3	3.1±0.5	0.184
FEV1%	92.2±17.4	86.3±17.0	0.268
FVC (L)	3.5±0.7	3.8±1.0	0.196
FVC%	94.2±21.0	91.0±22.8	0.630
FEV1/FVC ratio	98.2±13.4	93.6±16.4	0.317
FeNO ppb	22.0±13.0	18.6±8.5	0.338

*****Independent t- test used to compare variables (FEV1%, FVC%, FEV1/FVC ratio) between groups
*****Mann-Whitney test used to compare FeNO between groups

5.2.4. Baseline vitamin D levels

Vitamin D levels were assessed using serum levels of 25(OH)D and dietary intake estimation. Dietary intake was estimated at the baseline with FFQ

and a four-day diet record. Mean dietary vitamin D intake for all 42 participants was 4.2±3.3 μg per day estimated by the FFQ. Ninety-five percent of the participants consumed less than 10 μg of vitamin D daily which is the daily requirement in the UK (SACN, 2016). Only two participants consumed the daily recommendation 10-15 μg. Seventy-three percent of the participants consumed less than 5 μg of vitamin D per day. Mean average dietary vitamin D intake measured by the 4-day diet record was 2.4±1.8 μg per day. No significant difference was found between the intakes of vitamin D estimated by FFQ between males and females (*P* 0.404) or by 4-day diet record (*P* 0.233).

At baseline, no differences were found in serum 25(OH)D, dietary vitamin D intake estimated by FFQ or by four-day record between participants in the CG and IG (Table 5.5).

Table 5.5.Baseline serum 25(OH)D and dietary vitamin D intake in the CG and IG (means±SD)

Vitamin D status	**CG,(n=21)**	**IG,(n=21)**	***P* value**
Serum 25(OH)D (nmol/l)	35±25	40.2±39	0.970
Dietary vitamin D by FFQ (μg/d)	3.8±3.0	4.7±3.7	0.270
Dietary vitamin D by 24-h recall (μg/d)	2.6±1.8	2.2±1.6	0.555

*****Mann-Whitney test used to compare serum 25(OH)D, and dietary intake between the groups

In all participants, estimated dietary vitamin D intake determined by the 4-day diet record was significantly lower than the intake estimated by the FFQ (*P* <0.001) by Wilcoxon Signed rank test. However, Spearman rank correlation showed a significant correlation between the two methods, the FFQ and the 4-day diet record (*r*= 0.655, *P* <0.001; Figure 5.1).

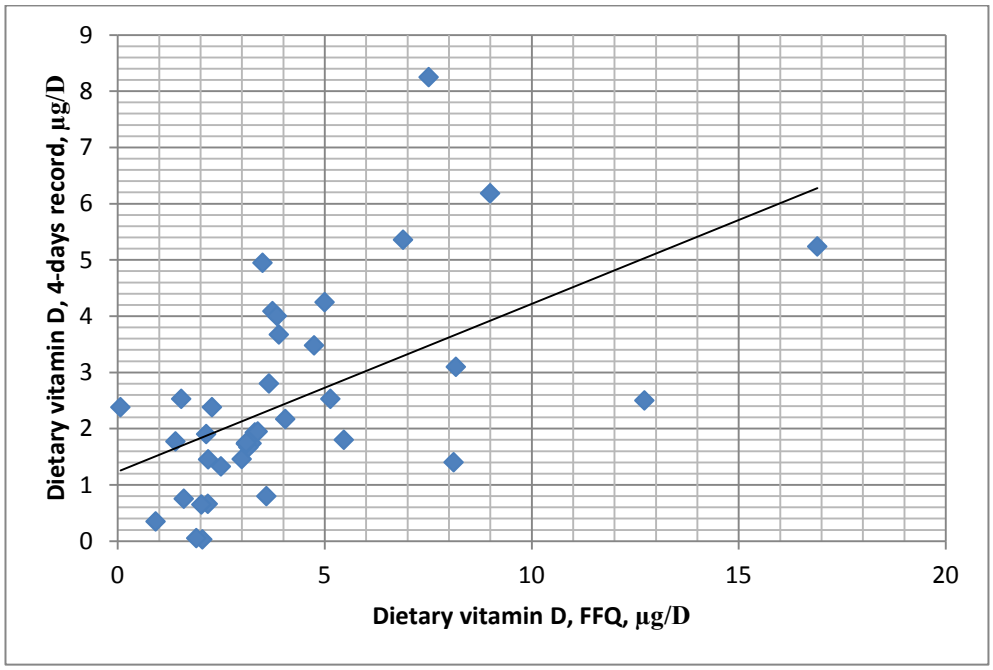

Figure 5.1. Correlation between dietary vitamin D intake estimated by the FFQ and the 4-day record

Baseline mean serum 25(OH)D of all participants was 37.7±32.4 nmol/l. Vitamin D status is classified a deficiency if the 25(OH)D is <30 nmol/l, insufficiency if the level is between 30-50 nmol/l and sufficient at >50 nmol/l. Fifty-five percent of the participants at the baseline were deficient, 19% were classified as insufficient and 26 % had normal serum vitamin D level (Table 5.6).

Table 5.6. Vitamin D status according to serum 25(OH)D levels among control (CG) and intervention groups (IG) at baseline

Vitamin D status	**All,(n=42)**	**CG,(n=21)**	**IG,(n=21)**
	n (%)	**n (%)**	**n (%)**
Deficiency , 25(OH)D <30 nmol/l	23 (55%)	10 (48%)	13 (62%)
Insufficiency , 25(OH)D =30-50 nmol/l	8 (19%)	6 (29%)	2 (10%)
Sufficient , 25(OH)D >50 nmol/l	11 (26%)	5 (24%)	6 (29%)

There was a weak positive correlation between dietary vitamin D intakes estimated by the 4-day diet record and serum 25(OH)D levels (*r* 0.483 *P* 0.002). No correlation was found between dietary intake of vitamin D estimated by FFQ and serum 25(OH)D (*r* 0.288 *P* 0.071).

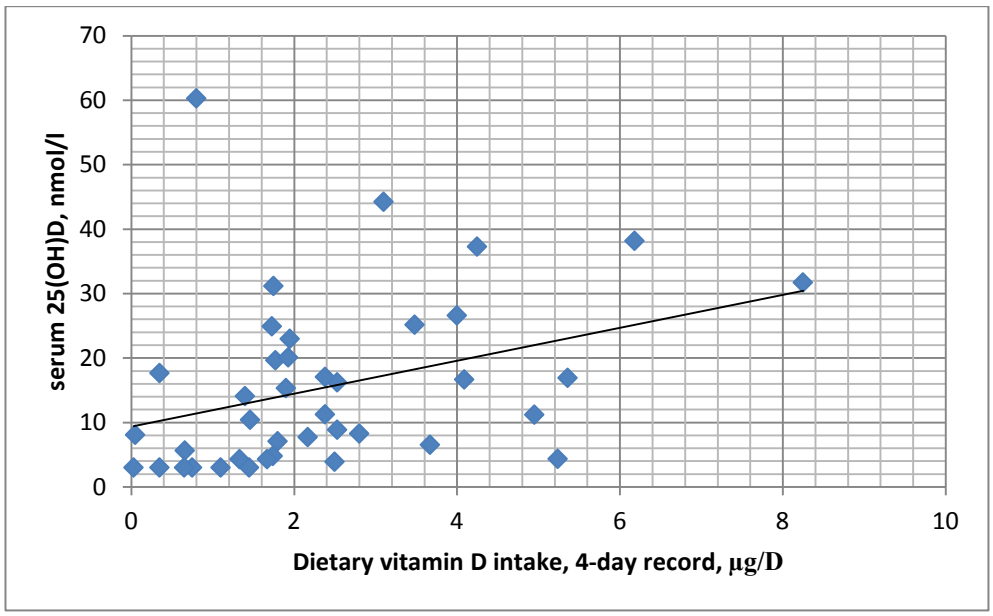

Figure 5.2. Correlation between serum levels of vitamin D and dietary intake (4-day record)

5.3. Effect of dietary intervention on serum 25(OH)D levels

The consumption of a 15 µg of daily dietary vitamin D for three weeks increased the level of 25(OH)D significantly from the baseline (*P* 0.002; Table 5.7). There was no difference between serum 25(OH)D at baseline and after three weeks in the CG. The change in 25(OH)D was significantly higher in IG than CG (7.7±11.1 nmol/l and -1±4.7 nmol/l) respectively (*P* <0.001).

Table 5.7. Serum 25(OH)D levels (nmol/l) at baseline and after three weeks (means±SD)

Groups	Baseline	After 3 weeks	*P* value
CG, n=21	35±25 nmol/l	34±24.2 nmol/l	0.366
IG, n=21	40.2±39.2 nmol/l	48±34.4 nmol/l	0.002*

*Significant difference by Wilcoxon Signed Rank test comparing vitamin D at baseline and after 3 weeks

Vitamin D deficiency among the participants was also reduced from 52 % to 33 % in IG while the percentage did not change after three weeks in CG (Table 5.8).

Table 5.8. Vitamin D status according to serum levels at baseline and after three weeks

Vitamin D status	**CG,(n=21)**		**IG,(n=21)**	
	Baseline n (%)	After 3 weeks n (%)	Baseline n (%)	After 3 weeks n (%)
Deficiency,25(OH)D <30 nmol/l	10 (48%)	9 (43%)	13 (62%)	8 (38%)
Insufficiency,25(OH)D = 30-50 nmol/l	6 (29%)	6 (29%)	2 (10%)	6 (29%)
Sufficiency, 25(OH)D >50 nmol/l	5 (24%)	6 (29%)	6 (29%)	7 (33%)

To ensure compliance to the dietary intervention, each participant who was enrolled into IG filled out a short questionnaire after the three weeks of the dietary intervention (Appendix 19). Seventy-five percent of the participants had consumed 100 % of the provided food items, 20% consumed 80-100 % of the foods and 5 % consumed 60 % of the provided foods. The type of food items that were provided to the participants was not easy to consume for 20 % of the participants and the amount of provided food items was difficult to consume for 35 %. The dietary items most preferred were beverages (all types) followed by the flavoured yogurt; the most disliked foods were the cheese fingers and canned sardines.

5.4. Effect of the dietary intervention on lung function

After three weeks of dietary intervention, no significant difference in lung function was found between CG and IG (Table 5.9). However, FVC (L) in IG was higher than CG after dietary vitamin D intervention for three weeks.

Table 5.9. Lung function after three weeks of the intervention in CG and IG (means±SD), differences between groups

Variables	CG,(n=21)	IG,(n=21)	*P* value
FEV1 (L)	3.1 ± 0.3	3.3 ± 0.5	0.073
FEV1%	94.6 ± 16.7	90.9 ± 9.2	0.385
FVC (L)	3.6 ± 0.6	4.2 ± 0.9	0.031*
FVC%	96.5 ± 20.6	98.1 ± 15.2	0.781
FEV1/FVC ratio	98.1± 15.8	94.1 ± 12.3	0.372
FeNO (ppb)	19.2 ± 5.1	18.8 ± 8.9	0.319

*Independent t-test used to compare variables (FEV1%, FVC%, FEV1/FVC ratio) between groups
*Mann-Whitney test used to compare FeNO between groups

No significant differences were found in FEV1 (L), FEV1 %, FVC (L) and FVC % after three weeks of dietary intervention in IG (Table 5.10). However a trend of increase in FVC % was found after intervention in the IG. The changes in FEV1 % and FVC % between CG and IG after three weeks were; for FEV1 % (2.33±12.2 % in CG and 4.7±13.4 % in IG, *P* 0.920) and for FVC % (2.33±17.7 % in CG and 7.2±17 % in IG, *P* 0.513).

Table 5.10. Lung function and FeNO at the baseline and after three weeks, differences within the group

Variables	CG			IG		
	Baseline	After 3 weeks	*P* value	Baseline	After 3 weeks	*P* value
FEV1 (L)	3.0±0.3	3.1±0.3	0.269	3.1±0.5	3.3±0.5	0.169
FEV1 %	92.2±17.3	94.6±16.7	0.390	86.3±16.6	91.0±9.2	0.135
FVC (L)	3.5±0.7	3.6±0.6	0.327	3.8±1.0	4.2±0.9	0.086
FVC %	94.2±20.9	96.5±20.6	0.553	91.0±22.8	98.1±15.2	0.066
FEV1/FVC ratio	98.2±13.4	98.0±15.8	0.960	93.6±16.4	94.1±12.3	0.896
FeNO (ppb)	22.0±12.8	19.2±5.1	0.371	18.6±8.5	18.8±8.9	0.900

*****Pared sample t-test used to compare variables (FEV1, FEV1%, FVC, FVC%, FEV1/FVC ratio) pre and post-intervention
*****Wilicoxon Singed Rank test used to compare FeNO pre and post-intervention

There was a trend for improvement in FEV1 % and FVC % in participant who had higher serum 25(OH)D at baseline (Table 5.11). Although there was a trend of improvement in lung function with higher vitamin D levels there was no significant correlation between lung function and 25(OH)D levels at the baseline for FEV1 % (*r* 0.241 *P* 0.125), FVC % (*r* 0.245 *P* 0.117), and FeNO (*r* 0.187 *P* 0.235).

Table 5.11. Lung function and FeNO in the different vitamin D groups (means±SD)

Variables **Means**	**Vitamin D deficiency** 25(OH)D <30 nmol/l n= 23	**Vitamin D insufficiency** 25(OH)D 30-50 nmol/l n= 8	**Vitamin D sufficiency** 25(OH)D >50 nmol/l n= 11	***P* value**
FEV1%	85±20	90.6±11.5	97±12	0.090
FVC%	87±24	94±14	101.6±20	0.249
FeNO	20.3±13	19.9±8.3	20.5±8	0.666
25(OH)D	5.9±2.8	16.7±1.6	33±12	<0.001

*Kruskal-Wallis test used to compare variables between groups

5.5. Discussion

The UK Nutrient and Food based guidelines (2007) stated that most of healthy individuals are able to consume their daily dietary requirements

through healthy and balanced diet (Food Standards Agency, 2007). However, Vitamin D presents itself in very few foods and many people do not consume the daily recommended amount (Spiro and Buttriss, 2014; O´Connor and Benelam, 2011). The prevalence of vitamin D deficiency in high latitude countries such as the United Kingdom is high, especially in the winter months. Lack of sun-exposure and low dietary intake of vitamin D can lead to vitamin D insufficiency or deficiency levels (Cashman and Kiely, 2014).

Vitamin D status is affected by seasons and in the Northern hemisphere, between October and March, vitamin D synthesis via sunlight is not enough to supply the amount needed per day and dietary intake becomes the only source of vitamin D (O´Connor and Benelam, 2011). The present study was conducted between February and April 2015. Measuring serum 25(OH)D at this period reflects vitamin D status after the winter months. The study aimed to minimize the effect of sunlight synthesis of vitamin D during the dietary intervention to better detect the effect of dietary sources of vitamin D on raising serum 25(OH)D.

In this study the aim was to estimate the dietary intake of vitamin D and to measure serum 25(OH)D levels among healthy people living in the UK. In addition, to investigate whether consumption of daily 15 µg of vitamin D as a dietary intervention for three weeks will increase serum 25(OH)D levels significantly and improve lung function and airway inflammation marker post-winter.

In the present study it was found that the daily vitamin D intake from diet measured by FFQ and by 4-day dietary record ranged from 2.4 µg to 4.2 µg per day in healthy adults. Similarly, a previous study found that dietary vitamin D intake in Aberdeen and Surrey ranged from 80 to 100 IU (2.0 µg - 2.5 µg) per day (Macdonald *et al.*, 2011). Two large studies in the UK reported that daily dietary intake of vitamin D in the UK population is about 2-3µg per day. They also found that the intake among men was

higher than women at 3.7 µg and 2.8 µg respectively. Older individuals (above 65) consumed slightly higher amounts of vitamin D than younger adults (4.1 µg for men and 2.9 µg for women) per day (Mavroeidi *et al.,* 2010; Gregory *et al.,* 2000). Moreover, the UK NDNS study found that the mean intake of vitamin D in Irish adults was 4.2 µg per day (Gregory *et al.,* 2000).

Although there are no daily recommendations for vitamin D intake for adults in the UK, the SACN stated that from 9 µg to 12 µg per day of dietary vitamin D is needed in winter to maintain serum 25(OH)D at 25 nmol/l or above (Buttriss, 2015). The US recommended dietary allowance (RDA) of vitamin D for healthy adults is 600IU (15 µg) per day and the estimated average requirement (EAR) is 400 IU (10 µg) per day (Ross *et al.*, 2011). Vitamin D intake from food is therefore generally not enough to compensate for lack of continuous synthesis in winter.

Low dietary intake of vitamin D could be due to number of reasons: few foods that are high vitamin D naturally, the food labels that shows the vitamin D content in the foods are not always available and the content of vitamin D of some foods such as salmon and sardines are not known when they were bought fresh from shops. A number of food sources such as animal organs are high in cholesterol and people prefer to avoid them from their diet. Also, food sources are more expensive than the natural source of sunlight exposure (Calvo and Whiting, 2006). Foods that are good source of vitamin D are more expensive than oral vitamin D supplementation. In this study for example to provide one participant with 15 µg of vitamin D per day cost from £ 5-6 (see the list of foods provided, table 2.1 in chapter 2) however, the oral vitamin D supplement for example, Fuiltum capsules, contain 20 µg per capsule cost £ 3.6 for 30 capsules, and £ 0.12 per capsule (BNF, 2016).

It is difficult to determine the exact amount of vitamin D that is synthesized from sunlight. Questionnaires, diaries and specific watches can be used to

estimate vitamin D that is obtained from sunlight exposure (Hoon Lee *et al.,* 2012; Rhodes *et al.,* 2010; Webb *et al.,* 2010). In the present study, the effect of sun-light exposure was small. Participants were exposed to the sunlight for a very short time, exposing small parts of their body to the sunlight, as it was winter. The weather during the study period was very often cloudy. In a study done in the UK the author stated that in winter, people usually only expose face, neck and hands and this equate to 6-10% of the body surface area BSA (Diffey, 2013). Exposing 5% of body surface to the sunlight can lead to no more than 14 ng/ml of serum 25(OH)D (Davie *et al.,* 1982). This support our results that showed low serum 25(OH)D at the baseline and there was no increase in serum 25(OH)D levels in the CG who did not consume high vitamin D sources after three weeks.

Lung function slightly improved after increasing 25(OH)D in the intervention group. This finding is similar to a large cross-sectional study (Berry *et al.,* 2011) done in the UK, which aimed to investigate the correlation between 25(OH)D and lung function in adults. They found that each 4 ng/ml increase in serum 25(OH)D levels increased FEV1 by 8 ml and increased the FVC by 13 ml in British adults. They stated that this correlation was not explained by reducing respiratory infection. However, they suggested that this positive correlation could be due to the positive effect of vitamin D on immunity and reducing inflammation, VDR are presents in lung epithelial cells and reduction the proliferation effect of airway smooth muscle cells (Berry *et al.,* 2011). Another study done among 646 elderly disabled women, which aimed to investigate the correlation between vitamin D and lung function found that higher 25(OH)D levels were associated with higher FEV1 and FVC (Semba *et al.,*2011). In our study, no significant improvement was found and this could be because participants had normal lung function at baseline. We did not find any significant improvement in FeNO levels and these findings could also be because participants had normal FeNO levels at baseline.

In summary, vitamin D deficiency is highly prevalent in the UK population after the winter months. Dietary vitamin D intake intends to be low among healthy participants living in Oxford. Dietary intervention involving consumption 15 µg per day for three weeks increased 25(OH)D levels significantly. Moreover, lung function was slightly improved after three weeks of vitamin D intervention, but not fractional exhaled nitric oxide. However, it was not very easy to consume 15 µg of vitamin D per day via diet and it was more expensive when compared to local available oral supplements.

Improving dietary vitamin D consumption from different food sources is the most important application of this study, especially after winter months as vitamin D levels are low in most healthy individuals in the UK. The American Dietary Guidelines (2014) reported that nutritional requirements should be obtained from diet. However, oral vitamin D supplementation could be also effective to improve 25(OH)D status. However, increase the awareness of dietary sources of vitamin D such as salmon and sardines is necessary in winter months as vitamin D from sunlight exposure is not enough to maintain 25(OH)D concentration at the desirable level.

The main limitations in this study were: short periods of dietary intervention due to time restriction (winter months); although all participants who were included in the study were living in the UK for at least six months, many ethnicities were included which could result in different responses to dietary intervention in raising 25(OH)D concentration and blood inflammatory biomarkers were not measured to give more information about the causality relationship between vitamin D and improved lung function. In addition, the FFQ used in this study to estimate vitamin D intake was not validated.

Future studies could investigate the effect of the same dose of vitamin D intervention using dietary sources and oral supplementation and to

compare the effect in raising serum 25(OH)D concentration between the two sources (diet vs supplement). The food frequency questionnaire to estimate vitamin D intake validation is also needed. Doing the same study with a longer intervention period could be also beneficial to investigate the effect at the baseline, after three weeks and after six weeks.

Abstract and poster presented in the Annual meeting of the British Society of Allergy and Clinical Immunology (BSACI), Telford, 2015

Dietary vitamin D intervention and lung function after the winter months

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Background: Vitamin D deficiency is a common problem, especially after the winter months in high latitude countries such as the United Kingdom. It has been reported that lower vitamin D levels are associated with lower lung function and lung capacity.

Objectives: (1) to estimate the dietary vitamin D intake and serum 25-hydroxy vitamin D (25OHD) levels among healthy adults in the UK; (2) to investigate the relationship between vitamin D and lung function in healthy UK adults; (3) to investigate the effect of a dietary intervention on serum 25OHD levels and lung functions in healthy UK adults.

Methods: Healthy adult participants were recruited from Oxford Brookes University and randomly allocated to either a control group (CG) or intervention group (IG). The intervention was consumption of 15 µg/day vitamin D through food items for three weeks. Serum25OHD, forced expiratory volume (FEV1%), and forced vital capacity (FVC%) were measured. Dietary vitamin D intake was estimated using food frequency questionnaire (FFQ).

Results: Forty-three participants, mean age 29±6.5 years, 21 CG and 22 IG, completed the study. At baseline for all participants, mean serum 25OHD was 15±13 ng/ml, 84% had vitamin D insufficiency (<25 ng/ml) and mean dietary vitamin D intake was 4.2±3.2 µg/D. In the IG, after 3 weeks of diet intervention, 25OHD increased significantly by 3.1 ng/ml (P=0.001) and lung function improved, although changes were not significant: FEV1% was 85%±17 at baseline compared to91%±9 after 3 weeks; FVC% was 111%±17 at baseline compared to 112%±14 after 3 weeks. No differences were found in CG.

Conclusion: Vitamin D deficiency prevalence is high after winter among healthy adults in the UK. Dietary intake may not be adequate to maintain 25OHD levels, thus a dietary intervention may be necessary to improve serum vitamin D levels and improve lung function.

Chapter 6

Comparison of two methods in measuring serum 25(OH)D

6.1. Justification and aims

Measuring serum 25(OH)D is important as it is the biological marker for vitamin D status in the body (Aspray *et al*., 2014). Vitamin D deficiency is associated with many disorders, particularly osteoporosis and rickets. In addition, other diseases have been found to be correlated with vitamin D deficiency such as diabetes, cardiovascular diseases, multiple sclerosis and asthma (Bischoff-Ferrari, 2010; Wang *et al.*, 2008). Measurement of 25(OH)D status is needed to investigate deficiency or very rarely toxicity. However, 25(OH)D levels are dependent on the test and the analysis methods used. Therefore, hospitals laboratories and research laboratories need a reliable method to measure 25(OH)D (Black *et al.,* 2015; Kocak *et al*., 2015).

A number of methods and kits are available to measure serum 25(OH)D and its metabolites such as chemiluminescence immunoassays (CLIA), radio immunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), high performance liquid chromatography (HPLC) and liquid chromatography-tandem mass spectrometry (LC-MS/MS). However, the two methods that are most commonly used in clinics and laboratories are immunoassays and HPLC (Wallace *et al*., 2010; Wootton, 2005). Each of these two methods has its strengths and limitations (see section 1.6). The requirements for laboratories that measure 25(OH)D are they should provide reliable, accurate and valid results and references and have a clear quality control systems, evaluation and repair services (Wootton, 2005). Additionally, the solutions and reagents need to be readily available and the method needs to be undertaken by skilled technicians (Wootton, 2005).

Chemiluminescence immunoassays are widely used in clinics such as orthopedic clinics and endocrinology clinics (Wootton, 2005). The HPLC

method is not suitable for routine tests and large number of samples due to high cost. However, the HPLC and the LC-MS/MS methods are considered the gold standard for measuring 25(OH)D status and give the most valid results when compared to other methods (Snellman *et al.,* 2010). HPLC is also recommended for the UK National Diet and Nutrition Survey (Lai *et al.,* 2011).

In this thesis, for studies completed in chapter 3, chapter 4 and chapter 5, serum 25(OH)D status was measured using chemiluminescence immunoassays by Cobas (Roche, USA). In this chapter we aimed to investigate the agreement of the results of serum 25(OH)D and the data from Study 3 (chapter 5) to the gold standard method LC-MS/MS.

6.2. Samples

A blood sample was taken and serum samples were extracted from participants from Study 3 (chapter 5). Vitamin D levels in 43 healthy individuals, 22 in the control group and 21 in the intervention group, were measured by chemiluminescence immunoassays at baseline and after three weeks of the supplementation. A sample of 4 ml of whole blood was taken and serum was separated and collected in 0.5 ml aliquots. One aliquot of serum was stored at -40°C for approximately four months until the last participant completed the study visits. It has been reported that 25(OH)D is stable and can be stored for up to a year at -20°C (Wootton, 2005). Participants were informed that the extra serum would be stored and used in the next stage of the study. Each participant signed a consent form before taking part in the study.

A total of 86 serum samples were sent to the Clinical Biochemistry Laboratory of City Hospital, Sandwell and West Birmingham Hospitals Trust. Here, total vitamin D status including D2 and D3 were measured for all samples using water LC-MS/MS. The column used was water Acquity UPLC BEH phenyl1,1.7 µm,2.1*50mm. The CV% of the method ranged from 9% to 21% (Wallace *et al.,* 2010). The lowest value of D2 detectable was 2.8

nmol/l. All participants had values less than 2.8 nmol/l, thus, for our participants D2 could not be detected.

6.3. Serum 25(OH)D of participants by the methods

Table 6.1: 25(OH)D values measured by CILA and LC-MS/MS methods

Participant´s code	25(OH)D at baseline		25(OH)D after three weeks	
	CLIA (nmol/l)	LC-MS/MS (nmol/l)	CLIA (nmol/l)	LC-MS/MS(nmol/l)
1	40.1	39.7	35.5	32.6
3	95.4	73.8	84.2	71.1
4	28.0	29.8	43.2	57.7
5	49.9	38.3	61.1	61.9
6	7.5	9.3	23.5	48.8
7	28.1	44.3	35.5	59.4
8	42.3	43.4	32.8	41.9
9	7.5	10.3	10.4	25.2
10	10.8	11.2	7.5	13.0
11	10.9	10.3	22.6	34.6
12	11.3	20.2	11.8	23.5
13	49.1	47.5	50.2	45.0
14	7.5	11.8	8.2	12.4
15	150.7	103.3	123.8	93.4
16	9.8	16.2	12.3	20.8
17	41.6	41.0	43.6	37.0
18	12.0	17.6	16.3	17.7
20	62.9	71.9	58.5	57.6
21	26.0	28.0	31.2	31.3
23	93.2	75.4	94.0	78.8
24	50.2	45.0	42.7	42.6
25	79.3	87.5	81.4	84.8
26	22.2	31.0	26.8	39.9
27	10.7	14.5	11.0	15.0
28	20.6	24.5	32.1	50.7
29	19.1	22.3	35.6	65.1
31	42.7	35.3	53.1	41.8
32	110.6	92.7	104.1	93.6
33	14.1	17.4	14.7	29.5
34	20.2	34.7	14.0	23.2
35	35.2	30.1	46.9	50.5
36	7.5	14.5	8.1	11.4
37	66.5	76.7	88.7	99.8
38	19.3	35.2	32.7	62.9
39	62.2	78.0	71.2	79.5
40	38.3	49.5	35.1	44.5
41	7.5	14.3	11.5	35.5
42	7.5	13.8	17.6	40.9
43	77.9	91.1	87.7	109.7
44	16.3	34.1	15.7	34.2
45	17.7	25.0	17.8	35.9
46	57.4	61.4	52.3	63.6
47	44.1	61.5	71.1	86.2

6.4. Correlation between CILA and CL-MS/MS methods

Strong significant correlations between the two methods were found, using Spearman rank correlation, at baseline (r 0.956 P <0.001) and after three weeks of the supplementation (r 0.905 P <0.001; Figure 6.1 and Figure 6.2).

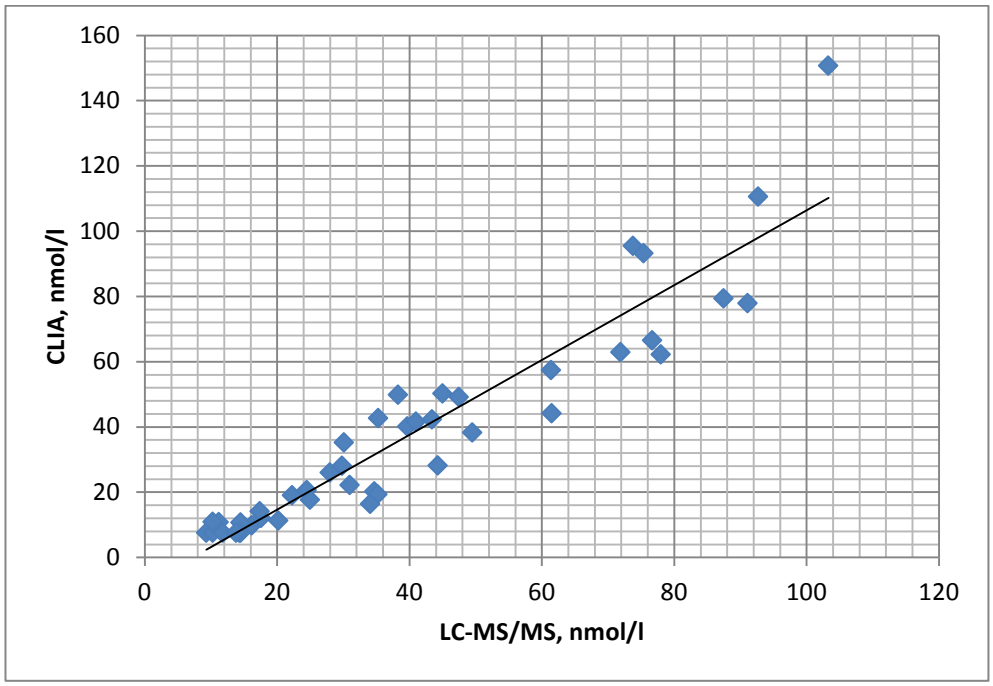


Figure 6.1. Correlation between CLIA method and LC-MS/MS method at baseline

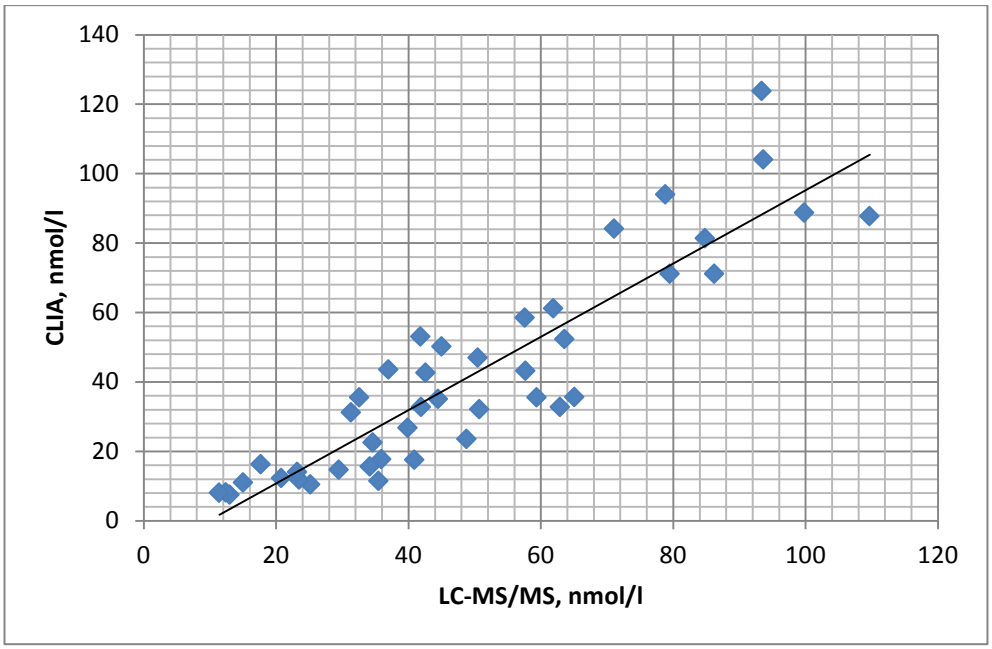


Figure 6.2. Correlation between CLIA method and LC-MS/MS method after three weeks of supplementation

Despite this strong correlation, it was found that fewer participants were classified as vitamin D deficient using the LC-MS/MS method (Table 6.2). Classifications of vitamin D status were as following; vitamin D deficiency 25(OH)D<30 nmol/l, vitamin D insufficiency 30-50 nmol/l and vitamin D sufficiency >50 nmol/l (Spiro and Buttriss, 2014). However, these differences in vitamin D status classifications were in acceptable agreement by Kappa analysis at baseline, (weighted Kappa 0.780, 95%CI 0.636 to 0.924) and after three weeks of the supplementation (weighted Kappa 0.619, 95%CI 0.456 to 0.782). Kappa values from 0.61 to 0.8 indicate good agreement (Altman, 1991).

Table 6.2: Vitamin D classification of participants using different methods

Vitamin D status	**Baseline, n (%)**			**After 3 weeks, n (%)**		
	CLIA	LC-MS/MS	Kappa value	CLIA	LC-MS/MS	Kappa value
Deficiency	23 (54%)	17 (40%)		17 (40%)	10 (23%)	
Insufficiency	9 (21%)	15 (35%)	0.780	12 (28%)	15 (35%)	0.619
Sufficiency	11 (26%)	11 (26%)		14 (33%)	18 (42%)	

6.5. Agreement between CLIA and LC-MS/MS methods

A Bland Altman test of the 43 serum samples measured by CLIA and LC-MS/MS methods shows acceptable agreement at baseline (mean difference -2.4 nmol/l, 95% CI -6.06 to 1.30, limits of agreement -25.8 and 21.08). However, the Passing-Bablok regression analysis (Intercept A -5.4585, 95% CI -10.2904 to -2.0655, Slope B 1.0982, 95% CI 0.9287 to 1.2656) shows that there was a systematic difference between the two methods (Intercept A not including zero), but there were no proportional differences between the two methods (Slope B included 1 value). However, there was no significant deviation from linearity by the Cusum test for linearity (*P* 0.82; Figure 6.3).

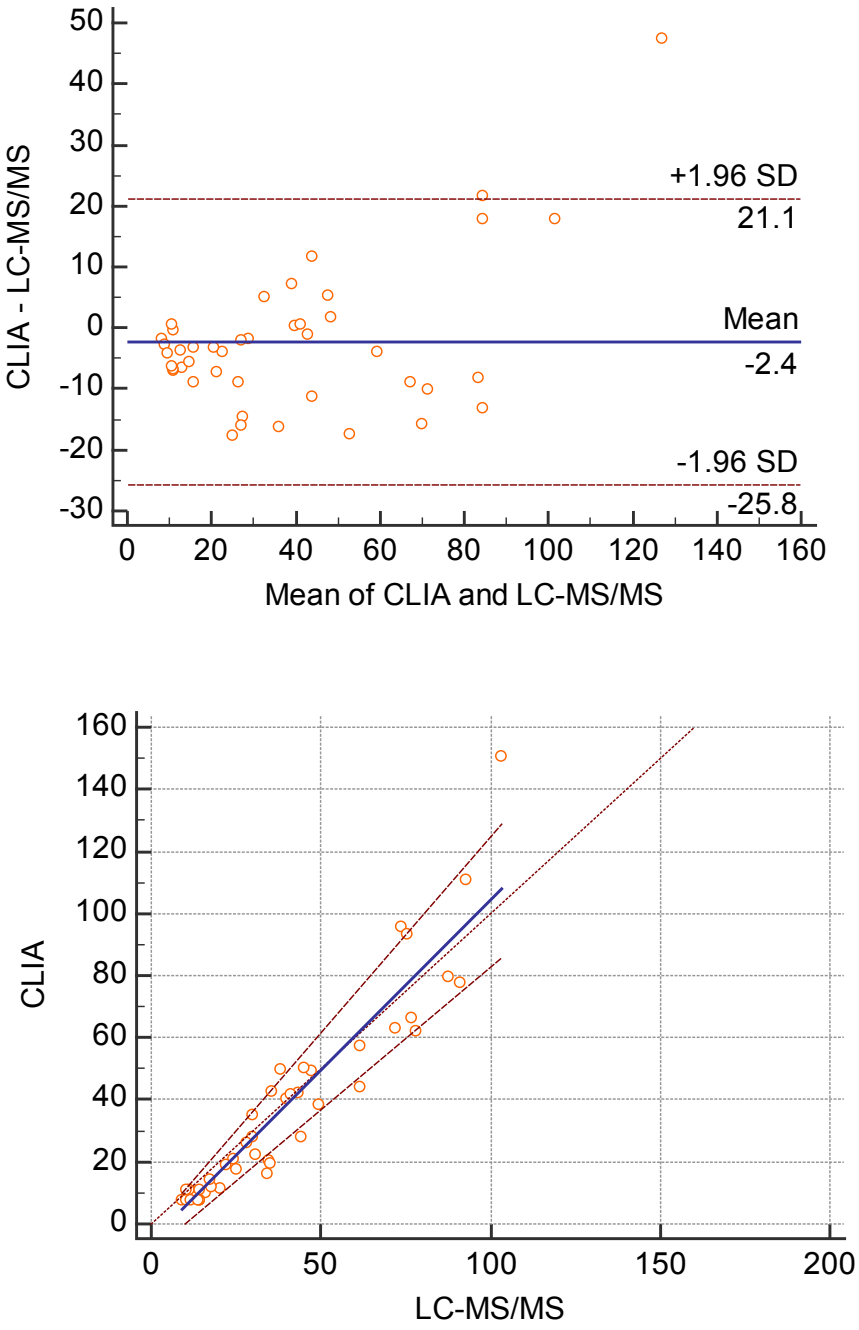
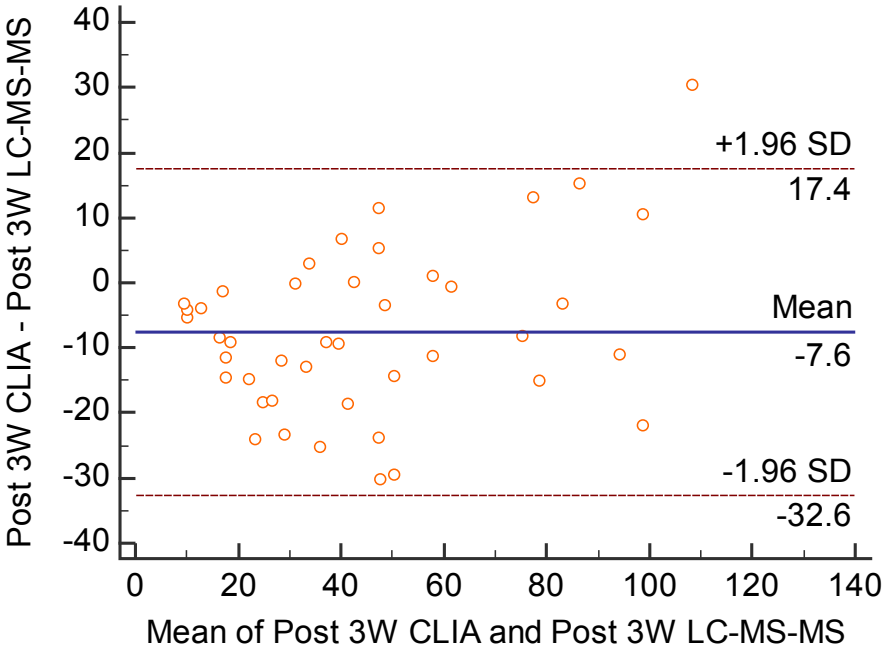


Figure 6.3 Bland Altman test and Passing-Bablok regression analysis between CLIA and LC-MS/MS methods at baseline (nmol/l)

For the 43 serum samples after three weeks of the supplementation by both methods, Bland Altman showed acceptable agreement (mean difference -7.6 nmol/l, 95% CI -11.53 to -3.67. limits of agreement -32.6 and 17.4). Passing-Bablok regression analysis (Intercept A -13.6005, 95% CI -21.7865 to -5.6828, Slope B 1.1062, 95% CI 0.9173 to 1.3016) shows that that there was a systematic difference between the two method (Intercept A not included zero), but there were no proportional differences between the two methods (Slope B included 1 value). However, there was no significant deviation from linearity by Cusum test for linearity (*P* 0.33; Figure 6.4).



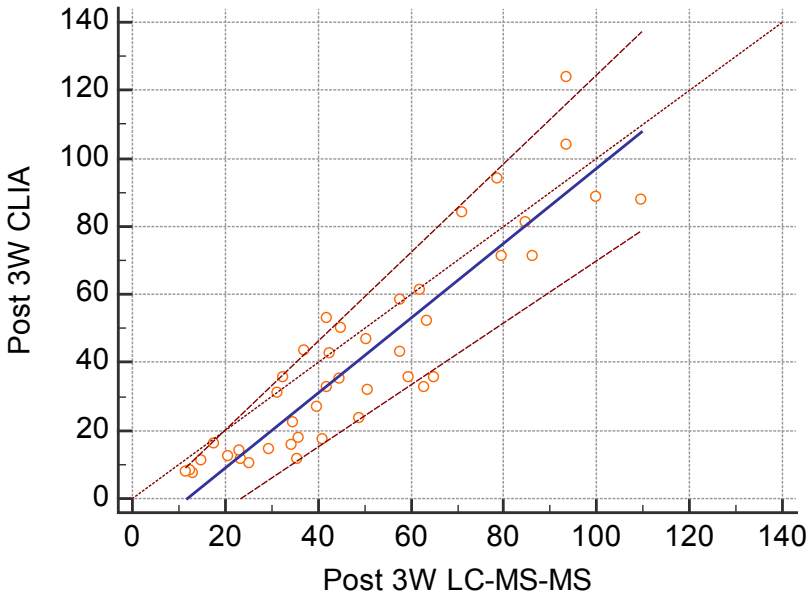


Figure 6.4 Bland Altman test and Passing-Bablok regression analysis between CLIA and LC-MS/MS methods after three weeks of supplementation (nmol/l)

6.6. Discussion

The present study shows that there was a strong correlation and good agreement between the two methods, CLIA and LC-MS/MS. However, more participants were classified as vitamin D deficient by the CLIA methods but these differences were acceptable by the Cusum test for linearity (Kocak *et al.,* 2015).

Previous studies and reviews have stated that Immunoassays are widely used in large laboratories; however, HPLC and LC-MS/MS methods are the gold standard for measurement of 25(OH)D levels (Kocak *et al.,* 2015; Lai *et al*., 2011). Despite this, these two methods are not suitable for routine clinical investigations or for large sample size studies and laboratories need high throughput or with large number of samples. HPLC and LC-MS/MS methods are more expensive, have a longer run time, require more well-skilled trained technicians and they are more complicated when compared to other methods (Lai *et al.,* 2011).

The results of the present study found a strong correlation between the CLIA and LC-MS/MS methods. However, the results showed that there were differences and variability in assigning vitamin D status between the two methods. The LC-MS/MS method tended to give higher values than CLIA. In our study we found that CLIA method had lower values of 25(OH)D and the average differences were 2.4 nmol/l at baseline and 7.6 nmol/l after three weeks of supplementation. The present results are in agreement with a study by Lai and colleagues (2011) who found that the LC-MS/MS method gave higher 25(OH)D values compared to DiaSorin Liaison (Chemiluminescent immunoassay) by 11.6 nmol/l. They also found that fewer participants were classified as having vitamin D deficiency when LC-MS/MS was used and that the Pearson correlation coefficient between the methods was 0.86 (Lai *et al.,* 2011). Another study by Kocak *et al* (2015) found good agreement between CLIA method and LC-MS/MS method in measuring 25(OH)D with a mean bias of -14% by the Bland Altman test. They also found that CLIA gave lower values of 25(OH)D than LC-MS/MS

and more participants were classified as vitamin D deficient with the CLIA method (Kocak *et al*., 2015). Black *et al* (2015) found the same results, LC-MS/MS method gave higher values of 25(OH)D (mean of values 65.5±22.7 nmol/l) when compared to CLIA method (mean of values 54.4±25.6 nmol/l) and more participants were classified as vitamin D deficient by CLIA (41%) compared to the LC-MS/MS (24%).

However, a study by Enko *et al* (2014) found a lower correlation between the LC-MS/MS method and CLIA in 133 participants in Austria (*r* 0.46) and they concluded that a standard method is needed in clinical practice. Another study curried out in Saudi Arabia found a significant difference between values measured by CLIA and by LC-MS/MS methods. They found that CLIA gave lower mean values (13.86±12.65 ng/ml) compared to LC-MS/MS (21.65±13.7 ng/ml) and a low correlation between methods (*r* 0.53), as well as more patients were classified as deficient when 25(OH)D concentrations was measured by CLIA (81%) compared to LC-MS/MS (57%). Moreover, the authors concluded that the high prevalence of vitamin D deficiency in Saudi Arabia could be due to low measurement values assessed by immunoassays that are commonly used in Saudi Arabia laboratories (Sadat-Ali *et al.,* 2014).

There are a number of possible reasons that could explain the different values of 25(OH)D measured by the LC-MS/MS and by the CLIA methods. Firstly, 25(OH)D is a lipophilic component and has a strong binding affinity to its binding protein. Separation of 25(OH)D from its binding protein is important to measure 25(OH)D concentration. The separation in the LC-MS/MS assay is done by solvent extraction while, in the CLIA method, blocking agents are used to displace 25(OH)D from its binding protein. Therefore, incomplete separation can lead to lower measurement values (Black *et al.,* 2015; Kocak *et al*., 2015). Secondly, the cross-reactivity between 25(OH)D and its metabolites in the sample may have an impact on the values. In immunoassays, the antibodies used in the method bind to the 25(OH)D carbon skeleton, in C1-C22 site and this binding can also

happen to 25(OH)D2 and any metabolites that have the same C1-C22 structure such as 24,25(OH)2D (Black *et al.,* 2015). Thirdly, the matrix effect may explain some of the difference in values. Roche calibrators (used in the CLIA method) include human serum as a matrix and confounding substances in the matrix such as lipid could lead to an interaction between the matrix, calibrators and the patient sample, which in turn could lead to high or low measurement values in 25(OH)D concentrations (Enko *et al.,* 2014; Black *et al*., 2015). Finally, a different calibrator used in each assay, method standardization, equipment maintenance and water quality could also affect 25(OH)D concentrations (Black *et al.,* 2015).

The main limitation of this study is that the reliability of the assay methods themselves was not investigated. Duplicate tests for the same sample may be useful to investigate the reliability of CLIA or the LC-MS/MS method. In this study the agreement between the two methods was analysed, but not within the same method (within assay). For future research, duplicate samples for each method would be recommended. Total 25(OH)D reflects the vitamin D status from food and sun-light and comprises the total value of both D2 and D3. In this study Cobas e401 was used to measure total 25(OH)D as it is not able to measure D2 and D3 separately, whereas the LC-MS/MS method measured both D2 and D3. The sum of D2 and D3 gives the total 25(OH)D (Snellman, 2010). However, in the present study none of participants had D2 of >2.8 nmol/l, which is the lowest value detectable by the LC-MS/MS method used. None of the participants were on vitamin D supplementation including D2 supplements form, in addition that all food sources of vitamin D contain D3 form except mushrooms that containing the D2 form (Holick, 2007; Holick *et al.*, 2011).

In conclusion, there was a strong correlation between the two methods with acceptable agreement; however, there was variability in the vitamin D classification of the participants. Immunoassays are widely used in clinical laboratories in order to analyze a large number of samples each day due to

high throughput, higher speed when compared to other methods and lower cost (Wallace *et al.,* 2010). Although LC-MS/MS has been considered a gold standard in measuring 25(OH)D due it sensitivity and accuracy, Black *et al* (2015) stated that the LC-MS/MS accuracy is dependent on the performance of the laboratory and considerable differences have been found between laboratories using the LC-MS/MS method compared to a laboratory that was certified to the standard reference method (by the National Institute of Standards and Technology and Ghent University). In addition to the high cost and the demand of highly skilled technicians, all of these reasons contribute to clinical laboratories using immunoassays more widely.

In conclusion, despite the wide usage of CLIA method in measuring vitamin D concentration, the LC-MS/MS method is still considered as the gold standard method and the recommended method to be used in the future studies due to its sensitivity and accuracy (SACN, 2016).

Finally, in the present study, both methods CLIA and LC-MS/MS gave an acceptable measurement of 25(OH)D concentrations. However, researchers and clinicians should be aware of the strength and limitation of each method and consider this when treating patients. They also need to be aware of the measuring method that is available in their care centre. Using same method is also needed when compare the patients results before and post treatment because underestimation of 25(OH)D lead to more patients classified as deficient, and maybe consumption unnecessary vitamin D supplementation, at the same time, overestimation of 25(OH)D values, patients who need an intervention could be left without treatment. Standardizing the methods used in laboratories against a certified laboratory may be a good solution to have valid and reliable values of 25(OH)D concentration and better treatment quality (Sadat-Ali *et al*., 2014; Black *et al.,* 2015).

Chapter 7

General discussion and conclusion

7.1. General discussion and findings

Vitamin D deficiency is a worldwide health issue (Holick and Chen, 2008). The prevalence of vitamin D deficiency is high in Gulf countries such as Saudi Arabia (Alharbi *et al.,* 2013) and in high latitude countries such as the United Kingdom (Ruston *et al.,* 2002).

Vitamin D is an important element for human health and is involved in many body functions such as bone calcification and calcium homoeostasis (Jovicic *et al.,* 2012; Weaver, 2007), prevention of diabetes (Al-Daghri *et al.,* 2012), cardiovascular diseases (Maki *et al*., 2011) and colon cancer (Feskanich *et al*., 2004). Vitamin D and its non-calcaemic effects have become a topic of interest for many researchers (Spiro and Buttriss, 2014; Bischoff-Ferrari, 2010). Previous studies have shown positive correlations between vitamin D and asthma outcomes as well as improving lung function (Bozzetto *et al.,* 2012; Chinellato *et al*., 2011). However, most of these studies are observational studies and only a few clinical trials have been conducted. Thus, more intervention studies and clinical trials are needed to study the causal correlation between vitamin D and asthma outcomes or other diseases (Aspray *et al*., 2014).

Four studies were conducted to complete this thesis. These consisted of a cross-sectional study (Chapter 3), two intervention studies (Chapters 4 and 5) and a comparison study (Chapter 6) to assess the measurement of serum 25(OH)D concentrations using the CLIA method and the gold standard method LC-MS/MS.

To the best of our knowledge, the current studies (Chapters 3 and 4) are the first to investigate the prevalence of vitamin D deficiency among adult asthma patients in Saudi Arabia and to undertake an intervention study

using two doses of oral vitamin D3 supplement. The cross-sectional study (Chapter 3) is important because it is one of the few investigating the outcomes and characteristics of adult asthma patients in Saudi Arabia. A number of studies have previously been undertaken, but these have been carried out in asthmatic children (Aldubi *et al*., 2015). Therefore, this cross-sectional study (Chapter 3) provided baseline characteristics of adult asthma outcomes in Saudi Arabia. The second intervention study (Chapter 5) in this thesis was a dietary intervention study aimed at investigating the effect of consuming 15 µg vitamin D via diet on the serum 25(OH)D levels after the winter months in healthy adults in the UK. This is of importance as there is currently no reference nutrient intake (RNI) for vitamin D for healthy adults aged 18 to 65 years in the UK as it is assumed that this age group is able to achieve adequate vitamin D levels from a balanced diet and sufficient sun exposure (SACN, 2015). However, new Public Health England (PHE) guidance published on 21st July 2016 suggested that a daily 10 µg of vitamin D is necessary for children and adults in the UK, especially during the winter and autumn months, and throughout the seasons in individuals who do not consume high vitamin D dietary sources (Public Health England, 2016).

Many individuals in the UK do not obtain sufficient sun light exposure during the winter months and during cloudy weather, which can be for half of the year (O´Connor and Benelam, 2011). Moreover, Ruston and colleagues (2002) in the National Diet and Nutrition Survey (NDNS) found that 24% adult males and 28% adult females in the UK have vitamin D deficiency. Thus, the importance of our study will be to determine if sufficient vitamin D can be consumed through diet alone to reduce the incidence of vitamin D deficiency after the winter months.

Cashman *et al* (2008) estimated that healthy adults in the UK require 7.2-41.1 µg dietary vitamin D per day to maintain serum 25(OH)D above 25-80 nmol/l during the winter season. However, Henderson *et al* (2003) found

that the mean dietary vitamin D intake in adults in the UK is about 3.7 µg per day in males and 2.8 mcg per day in females. Our study (Chapter 5) investigated the effect of consuming 15 µg per day of dietary vitamin D for three weeks on the total 25(OH)D and lung function adding the novelty of studying the effect of a dietary intervention on vitamin D level and lung function and the feasibility to consume this amount via diet only. To the best of our knowledge, this is the first study (Chapter 5) to investigate the effect of a dietary intervention on serum 25(OH)D, lung function and airway inflammation in the UK among healthy individuals.

After completion of the first study (Chapter 3), it was found that the prevalence of vitamin D deficiency among adult asthma patients from Saudi Arabia was 86 %. Dietary vitamin D intake was very low among these patients. Sunlight exposure was low among asthma participants due to hot weather and the traditional style dress in Saudi Arabia, which can lead to lower 25(OH)D concentration (Mansour and Alhadidi, 2012; Christie and Mason, 2011). No correlation was found between 25(OH)D and lung function or air way inflammation. However, forced vital capacity improved significantly among asthma participants after the high dose of vitamin D supplement (Chapter 4).

The first intervention study (Chapter 4) found that oral vitamin D3 supplementation as a single high dose increased serum 25(OH)D more than the continuous low dose after three weeks. However, after six weeks both single high dose and the continuous low dose were effective in significantly improving serum 25(OH)D. Raising serum vitamin D levels after supplementation by both doses also improved lung function in asthma patients. However, after three weeks of the single high dose of vitamin D supplement, FVC was significantly improved. Vitamin D supplementation did not affect airway inflammation or other inflammatory biomarkers. This finding could be explained by the small sample size and the sand storms that occurred during the study period. Sand storms contain bacteria and

fungi and may exacerbate asthma symptoms and increase the inflammation, which may lead to increase the FeNO levels (Meo *et al.,* 2013; Kwaasi *et al.,* 1998; Alangari *et al*., 2015).

Similar to the first study (Chapter 3), the third study (Chapter 5) found that the prevalence of vitamin D deficiency was high in the UK after the winter months with 81 % of healthy adults being vitamin D deficient. Daily dietary intake of vitamin D was also low. The dietary intervention of 15 µg per day of dietary vitamin D increased serum 25(OH)D significantly after three weeks among healthy adults in the UK after the winter months. Similar to the second study (Chapter 4), improved vitamin D status resulted in improved lung function, but did not reach the significant level. No correlation was found between 25(OH)D and FeNO levels.

The last study (Chapter 6) found a strong correlation between the values of serum 25(OH)D measured by CLIA and by the gold standard method LC/MS-MS (r 0.956, P <0.001). However, more participants were classified with vitamin D deficiency when the sample was measured by the CLIA method. In general, the CLIA method gave lower values than the LC/MS-MS method, although good agreement was found between the two methods using the Kappa analysis and Bland Altman test. This result was in agreement with previous research (Lai *et al.,* 2011).

The study has important implications and applications. Firstly, assessing vitamin D status by measuring 25(OH)D and estimating the dietary intake could be essential for asthma patients living in Saudi Arabia. Assessing vitamin D status could also be necessary for adults in the UK over the winter months. Secondly, health care providers and dietitians should encourage people to increase their sunlight exposure, as well as increasing their awareness of high vitamin D sources depending on the available food sources on the market.

Thirdly, over the counter vitamin D supplementation in both forms D2 or D3, such as tablets containing 10–15 µg vitamin D per day, could also be considered for individuals who are at risk of vitamin D deficiency; however, severe vitamin D deficiency may necessitate a high dose vitamin D supplement under a Physician's supervision. It was reported that vitamin D supplements as D3 form is more effective in raising serum 25(OH)D when consumed as a bolus doses compared to the form D2 (Tripkovic *et al,* 2012). It is also necessary for the food production market and policy makers to investigate different methods to fortify more foods with vitamin D.

Increasing vitamin D status could be effective in improving lung function in asthmatic patients and in healthy adults. Encouraging individuals to consume more vitamin D rich foods is necessary after winter months in the UK to maintain the desirable vitamin D status.

Finally, researchers and Physicians should be aware of the method used in their laboratory in measuring vitamin D status due to the differences in vitamin D status classifications. Although in this study it has been found that CLIA method and LC-MS/MS method showed a good agreement, the CLIA method gave lower 25(OH)D concentration compared to the LC-MS/MS method. Standardization of the method in measuring vitamin D is necessary.

The main limitations of this project were that: non-asthmatic participants as a control group in the first and second study were not included and blood inflammatory biomarkers were not measured for all participants due to financial constraints.

In the second study (Chapter 4), a small sample size was also a limitation and there was a large drop-out rate in participants due to a new virus that appeared in the hospitals in Saudi Arabia and most of participants were very anxious to complete their visits. The increases in inflammatory

biomarkers could be affected by the sand storms that happened during the study period. This may also have affected the results of these biomarkers in the second study (Kwaasi *et al.,* 1998; Alangari *et al.,* 2015).

Finally, the sunlight exposure questionnaire that was used in all studies to investigate the sunlight exposure pattern was not validated. Also, the developed food frequency questionnaire to estimate vitamin D dietary intake that used in studies was not valid. Using non-valid food frequency questionnaire to estimate vitamin D dietary intake may non-valid dietary vitamin D intake. However, there was no valid FFQ to estimate vitamin D intake in the UK population or to measure vitamin D intake in Saudi Arabia.

In the third study (Chapter 5), baseline data and post three weeks data were obtained. Post six weeks data was not measured due to financial constraints. Blood inflammatory biomarkers were also not measured in this study.

In the fourth study (Chapter 6, comparison of two method in measuring 25(OH)D concentration), duplicate measurements for each sample were not tested due to financial constraints. Duplicate measurements for each sample would add more information about the reliability of the methods used.

7.2. Conclusion

Vitamin D deficiency and insufficiency were highly prevalent with 86% in asthma participants in Saudi Arabia. Vitamin D deficiency and insufficiency were also highly prevalent with 81% in healthy adults in the UK after the winter months.

Vitamin D intervention using a single high dose supplement or a continuous low dose supplement for three weeks and six weeks was effective in raising serum 25(OH)D levels. A dietary intervention as consumption of 15 µg vitamin D per day for three weeks was also effective in raising 25(OH)D concentrations after the winter months.

Increasing serum 25(OH)D concentrations using the vitamin D supplementation in a single high dose or a continuous low dose and by rich vitamin D foods improved lung function in asthma participants and in healthy adults, but not airway inflammation or biomarkers.

Future studies that can provide more conclusive evidence could be:

- Investigating the correlation between vitamin D status and lung function as well as FeNO in asthma patients and non-asthma patients.
- Including a larger sample size to investigate the vitamin D intervention could provide better information about the effect of vitamin D supplementation on serum 25(OH)D and lung function.
- To estimate how much vitamin D as µg/day is needed to achieve and maintain vitamin D levels among people living in Saudi Arabia with minimum sunlight exposure.
- Validation study for a food frequency questionnaire to estimate vitamin D intake from diet, using the available food in Saudi Arabia and in the UK, as there is no valid FFQ to estimate vitamin D in these countries.
- Investigate the effect of the same dose of vitamin D given by oral supplementation or by high vitamin D foods to compare the effect on 25(OH)D levels.
- Investigate the effect of high vitamin D foods on 25(OH)D levels over a longer period (longer than three weeks).
- Comparing the methods used to determine 25(OH)D, such as the CLIA method and LC-MS/MS method with duplicate tests for the same samples would be useful to study the reliability of the methods.

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Appendices

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Vitamin D Deficiency and Asthma Outcomes in Asthmatic Adults from Saudi Arabia

Samih kokandi, Simon Wheeler and Emad Koshak
Oxford Brookes University, UK and King Abdul Aziz University, KSA

BACKGROUND

- Vitamin D is an important element for normal lung health
- Airway diseases, such as asthma, are characterized by an immunological mediated inflammation
- Few recent reports suggest the potential link between vitamin D deficiency and increased airways inflammation which can lead to poor asthma control

OBJECTIVE

- This study aimed to investigate the prevalence of vitamin D deficiency in individuals with asthma and to investigate the correlation between vitamin D level and asthma outcomes

METHODS

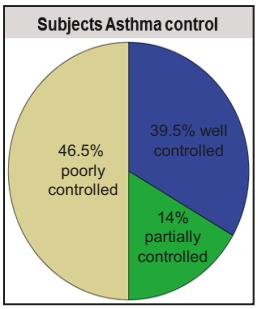
- Participants with asthma were recruited from the allergy clinic in King Abdul Aziz University Hospital at Jeddah, Saudi Arabia
- Lung function tests (FEV1, FVC), fractional exhaled nitric oxide and asthma control test were assessed
- Serum vitamin D level as 25(OH)D was measured
- Asthma control test (ACT) questionnaire was completed

Microfusion portable spirometer was used in this study

Niox Mino set was used to measure FeNO

RESULTS

- 49 adult asthmatics, age range 18-60 years with mean age of 35 ± 12 years, were included in the study
- 76% females and 24% males
- Mean serum vitamin D was **32.6(1.9) nmol/L**
- **55%** had vitamin D level **less than 30 nmol/L** (deficiency), 31% had levels from 30-50 nmol/L (insufficiency) and only 14% had levels higher than 50 nmol/L (sufficient)



Variables	FeNO <26 ppb	FeNO >26 ppb
Serum 25OHD, mean	34.66 nmol/l	29.6 nmol/l
FEV1%, mean	81%	76%
FVC%, mean	86%	84%

- Asthmatics who had **vitamin D deficiency** had **lower mean predicted FEV1** (**76% vs 82%**) and **higher FeNO** levels (**34 ppb vs 27 ppb**) when compared to patients who have vitamin D more than 30 nmol/l
- Patients who had FeNO levels >26 ppb (indication of airway inflammation) had lower vitamin D levels (**29.6 vs 34.66 nmol/l**), lower predicted % FEV1 (**76% vs 81%**) and lower predicted % FVC (**84% vs 86%**) when compared to lower than 26 ppb
- None of these differences reached statistical significant

CONCLUSION

- This study shows that low serum vitamin D is a very common finding in asthmatics (86%)
- Asthma was not well controlled in more than half of the participants
- There was a trend in association between low vitamin D and poor asthma outcomes

High Dose Oral Vitamin D3 Supplement Improves Lung Functions in Asthmatics

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BACKGROUND

Globally and locally, vitamin D deficiency is a common health issue. Few studies have been done to investigate the effect of vitamin D supplementation on asthma.

OBJECTIVES

The aim of this study was to investigate the effect of different doses of oral vitamin D_3 supplements on lung function and airway inflammation in asthma patients.

METHODS

Adult asthma patients were recruited from an allergy and asthma clinic in KAUH in Jeddah.

62 patients were screened and 32 were randomly enrolled to receive either a single high dose **(200 000 IU, n=15)** or low continues dose **(800 IU per day, n=17)** of vitamin D3 supplement.

Serum 25(OH)D, serum calcium, fractional exhaled nitric oxide (FeNO), forced expiratory volume in one second (FEV1) and forced vital capacity (FVC) were assessed at the <u>baseline</u> and after <u>three weeks</u>.

RESULTS

serum vitamin D increased significantly by **30.21** nmol (from 21.89±11.85 to 52.10±16.46) after three weeks of the high dose supplement ($p<0.001$) and increased by only **4.61** nmol (from 36.01±21.52 to 40.62±15.28) after continuous low dose ($p=0.071$).

Predicted FVC improved significantly from **88% to 98 %** ($p=0.025$) in the high dose group and from **82% to 87%** in the low dose group ($p=0.06$).

Predicted FEV1 increased from **82% to 86%** and from **77 % to 79 %** respectively.

Calcium level did not differ significantly in both groups.

FeNO level (inflammatory biomarker) increased significantly from **26** ppb to **37**ppb ($p=0.016$) after the high dose supplement and increased from **43** ppb to **46** ppb ($p=0.607$) after the low dose.

CONCLUSION

Vitamin D deficiency is common in asthmatics from Saudi Arabia. Oral high dose vitamin D intake is safe and well tolerated in deficient patients and could be helpful in improving lung functions but not inflammatory marker.

Microfusion portable spirometer was used in this study

Niox Mino set was used to measure FeNO

Variables	High dose group, single dose of 200 000 IU n= 15			Low dose group, daily dose of 800 IU n= 17		
	At baseline Means (SD)	After 3 weeks Means (SD)	Sig	At baseline Means (SD)	After 3 weeks Means (SD)	Sig
Vitamin D	21.89 (11.85)	52.1 (16.46)	<0.001*	36.01 (21.52)	40.62 (15.28)	0.071
Calcium	2.22 (0.06)	2.24 (0.11)	0.77	2.22 (0.11)	2.22 (0.08)	0.849
FEV1 %	81.64 (19.76)	86.07 (10.82)	0.261	77.29 (14.56)	79.12 (15.77)	0.302
FVC %	88.14 (19.01)	98.07 (14.03)	0.025*	81.94 (12.13)	86.47 (12.84)	0.06
FeNO (ppb)	26.29 (18.76)	37 (21.39)	0.016*	42.82 (44.38)	45.82 (43.52)	0.607

Dietary vitamin D intervention and lung function after the winter months

Samih Kokandi and **Helen Lightowler ,** Oxford Brookes University

BACKGROUND

- Vitamin D deficiency is a common problem, especially after the winter months in high latitude countries such as the UK
- It has been reported that lower vitamin D levels are associated with lower lung function and lung capacity

OBJECTIVE

(1) To estimate the dietary vitamin D intake and serum 25-hydroxy vitamin D (25OHD) levels among healthy adults in the UK

(2) To investigate the relationship between vitamin D and lung function in healthy UK adults

(3) To investigate the effect of a dietary intervention on serum 25OHD levels and lung function in healthy UK adults

METHODS

- Healthy adult participants were recruited from Oxford Brookes University after winter and randomly allocated to either a control group (CG) or intervention group (IG)
- The intervention was consumption of **15 µg/day** vitamin D through food items for three weeks
- Serum 25OHD, forced expiratory volume (FEV1%) and forced vital capacity (FVC%) were measured
- Dietary vitamin D intake was estimated using a food frequency questionnaire (FFQ)

RESULTS

- Forty-three participants, mean age 29±6.5 years, 21 in the CG and 22 in the IG, completed the study
- At baseline for all participants, mean serum 25OHD was **15±13 ng/ml; 84%** had vitamin D insufficiency (<25 ng/ml)
- Mean dietary vitamin D intake was **4.2±3.2 µg/D**
- In the IG, after 3 weeks of diet intervention, 25OHD increased significantly by **3.1 ng/ml** (P=0.001)
- In the IG, lung function improved, although changes were not significant:
  - FEV1% was 85%±17 at baseline compared to 91%±9 after 3 weeks
  - FVC% was 111%±17 at baseline compared to 112%±14 after 3 weeks
- No differences were found in the CG

Food used in the dietary intervention

CONCLUSION

- Vitamin D deficiency prevalence is high after winter among healthy adults in the UK
- Dietary intake may not be adequate to maintain 25OHD levels, thus a dietary intervention may be necessary to improve serum vitamin D levels and improve lung function