VITAMIN D STATUS IN YOUNG ADULTS IN HONG KONG: A BIOMARKER APPROACH TO A PUBLIC HEALTH CONCERN

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ABSTRACT

Low vitamin D status is very common across the world, mostly due to the modern "indoor" lifestyle that leads to low exposure to sunshine. Low vitamin D status is clearly associated with bone disease, and there is accumulating evidence of links with various non-communicable diseases (NCDs), which are the leading causes of death and disability globally. However, the link between vitamin D deficiency and NCDs is not yet confirmed. It is noted that most NCDs become obvious in late adulthood, but the process of disease development begins at a much younger age. The underlying biological changes such as poor glycaemic control, poor lipid profile, inflammation, DNA damage, and oxidative stress, form a "common soil" for NCD development. If the link between vitamin D deficiency and the elements of the "common soil" is confirmed, public health strategies to improve vitamin D status are warranted. In Hong Kong, local studies indicate that vitamin D deficiency is highly prevalent, but data on young people are lacking. Therefore, this study aimed to determine the vitamin D status in a group of young, apparently healthy adults living in Hong Kong, and to investigate the inter-relationships between vitamin D status and the risk of NCD through a biomarker approach in a two-part study.

In part 1 (the observational study), 196 (63 males and 133 females) young (18-26y), healthy, non-obese, non-smoking subjects were recruited with their written informed consent. Fasting venous blood and urine samples were collected from each subject. Plasma 25(OH)D concentration was measured as the indicator of vitamin D status, and a panel of sensitive biomarkers for glycaemic control, lipid profile, inflammation, DNA damage, and oxidative stress were measured. In part 2, a pilot supplementation trial was performed in a sub-group of those who were identified as being vitamin D deficient: 16

subjects received 2,400 IU vitamin D_3/day for 12 weeks, while 11 received matching placebo. The improvement of vitamin D status and biomarker response were determined.

The vitamin D status was found to be low: mean(SD) plasma 25(OH)D in the 196 subjects studied was 42(13) nmol/l, nearly all (194/196, 99%) had vitamin D insufficiency (defined as plasma 25(OH)D <75 nmol/l). Plasma 25(OH)D was found to be inversely associated with fasting plasma glucose and directly associated with the FRAP value ('total' and corrected for urate) for total antioxidant power ($p<0.05$). For those with plasma 25(OH)D <25 nmol/l (severe deficiency), significantly higher HbA1c, and total cholesterol/high density lipoprotein cholesterol ratio and lower high density lipoprotein cholesterol was seen (all $p<0.05$), in comparison with those with 25(OH)D ≥25<50 nmol/l and those with 25(OH)D ≥50 nmol/l. No significant association was seen between vitamin D status and DNA damage or any other biomarker. In the supplementation arm, correction of deficiency was linked to lower DNA damage ($p<0.05$), but no significant improvement was seen in any other biomarker. In addition, 146/196 of the subjects studied were found to have least one cardiometabolic disease related biomarker in the high risk category.

In conclusion, this study provides new data on the vitamin D status of apparently healthy young adults in Hong Kong, and presents novel findings on the association between vitamin D status and well-established biomarkers related to NCD risk in this group. The high prevalence of low vitamin D status in our young people is an important public health concern in Hong Kong, as this may have long-term impact on their health, and results provide support for adoption of public health strategies to improve their vitamin D status through, for example education, food fortification schemes, and health screening programmes to identify vitamin D deficient individuals for appropriate supplementation.

OUTCOMES ARISING FROM THE THESIS

Journal article

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Conference abstracts

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ABBREVIATIONS

$1,25(OH)_2D$	1,25-dihydroxyvitamin D
8-oxodG	8-oxo-7,8-dihydro-2'-deoxyguanosine
8-oxoGua	8-oxo-7,8-dihydroguanine
25(OH)D	25-hydroxyvitamin D
AD	Alzheimer's disease
ADP:	Adenosine diphosphate
ATP:	Adenosine triphosphate
BMD	Bone mineral density
BMI	Body mass index
CHD	Coronary heat disease
CI	Confidence interval
CPD	Cyclopyrimidine dimer
CVD	Cardiovascular disease
CYP	Cytochrome P450 enzyme
DIA BP	Diastolic blood pressure
DM	Diabetes mellitus
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EAR	Estimated average requirement
Endo III	Endonuclease III
FBS	Foetal bovine serum

Fpg	Formamidopyrimidine DNA glycosylase
FRAP	Ferric reducing antioxidant power
FRASC	Ferric reducing ascorbic acid assay
GPx	Glutathione peroxidase
GSH	Glutathione
HbA1c	Haemoglobin A1c
HCl	Hydrochloride
HDL-C	High density lipoprotein cholesterol
HOMA-B	Homeostasis model assessment for β cell function
HOMA-IR	Homeostasis model assessment for insulin resistance
HPLC	High performance liquid chromatography
HR	Hazard ratio
hsCRP	High sensitivity C reactive protein
IL	Interleukin
IS	Internal standard
LC-MS/MS	Liquid chromatography with tandem mass spectrometer
LDL-C	Low density lipoprotein cholesterol
MADB:	4-aminophenazone and N,N-bis(4-sulfobutyl)-3,5-dimethylaniline, disodium salt
MDA	Malondialdehyde
NAD^+:	Nicotinamide adenine dinucleotide
NADH:	Nicotinamide adenine dinucleotide
NER	Nucleotide excision repair
OGG1	8-oxoguanine DNA glycosylase 1

OGTT	Oral glucose tolerance test
OR	Odds ratio
oxLDL	Oxidative low density lipoprotein
PAD	Peripheral arterial disease
PBS	Phosphate buffered saline
PD	Parkinson disease
QUIKI	Quantitative insulin sensitivity check index score
RDA	Recommended dietary allowance
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RR	Relative risk
SD	Standard deviation
SOD	Superoxide dismutases
SPF	Sun protect factor
SYS BP	Systolic blood pressure
SZA	Solar zenith angle
T4EV	T4 endonuclease V
TAC	Total antioxidant capacity
TC	Total cholesterol
Tg	Triglycerides
TNF-α	Tumour necrosis factor alpha
TPTZ	2,4,6-tripyridyl-s-triazine
UL	Upper intake level
UVB	Ultraviolet B

VDR	Vitamin D receptor
VDRE	Vitamin D receptor element
WBC	White blood cell

CHAPTER 1

LITERATURE REVIEW

INTRODUCTION

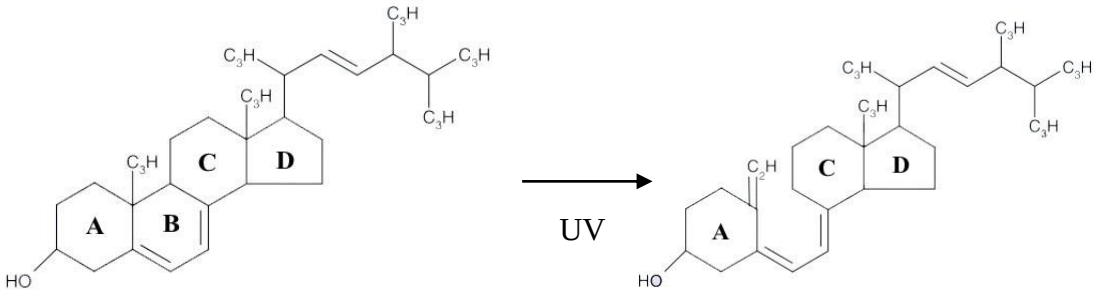
Vitamin D insufficiency is common in many parts of the world. The vitamin D status is unclear in the Hong Kong population, but there are some local data that indicate vitamin D insufficiency is common in pre-menopausal women. Although vitamin D is most commonly connected to bone health, there is accumulating evidence that it has an important role in general health promotion and protection, and that vitamin D deficiency increases risk of non-communicable disease (NCDs), including cardiometabolic disease (cardiovascular disease and diabetes), hypertension, cancer, poor immune function and multiple sclerosis. The mechanism by which vitamin D affects the function of non-skeletal tissues is unclear. Still, because of the high prevalence and the costs to long-term health, vitamin D deficiency is an important public health concern. However, vitamin D status is not routinely assessed during medical checkups, and the problem of vitamin D deficiency and its long-term impact on health is under-appreciated. Many of our young adults, who are likely to be deficient in vitamin D, may be at high risk of chronic disease later in life and do not realize it. There are many risk factors for chronic diseases, but high blood pressure, poor glycaemic control, dyslipidaemia, inflammation, oxidative stress and deoxyribonucleic acid (DNA) damage are biological features of damage that form a ‘common soil’ for their development.

In this literature review, current knowledge about vitamin D, the impact of NCDs and the risk factors leading to high risk of NCD and the relationship between vitamin D and NCDs, as well as the elements of the 'common soil' will be presented.

VITAMIN D SOURCES AND SYNTHESIS

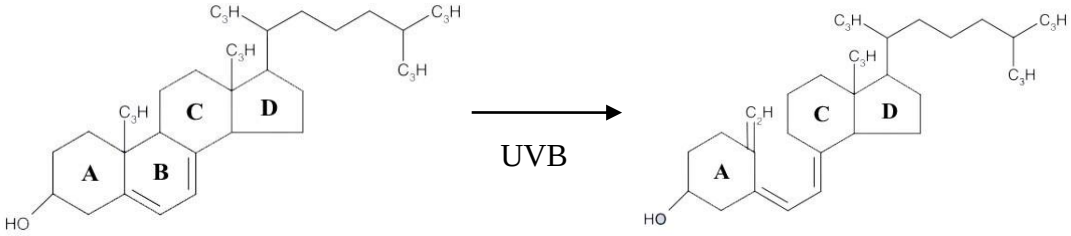
Vitamin D is a fat-soluble, steroid pre-hormone, which has long been known to be essential for bone health, and it is widely used as a food supplement for rickets and osteoporosis (Holick, 2007). However, in epidemiological studies, vitamin D deficiency has been shown to be associated with other diseases of various types, such as cardiovascular diseases (Pilz et al., 2016), cancer (Feldman et al., 2014), and diabetes (Afzal et al., 2013a; Khan et al., 2013; Song et al., 2013).

There are two types of vitamin D: vitamin D_2 and D_3. Vitamin D_2 is mainly synthesized by plants and fungi from ergosterol, and vitamin D_3 is synthesized from 7-dehydrocholesterol (7-DHC) in the human skin when it is exposed to sunlight, in particular ultraviolet B (UVB; 280–320 nm) (Webb et al., 1988; Holick, 2007), and also can be obtained from food of animal origin (Figure 1.1).



Ergosterol Vitamin D_2

Vitamin D_2 synthesis in plants and fungi



7-dehydrocholesterol Vitamin D_3

Vitamin D_3 synthesis in mammalian skin

Figure 1.1. The Synthesis of Vitamin D_2 and D_3

As noted, there are two main sources of vitamin D: dietary intake and endogenous, cutaneous synthesis (Holick et al., 2011). The food sources of vitamin D and the contents are listed in Table 1.1 (Holick, 2007). While it can be seen that oily fish and fortified foods provide ~500 units of vitamin D per day, this is much less than what we need and considerably less than what can be made in the skin as a result of 5-10 min of exposure to sunlight. It has been reported that skin can synthesis about 3,000 units (75 μg) per 0.5 minimal erythemal dose of UVB radiation, which would be the amount absorbed by the arms and legs by around 5 to 10 minutes of direct exposure (though this

varies with the time of day, season, latitude, and skin sensitivity) (Holick, 2007). Exposure to sunshine of 10~15 minutes everyday is enough for most healthy people to maintain vitamin D status (Macdonald, 2013). However, the efficiency of cutaneous vitamin D synthesis decreases as the process of ageing, and requires the presence of adequate amounts of the precursor (cholecalciferol) (Webb, 2006).

The Institute of Medicine (IOM) in the United States have given an "indicator" for the estimated average requirement (EAR, corresponding to the median intake needs of the population), recommended dietary allowance (RDA, covering the requirement of no less than 97.5% population, 2 standard deviation (SD) above the median needs), and tolerable upper intake level (UL). The recommendations are listed in Table 1.2 (Dietary reference intakes for calcium and vitamin D from the Institute of Medicine (IOM), US, 2011). It is noted that some groups, such as the elderly, renal failure patients and pregnant or lactating women require higher intakes (Hollis and Wagner, 2011).

Table 1.1. Food Sources and Content of Vitamin D (data from Holick, 2007)

Food Source	Typical Vitamin D content
Natural sources	
Fresh, wild salmon	600~1000 IU (15~25 μg) vitamin D_3/3.5 oz (99.2 g)
Fresh, farmed salmon	100~250 IU (2.5~6.25 μg) vitamin D_3 or D_2/3.5 oz (99.2 g)
Canned salmon	300~600 IU (7.5~15 μg) vitamin D_3/3.5 oz (99.2 g)
Canned sardines	~300 IU (7.5 μg) vitamin D_3/3.5 oz (99.2 g)
Canned tuna	~230 IU (5.75 μg) vitamin D_3/3.6 oz (102.1 g)
Canned mackerel	~250 IU (6.25 μg) vitamin D_3/3.5 oz (99.2 g)
Cod liver oil	400–1000 IU (10~25 μg) vitamin D_3/ 1 tsp
Fresh shiitake mushrooms	100 IU (2.5 μg) vitamin D_2/3.5 oz (99.2 g)
Sun-dried shiitake mushrooms	1600 IU (40 μg) vitamin D_2/3.5 oz (99.2 g)
Egg yolk	~20 IU (0.5 μg) vitamin D_3or D_2/3.5 oz (99.2 g)
Fortified foods	
Fortified milk	~100 IU (2.5 μg)/8 fluid oz (226.8 ml), usually vitamin D_3
Fortified orange juice	~100 IU (2.5 μg)/8 fluid oz (226.8 ml), usually vitamin D_3
Infant formulas	~100 IU (2.5 μg)/8 oz (226.8 g), usually vitamin D_3
Fortified yogurts	~100 IU (2.5 μg)/8 oz (226.8 g), usually vitamin D_3
Fortified butter	~50 IU (1.25 μg)/3.5 oz (99.2 g), usually vitamin D_3
Fortified margarine	~430 IU (10.75 μg)/3.5 oz (99.2 g), usually vitaminD_3
Fortified cheeses	~100 IU (2.5 μg)/3 oz (99.2 g), usually vitamin D_3
Fortified breakfast cereals	~100 IU (2.5 μg)/serving, usually vitamin D_3

Table 1.2. Vitamin D Dietary Reference Intakes Recommended by IOM by Life Stage (Ross et al., 2011)

Age group	EAR	RDA	UL
0~6 months	400 IU (10 μg)	600 IU (15 μg)	1000 IU (25 μg)
6~12 months	400 IU (10 μg)	600 IU (15 μg)	1500 IU (38 μg)
1~3 years	400 IU (10 μg)	600 IU (15 μg)	2500 IU (63 μg)
4~8 years	400 IU (10 μg)	600 IU (15 μg)	3000 IU (75 μg)
9~70 years	400 IU (10 μg)	600 IU (15 μg)	4000 IU (100 μg)*
>70 years	400 IU (10 μg)	800 IU (20 μg)	4000 IU (100 μg)

*This UL applies also to pregnant and lactating women.

The "indicator" given by IOM is useful in clinical application and experimental design. However, some studies suggested that the RDA may be too small, because people who are underlying conditions or have no or little exposure to sunlight may still be at risk of vitamin D deficiency, even though they take the RDA dose recommended by IOM (Holick, 2011; Bischoff-Ferrari et al., 2012; Spedding et al., 2013).

It is accepted that vitamin D supplements are generally safe. It is very unlikely that vitamin D toxicity can develop simply by sun exposure because of the protective adaption of the human body. For example, the melanin content of skin increases with sun exposure, blocking the sun rays, and conversion of precursor is less efficient when exposure is high (Wagner et al., 2002). Also, dietary intake from food is unlikely to increase vitamin D to toxic levels because most foods do not contain high amounts. Vitamin D toxicity can occur, but is rare, and is usually caused by chronic over intake

of vitamin D supplements without clear diagnosis of vitamin D insufficiency, or giving supplements to children, who need less vitamin D and show lower UL values than adults. When the plasma 25(OH)D level is higher than 375 nmol/l (150 ng/ml), it is very likely to show clinical signs of vitamin D toxicity (Ozkan, 2012; Alshahrani and Aljohani, 2013; Kaur et al., 2015).

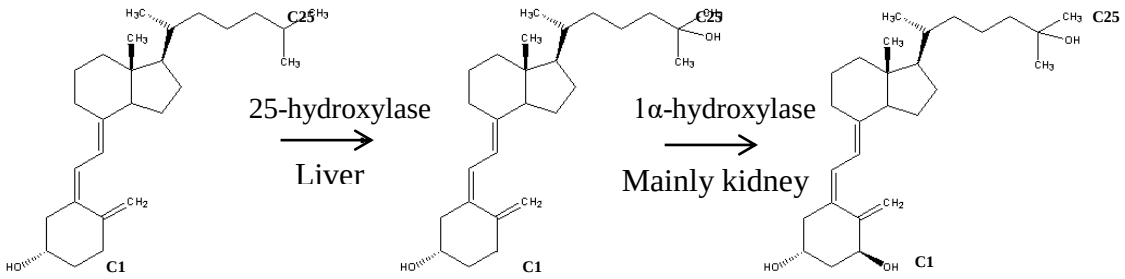
Vitamin D toxicity has serious consequences due to hypercalcaemia and subsequent hypercalcuria and nephrocalcinosis, with the symptoms of nausea and vomiting, dehydration and fever; even more seriously, it may cause bradycardia and hypertension (Kaur et al., 2015). However, the risk of death from vitamin D overdose is low. The mean lethal dose (LD_{50}) of vitamin D in humans is reported as 21 mg/kg (840,000 IU/kg), based on extrapolation from results from animal studies (Ozkan, 2012). To avoid vitamin D toxicity, monitoring of vitamin D level is critical in people taking vitamin D supplements as regular treatment or supplement.

VITAMIN D METABOLISM AND ACTION

Vitamin D metabolism

Vitamin D, from both dietary intake and cutaneous synthesis, is transported via blood to the liver, where vitamin D is hydroxylated by vitamin D-25-hydroxylase to 25-hydroxyvitamin D (25(OH)D), also known as calcidiol (Christakos et al., 2015). Most of the 25(OH)D is transported to the kidney by vitamin D binding protein (DBP) and further metabolized to the vitamin D active form 1, 25-dihydroxyvitamin D ($1,25(OH)_2D$, calcitriol) by 1α-hydroxylase (Figure 1.2). It is noted that not all of the

25(OH)D is hydroxylated in the kidney, some of the 25(OH)D is delivered to the target cells and transformed to the active form locally (Bikle, 2014). 1,25$(OH)_2$D metabolism is modulated mainly by parathyroid hormone (PTH), fibroblast-like growth factor-23 (FGF23) and 1,25$(OH)_2$D itself (Christakos et al., 2015).



Vitamin D_3 25(OH)D_3 1,25$(OH)_2D_3$

Figure 1.2. Transformation of Vitamin D_3 to 25(OH)D_3 and 1,25$(OH)_2D_3$

The metabolism of vitamin D is regulated by various cytochrome P450 enzymes (CYPs). CYP2R1 is the major enzyme which 25-hydroxylates both vitamin D_2 and D_3 in the liver (Cheng et al., 2004), but other enzymes also have 25-hydroxylation activity, for example, CYP3A4 also acts as an enzyme to convert vitamin D to 25(OH)D (Bikle, 2014). In CYP2R1 knockout mice, the serum 25(OH)D concentration was decreased by ~50%, compared with the 25(OH)D level of wild type mice, but the 25(OH)D level in the knockout mice was not decreased to zero, indicatint that CYP2R1 is not the sole enzyme to convert vitamin D to 25(OH)D (Zhu et al., 2013). For the 1α-hydroxylation of 25(OH)D, CYP27B1 is the sole enzyme for this transformation. The majoriy of CYP27B1 works in the kidney, but some 1,25$(OH)_2$D target cells, i.e. bone cells, immune cells and endocrine cells, also have CYP27B1, and the 25(OH)D can be

converted to 1,25$(OH)_2$D locally. The renal CYP27B1 activity is regulated by PTH (stimulation) and by FGF23 and 1,25$(OH)_2$D (inhibition) (Christakos et al., 2015). However, the regulation of CYP27B1 in non-renal cells is still unclear, and it was suggested that the mechanism is different from the renal CYP27B1. In a cell culture study, high calcium in human osteoblasts increased the transcription of CYP27B1 mRNA, while no significant impact was seen by PTH and FGF23 (van der Meijden et al., 2016).

Vitamin D binding protein (DBP)

Vitamin D binding protein (DBP) binds to vitamin D, 25(OH)D, and 1,25$(OH)_2$D, and its binding is very important for the stabilization of vitamin D and its metabolites in circulation. It is noted that although DBP binds to all members of vitamin D, the affinities are different. For vitamin D, the affinity of DBP to vitamin D_3 is stronger than that to vitamin D_2, thus, the clearance of vitamin D_2 is faster (Bikle, 2014). Regarding to vitamin D metabolites, the affinity to 1,25$(OH)_2$D is ~20 times lower than that of 25(OH)D (Christakos et al., 2015), however, the binding of 1,25$(OH)_2$D still increases the difficulty in 1,25$(OH)_2$D measurement (HeijBoer et al., 2012). With or without DBP binding makes no difference to the biological function of vitamin D, although the DBP binding indeed increases the stability of vitamin D (Bhan, 2014). However, it is noted that lower DBP expression does not always associate with worse vitamin D function. A study on vitamin D status and vitamin D activity on people with different skin tone showed that even though people with darker skin had lower plasma 25(OH)D level, but their lower DBP expression helps to increase the bioavailability of vitamin D, suggesting that the level of DBP may also be a factor that should be taken into consideration when assessing the vitamin D status (Powe et al., 2014).

Vitamin D action

Vitamin D binds to a specific receptor, the vitamin D receptor (VDR), which is found in many different cell types in the body (Table 1.3, Ross et al., 2011; Christakos et al., 2015).

Table 1.3. The Distribution of Vitamin D Receptor (Ross et al., 2011)

System	Tissues/cells
Musculoskeletal system	osteoblasts, chondrocytes, striated muscle
Cardiovascular system	cardiomyocytes, smooth muscle cells
Endocrine system	thyroid C-cells, parathyroid glands, islets of langerhans
Gastrointestinal system	stomach, oesophagus, intestine, liver
Immune System	thymus, T and B lymphocytes, bone marrow
Renal system	ascending portion of loop of Henle

The activated VDR (activated by calcitriol binding) interacts with the retinoid X receptor (RXR) to form a liganded VDR-RXR heterodimer, which recognizes vitamin D responsive elements (VDREs) in DNA, influencing the transcription of target genes, for example, Transient Receptor Potential Vanilloid 6 (TRPV6), PTH and osteocalcin (Haussler et al., 2012; Carvallo et al., 2013; Chow et al., 2013; Bikle, 2014). This interaction is controlled by $1,25(OH)_2D$ directly. For example, TRPV6 acts as an ion channel and plays an important role in absorbing calcium in the small intestine. The gene of TRPV6 also contains several VDREs. The level of $1,25(OH)_2D$ influences the

calcium absorption via TRPV6 in part (Haussler et al., 2012). The metabolism and action of vitamin D is summarized in Figure 1.3.

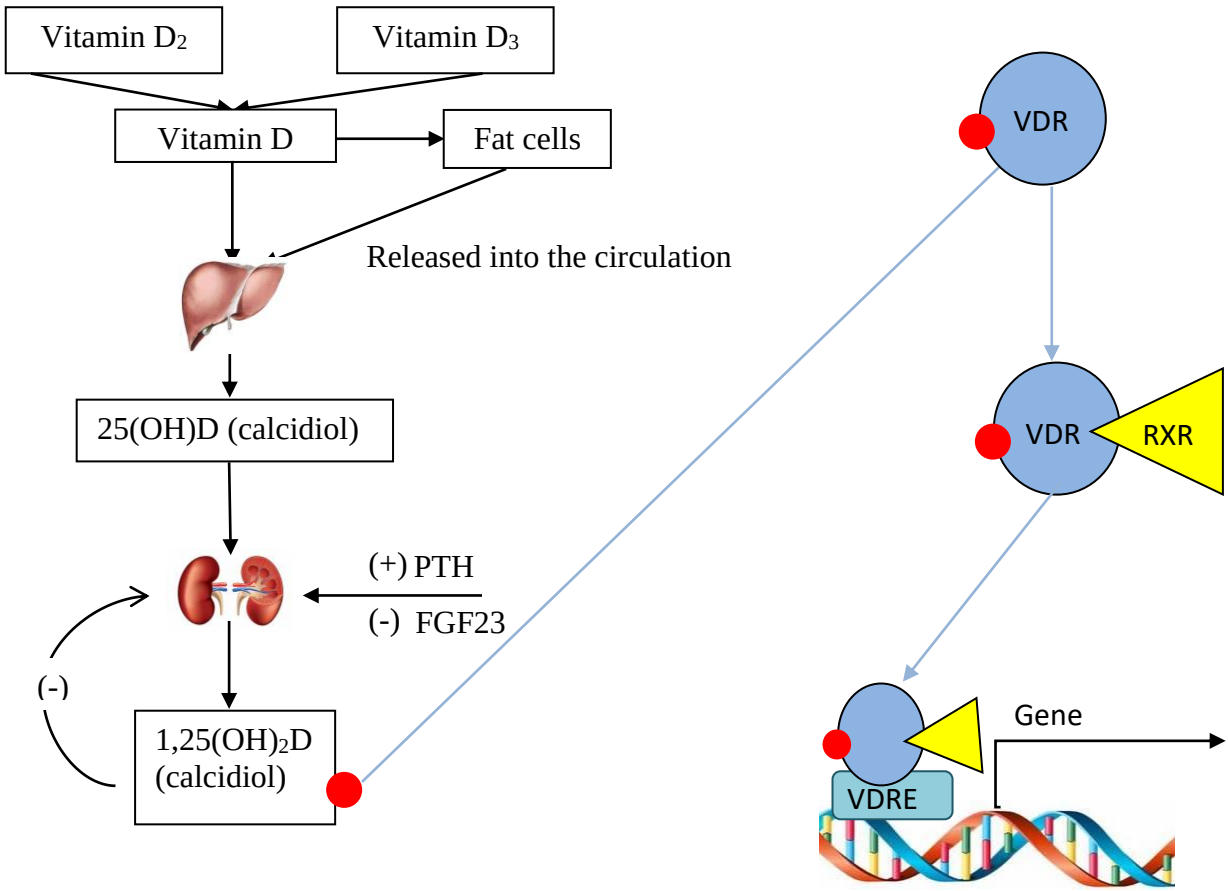


Figure 1.3. The Metabolism and Action of Vitamin D

The vitamin D status is tightly associated with the normal function of VDR, and its deficiency is linked to increase of PTH, decrease of calcium and phosphorus, and many long-term conditions, such as rickets, osteoporosis, cardiovascular disease, diavetes, hypertension, and cancer (Holick, 2007; Holick, 2011; Wang et al., 2012; Pludowski et al., 2013; Feldman et al., 2014; Krivošíková et al., 2015). Thus, the status of vitamin D is important for health maintenance.

DEFINITION OF VITAMIN D STATUS AND ITS MEASUREMENT

Definition of vitamin D status

The vitamin D status is defined by the plasma or serum 25(OH)D concentration rather than the concentration of the active form $1,25(OH)_2D$ (Holick et al., 2011; Ross et al., 2011). 25(OH)D has a longer half-life of between 10 and 19 days, as opposed to $1,25(OH)_2D$, which has a half-life of only ~15h or less (Schwalfenberg, 2007). $1,25(OH)_2D$ is not widely used as an indicator of vitamin D status, and there is little epidemiological evidence in defining clear cut-off point of vitamin D deficiency, insufficiency and sufficiency based on plasma/serum $1,25(OH)_2D$ concentration. Besides, the techniques for $1,25(OH)_2D$ measurement is very demanding. The tiny amount (in pmol/l level compared with nmol/l of 25(OH)D) in plasma and the involvement of vitamin D binding protein make the accurate measurement of $1,25(OH)_2D$ very difficult (Bikle, 2014). More importantly, as presented above, all vitamin D is metabolized to 25(OH)D in the liver, and part of the 25(OH)D in circulation is further metabolized to $1,25(OH)_2D$ in the kidney, and some of the 25(OH)D is converted to $1,25(OH)_2D$ in various cells locally, therefore, the $1,25(OH)_2D$ in plasma/serum does not reflect the vitamin D levels in the target cells, such as in osteoblasts (van der Meijden et al., 2016).

Vitamin D status varies a lot depending on geography, culture and lifestyle, thus, unlike most clinical standards, vitamin D deficiency/insufficiency/sufficiency is defined by "health based" but not "population based" data (i.e. the 95 percentile value of healthy people). There is no common agreement on the definition of vitamin D status categories, but it is generally accepted that plasma/serum 25(OH)D <25 nmol/l reflects

severe deficiency, and 25(OH)D >75 nmol/l is sufficiency (Holick et al., 2011; Ross et al., 2011). However, the in-between range is less well agreed upon (Figure 1.4). A report from Institute of Medicine (IOM) in US claimed that 50 nmol/l 25(OH)D can be used as the cut-off of vitamin D sufficiency (Ross et al., 2011). The threshold suggested by the Endocrine Society in US for sufficiency was 75 nmol/l, regarding to the beneficial role of vitamin D on PTH levels and bone health and calcium absorption (Holick et al., 2011).

Figure 1.4. Vitamin D Status Spectrum

The range between 25 nmol/l and 75 nmol/l is a 'grey' area. Some agencies (such as IOM) regard 50 nmol/l as the threshold of sufficiency. The range ≥25<50 nmol/l is identified as insufficiency range by IOM, but this range is defined as deficiency in guidelines from some others (such as the Endocrine Society). The range of ≥50<75 nmol/l is defined as sufficiency by IOM, but as insufficiency by the Endocrine Society (Holick et al., 2011; Ross et al., 2011).

From human studies, what is an 'optimal' Vitamin D status may be disease-specific. In regard to bone health, an inverse association was found between bone mineral density and vitamin D status from a meta-analysis involving 12 randomized, placebo-controlled clinical trials, and serum 25(OH)D at the range of 75-110 nmol/l was recommended by the author for people aged 65 or more to keep bone health and lower the risk of fracture (Bischoff-Ferrari et al., 2012). A significant inverse association between vitamin D

status and PTH levels was widely found in human studies, and PTH levels increase when 25(OH)D is lower than 75-80 nmol/l (Amini et al., 2013; Karefylakis et al., 2013). For non-skeletal health, relatively higher vitamin D status is also recommended, but because of the lack of well designed, large supplementation trials, optimal vitamin D status for maintaining non-skeletal health is not yet clear. A supplementation study on diabetic patients found that significant improvement of insulin sensitivity occurred only 6 months after supplementation, even though the subjects reached serum 25(OH)D of 80 nmol/l after 3 months' supplementation. The author concluded that the improvement in insulin sensitivity needs a more sustained period of high vitamin D status, and optimal serum 25(OH)D concentration for reducing the risk of insulin resistance was 80-119 nmol/l, suggesting that 25(OH)D of ≥80 nmol/l may be a recommended standard of vitamin D sufficiency (von Hurst et al., 2010). Apart from diabetes, a review recommended the minimum effective doses of 75, 80, 95 and 100 nmol/l were desirable for prevention of, respectively, premature mortality, CVD, respiratory infections and depression, and cancer (Spedding et al., 2013).

25(OH)D measurement

As noted above, plasma 25(OH)D in humans is in the nanomolar range, and a highly sensitive analytical method is required. There are various types of immunoassay for 25(OH)D, such as radioimmunoassay and chemiluminescent immunoassay, however, 25(OH)D is strongly hydrophobic and is largely (85-90%) bound to DBP within the plasma (Farrell et al., 2012). Thus, the antibodies used in immunoassay have difficulty binding to the DBP-25(OH)D complex. Furthermore, the specificity of some of the antibodies used in current 25(OH)D immunoassays is low, and 25(OH)D_2 and D_3 cannot be distinguished as the antibody used is not specific enough (Cashman and Kiely,

2008; Hedman et al., 2014). High performance liquid chromatography (HPLC) combined with tandem mass spectrometry (LC-MS/MS) offers high sensitivity as well the required high level of specificity. The LC-MS/MS approach is regarded as the "gold standard" in 25(OH)D measurement, because it is more accurate and reliable than immunoassay (El-Khoury et al., 2011; Farrell et al., 2012). In a study that compared accuracy of 203 plasma and serum samples measured by five commercial automated immunoassay methods including Architect i2000SR, Centaur XP, iSYS, Liaison and Modular Analytics E170 and radioimmunoassay method against the reference LC-MS/MS method, significant deviations were found between automated methods and RIA from the LC-MS/MS method. The difference from the LC-MS/MS method was large in some methods, results from Centaur XP were generally ~50% lower compared with the LC-MS/MS results, and RIA method was ~20% lower, indicating a large variance caused by method used (HeijBoer et al., 2012). However, even the same method was applied, the results from different laboratories with the same mehtod also showed significant difference across centres (Black et al., 2015). Thus, proper attention to quality control must be taken in 25(OH)D measurement.

LC-MS/MS measures vitamin D concentration in plasma, serum and other biological fluids, such as milk. However, the lipophilic nature and lack of easily chargeable groups in vitamin D make the measurement complex, and sample pretreatment is required before the chromatographic separation, for example, release 25(OH)D from binding proteins in the biological sample, and to extract the lipophilic 25(OH)D into an organic solvent. In order to correct for the loss of 25(OH)D during the sample workup, internal standard (IS) such as 3-epi-25-Hydroxyvitamin D_3 should be introduced and this improves the accuracy of results. The IS is added in a known amount to every sample,

control and calibrator before sample pre-treatment begins and the ratio of sample 25(OH)D to IS is calculated and used to obtain the corrected 25(OH)D result from the LC-MS/MS data (Ong et al., 2012).

WORLDWIDE VITAMIN D STATUS

Even though there is still no common agreement on the definition of vitamin D status cut-off points, vitamin D status is generally poor in the various regions of the world. A systematic review that involved over 168,000 subjects from 44 countries showed that the worldwide vitamin D status varies a lot (Hilger et al., 2014). From studies involved in this systematic review, mean value of 25(OH)D concentration ranged from 4.9 to 136.2 nmol/l. No significant gender difference was found in this study, even though females in Asia/Pacific and Middle East/Africa tend to have lower vitamin D status (Hilger et al., 2014). Low vitamin D status was found to be very common. Among the subjects studied, 88.1% of the studied samples had 25(OH)D concentration <75 nmol/l, 37.3% had levels <50 nmol/l and 6.7% were <25 nmol/l (Hilger et al., 2014). More worryingly, vitamin D status was found to be relatively lower than before (Dawson-Hughes et al.,1997; Shea et al., 2009). In the US, Dawson-Hughes *et al.* in 1997 reported that the mean 25(OH)D value of 209 elderly people who were living in Boston (42 ^{o}N) was 68.9 nmol/l (Dawson-Hughes et al.,1997). However, in 2009, another study which was conducted in the same area, 919 elderly people were found to have mean 25(OH)D concentration of 49.2 nmol/l (Shea et al., 2009). The mean 25(OH)D had decreased by about 20 nmol/l in elderly people who were living in the same place.

VITAMIN D DEFICIENCY: WHY DOES IT OCCUR?

Vitamin D deficiency is associated with many factors, including poor dietary intake, geographical latitude, and lack of exposure to sunlight. The main source of vitamin D is sun-exposed skin. However, the modern lifestyle is characterized by more time indoors rather than activity outside. This increases the chances of vitamin D deficiency developing. However, deficiency can be severe and yet no clear clinical signs or symptoms of disease show. In this section, the factors that can lead to vitamin D deficiency are discussed briefly.

Poor dietary intake

Compared with the amount of vitamin D from cutaneous synthesis, the amount from dietary intake is relatively low. Nonetheless, diet is an important source of vitamin D, especially for those who are not able to receive sunshine exposure or living in high latitude during the winter time (Holick, 2007). Even though fungi is a source of vitamin D_2, dietary vitamin D mainly comes from oily fish and fortified dairy products (Table 1.1). Therefore, a strict vegetarian diet is more likely to lead to vitamin D deficiency. In a study conducted by Lamberg-Allardt et al. (1993), it was found that compared with omnivorous controls, dietary vitamin D intake was significantly lower in strict vegetarians and lacto-vegetarians ($p < 0.001$), and the vitamin D status was found to be significantly lower ($p < 0.05$) in both strict and lacto- vegetarians, mean(SD) serum concentrations were 27(19) and 29(16) nmol/l, compared with the 47(15) nmol/l in omnivorous controls (Lamberg-Allardt et al.,1993).

Lack of vitamin D fortification of foods also increases the risk of vitamin D deficiency, especially in winter in high latitude. In Denmark, fortified milk and bread was found to lower the risk of vitamin D deficiency (Madsen et al., 2013). In this study, 355 participants from 96 families received fortified milk and bread while 371 participants from 99 families received non-fortified food. Median vitamin D intakes were 376 IU/d and 88 IU/d in fortified group and control group, respectively. The vitamin D status in the fortified group was significantly higher than the control group after a 6 months' intervention, with mean values of 67.6 and 41.7 nmol/l in fortified group and control group respectively ($p<0.001$) (Madsen et al., 2013). In a German study, vitamin D fortified milk was also found to be effective in preventing vitamin D deficiency during the winter time in children (Hower et al., 2013). The median 25(OH)D in the children (n=39) taking fortified milk was 62 nmol/l, compared with 34 nmol/l in the control group in late February. Besides, fortification of 2.85 μg/100 ml in milk was shown to be safe even during the summer time, as the 25(OH)D concentration remained in the normal range (ranged 47-101 nmol/l) (Hower et al., 2013).

Latitude and season

UVB radiation is necessary for vitamin D synthesis, and its intensity impacts on vitamin D synthesis efficiency directly. The amount of photons (the energy of the sunlight) reaching the Earth's surface is greatly affected by the angle at which the sun's rays reaches the Earth. The solar radiation has a relatively short path length through the atmosphere at smaller solar zenith angle (SZA), which is equatorial region of the Earth (Figure 1.5). At this region the photonic energy, which is essential for cutaneous vitamin D synthesis, is more concentrated because of less attenuation. In contrast, UV light is more attenuated at higher latitudes and can easily become insufficient for

vitamin D synthesis (Webb, 2006). It was found that every degree increase in latitude was associated with 1.0 nmol/l serum 25(OH)D decrease (van der Mei et al., 2007).

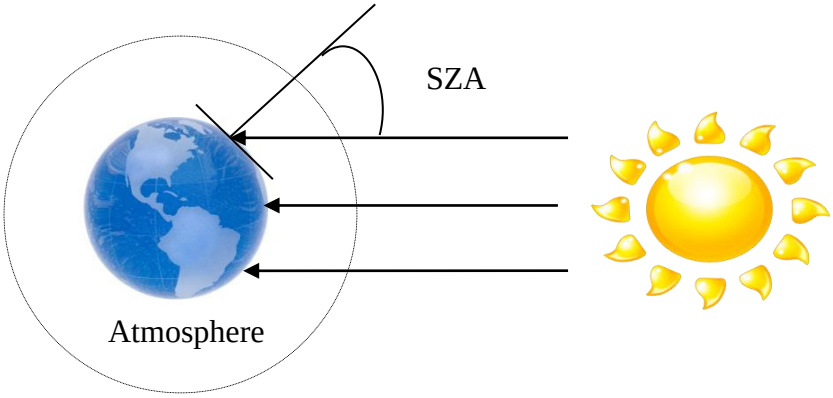


Figure 1.5. The SZAs and the Pathlength through the Atmosphere

Higher latitude is also associated with lower effective UV radiation. UV radiation with wavelength 280–320 nm is the most effective for vitamin D synthesis (Webb et al., 1988), and the efficiency rapidly decreases when the wavelength increases to 315 nm. The vitamin D synthesis available UV light has no significant difference across seasons in regions near the quator. However, in regions located at 40^{o}N, little or no vitamin D effective UV radiation is available in December and January, although the effective UV radiation is similar level with that at the equator during the summer time (June to August). For the regions located at 90 ^{o}N, no vitamin D effective radiation can be detected for most of the year (from September to next May) (Kimlin, 2008). Furthermore, because of lack of sunlight during winter in some regions of the world, those who live at north or south latitudes of 51^{o} or higher will not be able to synthesize vitamin D during wintertime (Engelsen et al., 2005).

Season is another factor that influence the vitamin D status. Two large observational studies found consistently lower vitamin D status in winter in US and Australia. In US, data from 18,875 subjects showed that the people recruited during November to March (winter) had significantly lower 25(OH)D concentration than those who were recruited from April to October (summer). This finding was consistently found in all race groups, including non-Hispanic whites, non-Hispanic blacks and Mexican Americans (Looker et al., 2007). In Australia, Boyages and Bilinski found that among 24,819 subjects recruited, vitamin D status was seen to be highest in the subjects recruited in January (summer) and lowest in those recruited in September (winter), consistently in both females and males, in any age groups (Boyages and Bilinski, 2012).

As mentioned above, during wintertime, less energy from sunshine can be used because of the relatively larger SZA. Besides, less vitamin D effective UV radiation is available in winter, especially in high latitude regions, and the effect of season on vitamin D status found to be larger in high latitude regions (Kimlin et al., 2007). Climate and weather is another important factor associated with lower vitamin D status during winter time. The heavy cloud, which is more common in winter time, blocks the UV light (Engelsen, 2010). The UVB radiation was found to be decreased by ~14% in partly cloudy weather (50% cloud) and by ~70% in cloudy weather (100% cloud) (Kimlin, 2008). The heavier clothes worn in cold weather in winter is also a risk factor of low vitamin D status. Cold air drives people to wear more clothes and covering more body parts in winter, reducing the available skin area for vitamin D synthesis (Macdonald, 2013).

Figure 1.6 shows an estimated time of sunshine exposure to obtain sufficient vitamin D. The red areas reflect when and which latitude that no available UVB for cutaneous vitamin D synthesis, dietary vitamin D and/or supplementation are needed for vitamin D maintenance. In the black area, sufficient vitamin D can be obtained in a few minutes of exposure to sunshine (Webb and Engelsen, 2006). However, it is noted that this figure is not the "golden rule" for everyone. This guideline was estimated under the condition that people have light skin colour, expose ~10% of body (face, neck and hands) directly during noon with clear sky (Webb and Engelsen, 2006). For those who have darker skin or on a cloudy day, the time of UV exposure has to be extended. Besides, if time spent under sunlight is in early morning or towards nightfall, more UV exposure time is also needed (Macdonald, 2013).

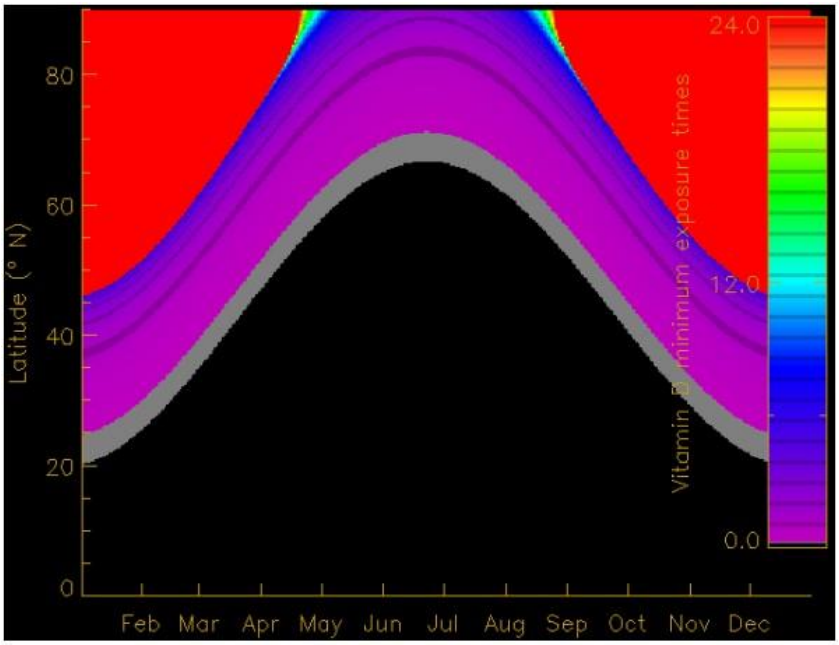


Figure 1.6. Estimated UV Exposure Time Depending on Latitude and Season (from Webb and Engelsen, 2006)

It is noted that in some studies, the vitamin D status does not reflect the impact of latitude. For example, a multi-centre (Urumqi (located 43^{o}N), Beijing (40^{o}N), Dalian (39^{o}N), Hangzhou (30^{o}N) and Guangzhou (23^{o}N)) study in China found that the vitamin D status in these five cities did not correlate with the corresponding latitude. The highest mean 25(OH)D concentration was found in people living in Dalian (55 nmol/l), which is at a higher latitude than Guangzhou (54 nmol/l) and Hangzhou (48 nmol/l). People in Beijing has the lowest mean 25(OH)D concentrations, 41 nmol/l. The mean 25(OH)D concentration of people in Beijing is ~25% lower than that in Dalian, which has similar latitude with Beijing (Yu et al., 2015). It is noted that Beijing is highly air polluted, and the finding from this study suggests a possibility that the air pollution, which blocks UVB, may be a risk factor of vitamin D deficiency.

Obesity

In a meta-analysis that involved 34 studies with a representative adult population (n= 17,135), a significant inverse association between serum 25(OH)D levels and body mass index (BMI) was found, and this association was consistently significant in both men and women (Saneei et al., 2013). Another meta-analysis of 21 studies with children, adolescents, adults and elderly also found 35% higher prevalence of vitamin D deficiency in obese people, compared with the normal BMI controls ($p<0.05$) (Pereira-Santos et al., 2015).

The link between vitamin D deficiency and obesity can be explained by a lower response to UV radiation as well as oral vitamin D intake (Wortsman et al., 2000). Obese children had significantly lower baseline ($p<0.05$) vitamin D status compared with the normal controls. After receiving the same dose of UVB (290~320) light (27-

mJ/cm^2 suberythemic dose), the improvement of 25(OH)D concentration after 24 hours was also lower in the obese group, the mean value was 17.4 nmol/l, compared with 38.3 nmol/l in the normal group ($p<0.01$). In vitamin D supplementation trials, obese children also had significantly lower vitamin D status 24 hours after taking 50,000 IU (1.25 mg) vitamin D_2, the mean values were 50 and 85 nmol/l, in obese and control children respectively, $p < 0.05$ (Wortsman et al., 2000). A supplementation study found that serum 25(OH)D was inversely, but weakly associated with total body fat mass at baseline, $r=0.19$; $p < 0.0001$, and people with smaller BMI had better response to high and medium doses of vitamin D (3200-4800 IU and 1600-2400 IU per day), however, the response to the low vitamin D supplementation group (400, 800 IU per day) was not significantly associated with BMI (Gallagher et al., 2013).

These studies indicate that obese people have higher risk of developing vitamin D deficiency, and their responses to both dietary intake and UV radiation are poorer than people with normal BMI. This is usually explained by the sequestration in adipose tissue of vitamin D (Saneei et al., 2013). The association between higher BMI and lower vitamin D status may also be associated with the lifestyle. Insufficient sunshine exposure of some obese people is also associated with their limited mobility and lack of outdoor activities (Brock et al., 2010; Saneei et al., 2013).

Dark skin

A meta-analysis found that the 77% of the immigrants with dark skin had 25(OH)D <50 nmol/l, which is much higher than the overall global prevalence (37% have 25(OH)D <50 nmol/l). Most of the subjects involved were immigrated to Europe, North America or Austrilia (regions with relatively higher latitude) (Hilger et al., 2014; Martin et al.,

2016). People with dark skin need 3-5 times more exposure to sunlight to produce the same amount of vitamin D, because of the natural protection afforded by melanin (Nair and Maseeh, 2012). An intervention study found that vitamin D synthesis efficiency is highly dependent on the degree of skin pigmentation. After receiving a single total body UVB exposure (22 mJ/cm^2), 25(OH)D levels were significantly ($p<0.0001$) increased in fair-skinned volunteers (n=20) one day after UV radiation treatment, while there was no significant change in black-skinned people (n=11) (Libon et al., 2013). Another study with 69 volunteers of different skin colour (skin lightness was assessed by reflective meter) also found that the response of 25(OH)D to UVB light was significantly associated with skin lightness ($p<0.05$), and this improvement was found to be independent from their baseline vitamin D status (Armas et al., 2007). The low efficiency of cutaneous vitamin D synthesis in darker skinned people is associated with the lower response to UVB light. The melanin in skin absorbs UVB and thus less UVB is available for vitamin D synthesis (Webb, 2006). Type 1 skin is the lightest skin tone, it is very sensitive to UVB radiation, no matter for sunshine burn or vitamin D synthesis. In a US study, the standard vitamin D dose (SDD, corresponding to the UV equivalent of an oral dose of ~1000 IU vitamin D) was found to be induced by only 37.2 J/m^2 for minimum 16 minutes radiation for type I skinned people. However, under the same condition, for people with the darkest skin (type VI skin), this dose was increased to 185.1 J/m^2 for a minimum of 83 minutes (Fitzpatrick,1988). The huge difference in the UVB response leads to a much higher risk for people with dark skin for vitamin D deficiency, especially for the immigrants to regions located at higher latitudes. Higher dose of vitamin D intake to maintain normal vitamin D status is recommended to people with darker skins (Hall et al., 2010).

Disease

Bowel disease

Vitamin D status was found to be significantly lower in inflammatory bowel disease patients and it was inversely associated with the severity of the disease (Ulitsky et al., 2011; Del Pinto et al., 2015). The mechanism by which bowel diseases causes low vitamin D status is not well explained, however, it is widely accepted that it was caused by low absorption of vitamin D from dietary intake. However, considering the small contribution of dietary intake to total vitamin D status, this link was also considered as the results of both air pollution and low outdoor activities on both vitamin D deficiency and bowel disease (Del Pinto et al., 2015).

Liver disease

Lower vitamin D status was found in various kinds of liver disease, including cholestatic hepatitis, non-alcoholic fatty liver disease, and alcoholic liver disease (Eliades et al., 2013; Stokes et al., 2013; Trépo et al., 2014). The response to vitamin D supplementation was also found to be poorer in children with cholestatic hepatitis (Heubi et al.,1989). The liver is the only organ that hydroxylates vitamin D to 25(OH)D, thus impaired liver function leads to low vitamin D hydroxylation and consequently lower 25(OH)D (Stokes et al., 2013). However, for vitamin D status maintenance, the liver plays a more important role, not just limited to the metabolism of vitamin D. Less bile salts are produced in cholestatic liver diseases patients, leading to poorer vitamin D absorption (Stokes et al., 2013). In addition, the liver is an important site for protein synthesis, including vitamin D binding protein. As noted before, vitamin D was significantly associated with serum albumin levels and vitamin D binding to protein increased the stability of vitamin D, 25(OH)D and $1,25(OH)_2D$ (Gallieni et al.,

2009). Damaged liver function leads to lower protein production and insufficient vitamin D binding protein, resulting to low vitamin D status (Gallieni et al., 2009).

Kidney disease

The kidney is the main function site of the 1α-hydroxylase, which is responsible for production of the majority of circulating $1,25(OH)_2D$ from 25(OH)D. For patients with kidney diseases, their 25(OH)D concentrations can be maintained to the same or even higher levels than healthy people, however, kidney disease strongly influences the bioavailability of vitamin D, even under normal 25(OH)D status (Holick, 2007). Besides, the inhibitor of 1α-hydroxylase, FGF23, increases in patients with chronic kidney disease, also leads to less $1,25(OH)_2D$ production (Quarles, 2012). Thus, the impact of kidney disease is not directly to the indicator of vitamin D status, 25(OH)D, but more to the activity of vitamin D – the concentration of $1,25(OH)_2D$.

Others

Vitamin D deficiency can develop also because of low sun exposure in certain religious or cultural groups, especially in women, even when the amount of sunlight reaching the Earth's surface is high (Al-Mogbel, 2012). Ageing is another factor associated with vitamin D deficiency. The ability to make vitamin D in the skin decreases with age, because of the decrease of 7-DHC (the precursor of vitamin D_3) (Webb, 2006). Furthermore, people often have less outdoor activity and be more likely to be hospitalized as the process of ageing. These factors further enhance the risk of vitamin D deficiency (Van Schoor et al., 2014). The application of sunscreen would also lower the ability of vitamin D synthesis. Sunscreen of sun protect factor (SPF) 8 applied to skin with 2 mg/cm^2 blocks 97.5% UVB, and the block efficiency increases with the SPF

(Matsuoka et al., 1987). The increasing awareness of the skin damage and ageing caused by sunshine also associates with avoidance to sunshine exposure, leading to the compromised vitamin D status.

In summary, there are various environmental, cultural, lifestyle, aged and disease-related factors leading to vitamin D deficiency, however, it is noted that for most people, the most important factor leading to vitamin D deficiency is insufficient sunshine exposure. In many countries, such as Canada and US, there is mandatory vitamin D food fortification, aimed to decrease the risk of vitamin D deficiency, especially during the winter time (Madsen et al., 2013; Cashman, 2015). However, it is noted that there is no such compulsory vitamin D food fortification programme in Hong Kong.

NON-COMMUNICABLE DISEASE (NCD)

The global burden of NCDs

NCD is the leading cause of death globally, and ~65% of mortality is caused by NCDs worldwide (World Health Organization (WHO), Noncommunicable disease fact sheet, data accessed in June, 2016). Among the deaths caused by NCD, 80% are in low- or middle-income countries, causing a great medical and financial burden to those who are least able to afford it (Beaglehole et al., 2011). From the data provided by the WHO, 36 million people died from NCDs each year, and among them, cardiovascular disease (CVD) is the most frequent cause of death (17.5 million, 48%), followed by cancers

(7.6 million, 21%), respiratory disease (4.2 million, 12%) and diabetes (1.3 million, 4%) (WHO, Global Health Observatory (GHO) data, data accessed in June, 2016).

Apart from high mortality, NCDs also cause heavy financial burdens. In the World Economic Forum, the rise of NCDs was regarded as one factor which could amplify the global economic risk (World Economic Forum, the Global Risks Report 2016). In more detail, the estimated cost for new cancer cases in 2010 was 289,737 million US dollars, including 153,697 million dollars in medical costs, 67,072 million dollars in non-medical costs, and 68,969 million dollars in income losses; for CVD, the global cost in 2010 was 863 billion US dollars, 474 billion dollars were direct medical care cost and 389 billion dollars was for the productivity loss; for diabetes, the global cost in 2010 was ~500 billion US dollars. In total, the economic burden of life lost caused by NCDs was estimated to be 22.8 trillion US dollars in 2010. More worryingly, the increase of NCDs also causes rapid increase in the economic burden. It was estimated that in 2030, the overall cost for new cancer cases would increase to 457,857 million US dollars, with 218,322 million dollars in medical costs, 94,658 million dollars in non-medical costs and 144,876 in income losses. As for CVD, the global cost was estimated to increase by ~20% to 1,040 billion US dollars, and this figure was increased to 745 billion dollars for diabetes. The total economic burden of life lost caused by NCDs was estimated to be 43.3 trillion US dollars (Bloom et al., 2012).

The development of NCDs is highly associated with age, under the stress of smoking, high diet salt, alcohol over-consumption, obesity and physical inactivity (Figure 1.7), but it is also noted that nowadays, NCD is not just prevalent in elderly people. Around 30% of the deaths caused by NCDs occured in people <60 years (Beaglehole et al.,

2011). As the trend of ageing in populations occurs all over the world, the burden brought by NCDs is also increasing. Lifestyle and behavioural factors from early adult life are important determinants of long-term health in a later stage of life. For example, results of a prospective study of 3,154 men and women, free of CVD and aged 18-30 years at entry, showed that maintenance of a healthy lifestyle is associated with markedly lower CVD risk after 20 years of follow-up (Liu et al., 2011). Five 'healthy lifestyle' factors were recorded within the group at entry, and also after 7 and 20 years. These factors were normal body weight (BMI $<25 kg/m^2$), no or moderate alcohol intake, higher healthy diet score, higher physical activity score and being a non-smoker. A low CVD risk profile (defined as absence of CVD or diabetes, desirable total cholesterol and blood pressure values without the aid of medication, and non-smoking status) in year 20 was highest in those with most healthy lifestyle factors. In those with 0-1, 2, 3, 4, 5 healthy lifestyle factors (averaging baseline, year 7 and year 20 data) the prevalence of low CVD risk profile was, 3.0%, 14.6%, 29.5%. 39.2% and 60.7%, respectively (p for trend <0.001) (Liu et al., 2011).

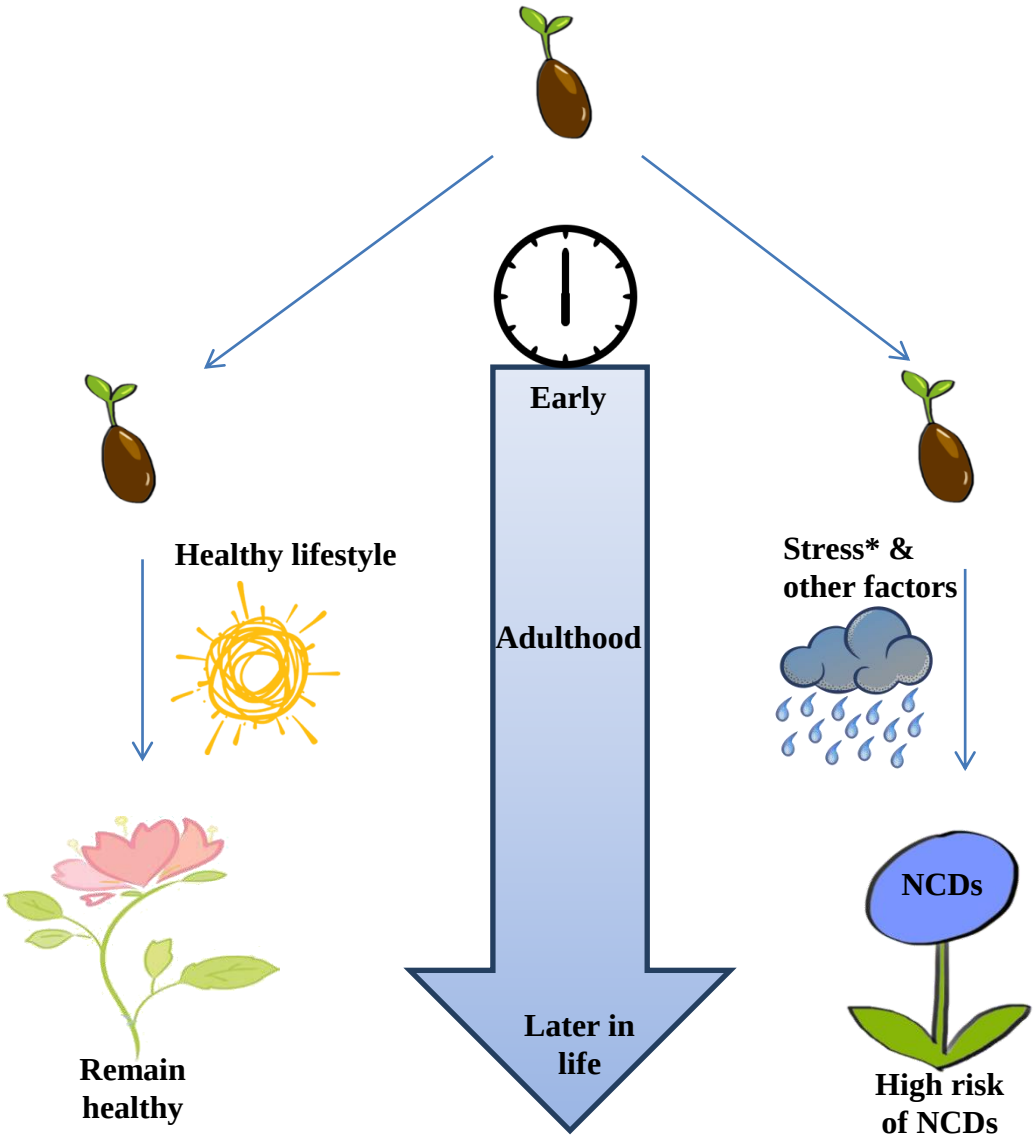


Figure 1.7. The Development of NCDs

*The development of NCDs is an accumulation of long-term exposure to stress, including smoking, high diet salt, alcohol over-consumption, obesity and physical inactivity.

Even though most of the NCDs become overt in a later stage of life, the underlying changes that pre-date disease can be detected by the use of sensitive biomarkers. If these

biomarkers reveal increased risk of NCDs developing as ageing, the early prevention measures can be introduced, especially to those who are found to have NCD-related biomarkers in the high risk range. Thus, to monitor the early changes through a biomarker method is an effective, and importantly, low cost strategy to lower the risk of NCDs and promote good health in a later stage of life. In the following section, some of the well established biomarkers related to risk of NCDs will be presented.

Cardiometabolic risk factors

Cardiovascular disease (CVD) is a group of diseases with heart and/or vessels disorders, including coronary heart disease (CHD), cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, and deep vein thrombosis and pulmonary embolism (WHO, CVD fact sheet, data accessed in April, 2016). Over 75% of the CVD cases are found in low or middle income countries, leading to financial stress. CVD causes the heaviest economic burden in health care in most countries, 12~16.5% of the finance input in health care was injected into CVD (Muka et al., 2015). For diabetes, which is a major risk of CVD, the prevalence has increased rapidly during the last two decades. In 1980, the global prevalence was 4.1%, and increased to 8.5% in 2014. Again, the increase in prevalence of diabetes was faster in low and middle countries and has brought heavy financial burden and labour loss to these countries (WHO, Global Report on Diabetes, data accessed in June, 2016). Therefore, strategies to address the cardiometabolic disease problem are needed.

Hypertension, dyslipidaemia, inflammation and impaired glycaemic control are well established CVD risk factors. For example, middle-aged people free of CVD but with systolic blood pressure (SYS BP) of 130-139 mmHg or diastolic blood pressure (DIA

BP) of 85-89 mmHg, were found to have 2.5 fold (95%CIs:1.6, 4.1; $p<0.05$; in women; n=3,892) and 1.6 fold (95%CIs:1.1, 2.2; $p<0.05$; in men; n=2,967) higher Hazard Ratio (HR) of developing CVD in the following 12 years, compared with those with baseline optimal blood pressure (SYS BP<120 mmHg and DIA BP <80 mmHg) (Vasan et al., 2001). In regard to lipids and inflammation, a prospective study of initially healthy women in US (n=15,632) showed that those with higher total cholesterol (>5.6 mmol/l), or elevated low density lipoprotein-cholesterol (LDL-C) (>3.97 mmol/l), or decreased high density lipoprotein-cholesterol (HDL-C) (<1.0 mmol/l) or elevated high sensitivity C reactive protein (hsCRP) (>0.5 mg/l) had >50% higher Relative Risk (RR) ($p<0.05$) of CVD developing in the 10 years of follow-up (Ridker et al., 2005). High hsCRP is associated with higher risk of CVD mortality in men also (Laaksonen et al., 2005). In men with baseline hsCRP of 3.0-9.9 mg/l, CVD mortality during a median 14.6 years of follow-up was 4.1 fold higher than those with baseline hsCRP <1.0 mg/l (Laaksonen et al., 2005).

Small decreases in HDL-C appear to have higher impact on CVD risk than similar levels of increase in LDL-C or hsCRP. The Framingham study found that people with HDL-C ≥1.68 mmol/l had an 8-fold lower risk of CHD than those with HDL-C <0.90 mmol/l, and that the association between CHD and HDL-C is stronger than for other individual lipid parameters (Gordon et al., 1977). It has also been found that an increase of 0.026 mmol/l in HDL-C is associated with 2-3% decrease in the risk of CHD in both men and women (Link et al., 2007).

Because of the strong influence of HDL-C on CHD risk, some studies have also included TC/HDL-C ratio (Ridker et al., 1998). In a nested case-control study of 245

subjects who developed myocardial infarction and 372 who remained healthy during a follow-up period averaging 9 years, it was found that the baseline TC/HDL-C is also an important predictor of incident cardiovascular disease. The myocardial infarction cases had a mean ratio of 5.5, compared to 4.5 in those who remained healthy ($p<0.0.001$) (Ridker et al., 1998). However, in the Systematic COronary Risk Evaluation project in which 12 studies in Europe encompassing data on 117,089 men and 88,080 women, including TC or the TC/HDL-C ratio in a predictive model gave virtually interchangeable results in relation to the 10-year risk of developing fatal cardiovascular disease (Conroy et al., 2003). It was found that the 10-year risk of fatal CVD in people with a TC/HDL-C ratio of 7 was doubled, compared to those with a TC/HDL-C ratio of 3, independent of age, gender, smoking habit or diabetes (Conroy et al., 2003). Therefore, the TC/HDL-C ratio may still provide additional useful information about future CVD risk, and was used in this current study.

In regard to Triglycerides (Tg), these were found to be positively associated with the risk of CVD. A prospective study involving 13,981 subjects (6,394 men and 7,587 women) found that the non-fasting Tg at baseline is associated with the development of myocardial infarction and ischaemic heart disease in both men and women during an average follow-up of 26 years (Nordestgaard et al., 2007). For those with baseline Tg of 1.0-1.9 mmol/l, the age adjusted HRs of myocardial infarction and ischaemic heart disease were increased to 2.2 and 1.7 in women and 1.6 and 1.3 in men, respectively, compared with those with baseline Tg <1.0 mmol/l ($p<0.05$). The HR figures were >4.5 in both genders for myocardial infarction and >2.0 for ischaemic heart disease when baseline Tg was >5.0 mmol/l, all $p <0.05$ (Nordestgaard et al., 2007).

Poor glycaemic control is the direct indicator of diabetes, but it also has a high impact on CVD risk. A meta-analysis of 17 prospective cohort studies involving a total of 237,468 men and women in the Asia-Pacific region found that for each reduction of 1 mmol/l in fasting plasma glucose concentration, there was a 23% lowering of risk of incidence of and mortality from ischaemic heart disease and 19% lower risk of CVD death during a mean follow-up of 6.5 years (Lawes et al., 2004). The incidence of CHD is found to be associated also with haemoglobin A1c (HbA1c), a biomarker of average plasma glucose in the previous 3 months (Peterson et al., 1998). The HR of CHD developing within the next 15 years in people with baseline HbA1c of 5.5-5.9%, 6.0-6.4% and 6.5% or higher increased by 23%, 78% and 95% , respectively (all $p<0.05$), compared with those with baseline HbA1c of 5.0-5.4% (Selvin et al., 2010).

Uric acid is not commonly measured in CVD risk assessment, but elevated uric acid is also found to be associated with hypertension, poor glycaemic control and increased risk of CVD (Benzie and Strain, 1996a; Feig et al., 2008). An increase of 59.48 μmol/l in plasma uric acid concentration is associated with 9% and 26% higher HRs in men and women, respectively, as found during an average of 16.4 years follow-up ($p<0.05$) (Fang and Alderman, 2000).

DNA damage and the comet assay

DNA damage is an inevitable consequence of normal cell metabolism, but if the DNA damage is not properly repaired, there is higher risk of mutations and genotypic changes, and high level of DNA damage causes cellular function loss or even cell death (Cooke et al., 2003). DNA damage was found to be an independent risk factor of various NCDs, especially for cancer development, thus, to maintain DNA damage to a

low level is very important for health (Hoeijmakers, 2009). The most commonly used method for DNA damage qualification is the single cell gel electrophoresis assay, also known as the comet assay (Collins et al., 2014).

The comet assay is a relatively simple and reliable way for measuring DNA damage and repair, depending on the choice of different versions of the comet assay (Collins et al., 2014). Compared with the traditional chromatographic method, the comet assay is more accurate, as the sample preparation and procedure of comet assay is relatively simple, thus, less extra DNA damage is induced during measurement (Collins et al., 2014). Basically, the comet assay detects the amount of DNA strand breaks after the relaxation of DNA, and these are detected by gel electrophoresis (Azqueta and Collins, 2013). The image of the cell under fluorescent microscope looks like a comet (this is where the name comes from), the loops of DNA around the breaks migrate faster and form the tail of the 'comet', the higher amount of DNA in the comet tail indicating a higher level of DNA damage (Collins, 2004; Collins et al., 2014). Various kinds of damage and repair can be quantified with different versions of the comet assay, this will be discussed briefly below.

Alkaline comet assay

The alkaline comet assay is the basic version of comet assay developed by Ostling and Johanson in 1984, and further modified by Singh et al. (Ostling and Johanson, 1984; Singh et al., 1988). The basic alkaline comet assay detects pre-existing strand breaks (Collins, 2004). In this assay, cells are embedded in low melting point agarose, and the cell membranes and histones are removed by lysis, forming nucleoids. The DNA in the nucleoids is unwound in high pH solution, and the relaxed DNA is pulled towards the

anode under electrophoresis in high pH (~10) electrophoresis solution (Singh et al., 1998; Collins et al., 2014). After staining, the image of DNA can be seen under the fluorescent microscope, and the strand break DNA damage can be quantified by assessing the amount of DNA in tail.

Enzyme-assisted comet assay

Apart from strand breaks, there are still many kinds of DNA damage which can not be detected by the alkaline comet assay. Specific enzymes can be used to react with specific lesions, forming strand breaks which can be seen in the comet assay (Collins and Dušinská, 2002). The enzymes used in practise include but are not limited to Endonuclease III (Endo III), T4 endonuclease V (T4EV) and Formamidopyrimidine DNA glycosylase (Fpg) (Collins et al., 1993; Wong et al., 2011; Ho et al., 2013). For example, Endo III detects oxidised pyrimidines; T4EV detects cyclobutane pyrimidine dimers induced by UVB; and Fpg, which was used in this current study, detects oxidation induced DNA damage including 8-oxo-7,8-dihydroguanine (8-oxoGua) and formamidopyrimidines, which are ring-opened purine derivatives including 2,6-diamino-4-hydroxy-5-formamidopyrimidine, and 4,6-diamino-5-formamidopyrimidine (Collins, 2009; Wong et al., 2011; Ho et al., 2013; Collins et al., 2014).

Challenge comet assay

Apart from detecting existing DNA damage, the comet assay can also be used to quantify the cellular resistance to challenges (Collins, 2014). Compared with the basic alkaline comet assay, the oxidation challenge (usually caused by hydrogen peroxide (H_2O_2) using controlled concentration) or other challenges (induced by UV light or chemicals such as heavy metals) are induced in the cells before they are embedded into

low melting point agarose (Anderson et al., 1994; Fairbairn et al., 1995; Wachtel-Galor et al., 2005). The challenge introduced similar levels of stress to all cells but the final DNA damage can be lowered by higher resistance to these challenges. As a result, the lower DNA in tail from this kind of study is associated with higher resistance to the challenge (Anderson et al., 1994; Fairbairn et al., 1995; Han et al., 2010).

DNA repair comet assay

In the DNA repair process, there are several pathways, including nucleotide excision repair and base excision repair, but both need the involvement of enzymes. For example, 8-oxoguanine DNA glycosylase 1 (OGG1) is an important enzyme that removes the 8-oxoGua in DNA, and its activity is very important for the repair of oxidation-induced DNA damage (German et al., 2013). In the comet assay, the OGG1 activity can be measured by applying a cell extract to 'substrate' cells with certain amount of oxidation-induced DNA damage, the amount of strand breaks in the substrate cell treated with the cell extracts (containing OGG1) reflects the activity of OGG1 in removing the 8-oxoGua (Collins and Gaivão, 2007; Choi et al., 2015).

Antioxidant status and oxidative stress

Oxidative stress is regarded as the one of the most common causes of disease (Camps and García-Heredia, 2014). For humans, oxygen is essential for life maintenance, and is involved in the aerobic respiration and release energy (such as the adenosine triphosphate (ATP) formed from aerobic respiratory), but on the other hand, the reactive oxygen species (ROS) released from normal cellular metabolism (such as superoxide anion) cause damage to important biological molecules like DNA, lipids, and protein (Benzie, 1996; Benzie, 2000; Halliwell and Gutteridge, 2007; Benzie and Choi, 2014).

Oxidative stress is influenced by two factors, lack of antioxidant defence and excess ROS (Halliwell and Gutteridge, 2007). There are some well established sensitive biomarkers used to assess the antioxidant status and oxidative stress, and some of them are introduced below.

The ferric reducing antioxidant power (FRAP) assay (described in detail in Chapter 3) is a well-validated method to assess the "total antioxidant power" of biological fluids (Benzie and Strain, 1996b; Benzie and Strain, 1999, Szeto et al., 2002; Benzie and Choi, 2014; Asemi et al., 2013; Gawlik et al., 2016). FRAP value does not measure individual antioxidants, but is a measurement of the combined, or total, amount of electron donation of the reductive antioxidants with less positive redox potential than ferrous ion, including uric acid, ascorbic acid and alpha tocopherol (Benzie and Strain, 1996b). It is noted that even though uric acid performs as an antioxidant under physiological condition, the high level of uric acid is associated with higher risk of gout and CVD (Benzie and Strain, 1996a). Thus, the FRAP value corrected for urate is also given in this current study.

Ascorbic acid (vitamin C) is essential for collagen synthesis, and vitamin C deficiency causes scurvy, which is now rarely seen but nevertheless exists (Ma et al., 2016). Low ascorbic acid is associated with cardiovascular disease and cancer, and other biological dysfunctions (Gey, 1998; Benzie, 1999). A large epidemiological study suggested that the plasma level of >50 μmol/l vitamin C is beneficial for CVD prevention (Gey, 1998). Unlike vitamin D, ascorbic acid (vitamin C) is not synthesized in the human body, and the only source of ascorbic acid is the diet. Fruits and vegetables are rich in ascorbic acid, though concentrations vary substantially. A study by our group showed that the

ascorbic acid content is much higher in strawberry (540 mg/kg) and kiwifruit (520 mg/kg), than some very commonly consumed fruits like apple (<50 mg/kg) and pear (<10 mg/kg). Besides, the inappropriate storage condition and the breakdown of food also cause a rapid loss of vitamin C (Szeto et al., 2002). Ascorbic acid is the also a key antioxidant in oxidative stress resistance, and contributes ~15% of the total plasma FRAP value (Benzie and Strain, 1996b; Benzie, 1999).

Uric acid is the product of purine metabolism, and can also perform as an antioxidant (contributing ~60% in FRAP value in human plasma) (Benzie and Strain, 1996b). In humans, allantoin is the non-enzymatic oxidation product of uric acid, formed by the redox reaction with ROS or reactive nitric species, however, its concentration is independent from the uric acid level (Czerska et al., 2015). Allantoin has been used as a biomarker that reflects oxidative stress, and it was found to be higher in those with Down's syndrome, Rheumatoid Arthritis, type 2 diabetes patients, overweight, and hypercholesterolomic people (Benzie et al., 1999; Zitnanová et al., 2003; Yardim-Akaydin et al., 2004; Choi et al., 2008; Chung and Benzie, 2013).

8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) is the product from the oxidized guanine repair progress, and its urinary concentration is the most frequently detected biomarker reflecting overall oxidation-induced DNA damage to the nucleotide pool (Lee et al., 2010; Poulsen et al., 2014). In DNA, 8-oxodG damage is linked to G→T mutation, and this mutation is linked to the development of cancer, especially when it occurs in important genes like tumour suppressor *p53* (Brancato et al., 2016). Furthermore, high level of 8-oxodG is also found to be associated with higher risk of atherosclerosis, heart failure and depression (Kroese LJ, Scheffer PG, 2014; Basu et al.,

2015). In human studies, the 8-oxodG concentration in urine is more frequently used than that in plasma, as it is more stable in urine and the concentration is independent from the direct impact of diet (Halliwell and Gutteridge, 2007; Lee et al., 2010). As the concentration of 8-oxodG in urine is influenced greatly by the water intake before sample collection, the concentration must be standardized by the urine creatinine concentration (Lee et al., 2010).

Antioxidant enzymes are also very important in human body to decrease oxidative stress, for example, Superoxide dismutases (SOD) and Glutathione peroxidase (GPx) (Halliwell and Gutteridge, 2007). There are two kinds of SOD, Cu/Zn-SOD is found in cytoplasm, while the Mn-SOD is mainly found in mitochondria. SOD reacts specifically with superoxide (a free radical), changing it to H_2O_2. It is noted that the H_2O_2 produced by SOD is also a kind of ROS, and this can react with glutathione to form water under catalysis by GPx (Halliwell and Gutteridge, 2007).

VITAMIN D AND HEALTH

Vitamin D is known to be necessary for bone health (Holick, 2007). However, there is growing evidence that vitamin D deficiency is a risk factor for diseases of various types, including cardiovascular disease, cancer, diabetes and neurological disorders (Wang et al., 2012; Pludowski et al., 2013; Feldman et al., 2014; Krivošíková et al., 2015; Pliz et al., 2016). The link between vitamin D and non-skeletal tissues is supported by the finding of a wide distribution of vitamin D receptor, which can be found in the nucleus of cells in over 30 human tissues (Table 1.3). This indicates that vitamin D is not only involved in metabolism of bone cells, but also contributes to the function and

modulation of other tissues (Larussa et al., 2012; Christakos et al., 2015). Other evidence that vitamin D is important for health comes from epidemiological studies and supplementation trials. Some of this evidence is presented and discussed below.

Vitamin D and CVD

There are many population-based studies that have looked at associations between vitamin D and CVD and its risk factors (Schleithoff et al., 2006, van de Luijtgaarden et al., 2012; Wang et al., 2012; Kim et al., 2013; Pilz et al., 2016). Studies have investigated the relationship between vitamin D status and the incidence and severity of CVD, and with risk factors such as inflammation and lipids (Wang et al., 2012). The effect of vitamin D supplementation on cardiac function and CVD risk factors has also been investigated. Some of these studies are described in more detail below.

A meta-analysis found that low vitamin D status is associated with higher risk of CVD, every decrease of 25 nmol/l in plasma/serum 25(OH)D was linked to 18% higher relative risk (RR) of CVD incidence (95%CIs: 1.07, 1.29). In more detail, compared with those in the highest vitamin D category, those in the lowest category had 52%, 42%, 38% and 64% higher risk of, respectively, total CVD, CVD mortality, CHD and stroke, $p<0.05$ (Wang et al., 2012).

A study in the Netherlands investigated vitamin D status in 490 patients with peripheral artery disease (n=254) or thoracic and/or abdominal aortic aneurism (n=236) (van de Luijtgaarden et al., 2012). Results showed significant associations ($p<0.05$) between vitamin D status and atherosclerotic markers (ankle-brachial index, carotid artery

intima-media thickness, hsCRP, indicating the poorer the vitamin D status, the more severe the arterial disease (van de Luijtgaarden et al., 2012).

For supplementation trials, no significant decrease in CVD incidence was seen with vitamin D and/or calcium supplementation in a meta-analysis involving five trials (Wang et al., 2010a). Furthermore, no significant effect of vitamin D supplementation was seen on heart failure risk (400 IU vitamin D plus 1,000 mg calcium for a median follow-up of 7.1 years on 35,983 postmenopausal women; Donneyong et al., 2015), or blood pressure or artery stiffness (400 IU or 2,500 IU vitamin D/day for 6 months on 98 postmenopausal women; Gepner et al., 2015), but there was significant improvement on inflammation and lipid profile seen in two supplementation trials (Schleithoff et al., 2006; Kim et al., 2013)

In Germany, 93 congestive heart failure patients completed a double-blind, randomized, placebo-controlled trial. The patients received 50 µg (2,000 IU) vitamin D plus 500 mg calcium per day (D(+) group, n=52), or vitamin D placebo plus 500 mg calcium per day (D(–) group, n=51). The intervention lasted for 9 months. Results showed that at entry, the median serum 25(OH)D overall was ~37 nmol/l and was similar in both groups. After 9 months, the median serum 25(OH)D in the D(+) group had increased by 67 nmol/l (by 186%, $p<0.001$) and the anti-inflammatory cytokine interleukin 10 (IL-10) had increased (by 43%, $p<0.05$). The tumour necrosis factor alpha (TNF-α) concentrations did not change significantly in the D(+) group, but had increased by 12% ($p<0.05$) in the placebo group. Results indicate that vitamin D supplementation may help limit inflammation in CHF patients by suppressing the release of the pro-

inflammatory cytokine TNF-α, and increasing synthesis of the anti-inflammatory cytokine IL-10 (Schleithoff et al., 2006).

A Korean study investigated the relationship between vitamin D status and a variety of CVD risk biomarkers and glycaemic control in 171 middle-aged, apparently healthy, but overweight or obese adults (body mass index (BMI) >23kg/m2) (Kim et al., 2013). Results showed that 114 (67%) of the subjects were vitamin D deficient (serum 25(OH)D ≤50 nmol/l), but significant differences in CVD risk factors between the two groups (according to vitamin D status) were found only for serum triglycerides and the inflammatory biomarker interleukin 6 (IL-6). No differences in fasting glucose or insulin resistance (by homeostasis model assessment for insulin resistance (HOMA-IR)) were seen between the two groups. There were weak but statistically significant ($p<0.05$) correlations found between vitamin D status and triglycerides ($r=-0.203$) and HLD-C ($r=0.179$) (Kim et al., 2013).

In summary, there is evidence that vitamin D plays a role in cardiovascular health, possibly through an anti-inflammatory effect or effects on lipids. However, the evidence is not clear cut, and experimental evidence from randomized, controlled intervention trials is lacking.

Vitamin D and cancer

Association between vitamin D status and risk of various cancers has been investigated in observational studies owing to the reported anti-proliferative effects of vitamin D in colon, breast, prostate and liver cancer cells (Nair-Shalliker, et al., 2012a; Vuolo et al., 2012). Some of the extensive epidemiological data of the association between vitamin D

status and risk of various cancers are presented below, along with results of randomized placebo-controlled supplementation studies with vitamin D.

Epidemiological studies

A case control study conducted by Theodoratou et al. recruited 2,001 patients with adenocarcinoma of colorectum and 2,237 healthy controls in Scotland. The authors reported that increased risk of colorectal cancer was significantly ($p<0.001$) associated with low plasma 25(OH)D level (<25 nmol/l) (Theodoratou et al., 2012). Vitamin D status was also associated with urothelial bladder cancer (UBC) (Amaral et al., 2012). In this Spanish case control study of 1,130 UBC patients and 1,038 healthy controls, plasma 25(OH)D was significantly lower in the cancer patients than in controls (respectively, median 34.75 and 37.5 nmol/l; $p=0.001$). Plasma 25(OH)D <50 nmol/l was associated with >50% increase in UBC risk. However, on closer examination it was found that the association between vitamin D status and UBC was only significant in smokers (Amaral et al., 2012).

A significant inverse association between vitamin D status and tobacco-related cancer risk was found in a Danish study (Afzal et al., 2013b). The main study (Copenhagen City Heart study) involved 12,175 subjects whose smoking habits were evaluated from questionnaire. Plasma 25(OH)D was measured in a sub-group of 9,791 subjects who were free of cancer at entry. Those who smoked were more likely to have low vitamin D status. During follow-up for up to 28 years, 1,081 cases of cancer developed. Risk of lung cancer, head and neck cancer, bladder cancer and kidney cancer were significantly increased with a 50% plasma 25(OH)D reduction: the HR (95%CIs) for all cancers were 1.19 (1.09, 1.31), 1.44 (1.19, 1.73), 1.28 (1.06, 1.54) and 1.34 (1.04, 1.73), respectively

(Afzal et al., 2013b). These findings suggest that higher vitamin D status may help lower risk of tobacco-associated cancers.

A Mexican study recruited a total of 1,000 breast cancer patients (pre- and post-menopausal) and 1,074 matched controls, and serum 25(OH)D was measured in a sub-set of 573 cases and 639 controls (Fedirko et al., 2012). Women with breast cancer had significantly lower serum 25(OH)D than controls; mean 47.5 versus 55.5 nmol/l; $p<0.0001$. Lower serum 25(OH)D was significantly associated with higher breast cancer risk in both pre- and post- menopausal women ($p<0.05$). The odds ratio (OR) (95% CIs) in the highest tertile (25(OH)D >75 nmol/l) compared to 'reference' group of the lowest tertile of serum 25(OH)D (25(OH)D <50 nmol/l) was 0.53 (0.36, 0.78, $p<0.05$). The dietary vitamin D intake (evaluated by questionnaire) was also inversely associated with breast cancer risk ($p=0.054$) (Fedirko et al., 2012).

Supplementation trials

From a meta-analysis which involved 18 supplementation trials, vitamin D supplementation was found to associate with 12% lower risk of cancer mortality (RR: 0.88, 95%CIs: 0.78, 0.98), but no significant effect on cancer incidence, regardless of the baseline vitamin D status or supplemented with vitamin D or its metabolites (Bjelakovic et al., 2014).

One large, randomized placebo-controlled well designed study in US involved 1,179 women aged over 55 years who took one of three treatments for 4 years (Lappe et al., 2007). The double blinded treatments were: 1000 IU vitamin D and 1400 mg calcium per day (n=446); calcium plus vitamin D placebo (n=455); or double placebo (n=288).

The risk of incident cancer in the 2-4 years of follow-up was significantly lower in the vitamin D group: RR (95%CIs) 0.232(0.09, 0.6; p<0.005), but no significant effect of calcium alone was seen: RR (95%CIs) 0.586 (0.29, 1,20). It is noted that the mean serum 25(OH)D levels at baseline in all three treatment groups were ~72 nmol/l, and remained at this level in the placebo and calcium only groups, but that after 12 months the level in the vitamin D group had increased to 96 nmol/l. A larger placebo-controlled study also in US was performed by Wactawski-Wende et al., (2006). A total of 36,282 postmenopausal women aged 55-79 years and cancer-free in 10 years before entry were randomly allocated to take 1000 mg calcium plus 400 IU vitamin D per day (n=18,176), or placebo (n=18,106). After a follow-up of 7 years' mean duration, no significant effect on total cancer or colorectal cancer incidence was detected: HRs (95%CIs) were 0.98 (0.91, 1.05) and 0.1.08 (0.86, 1.34), respectively Wactawski-Wende et al., (2006). A nested control-case study which was embedded in this study measured serum 25(OH)D concentration at baseline (but not after treatment) in 306 matched pairs. Vitamin D status at baseline was not different between the two treatment groups. However, a significant inverse trend between baseline vitamin D status and colorectal cancer incidence was seen. Taking serum 25(OH)D of ≥58.4 nmol/l as reference, those with lower vitamin D status at baseline had around two-folds increase in invasive colorectal cancer developing during follow-up (Wactawski-Wende et al., 2006). This study also investigated invasive breast cancer incidence as an outcome, but no relationship between baseline vitamin D status and invasive breast cancer was seen, and there was no difference across the two treatment groups in terms of development of invasive breast cancer (Chlebowski et al., 2008). There are two points to note about this study: the dose of vitamin D was low (400 IU/d), and changes in serum vitamin D as a result of the supplement were not measured. Therefore, whether the lack of effect on

incidence of colorectal or invasive breast cancer was due to insufficient dosing with vitamin D is not clear.

In summary, the effect of vitamin D status on overall cancer risk is still unclear. The relationship between poor vitamin D status and increased risk of colorectal cancer is consistent in epidemiological studies, and there is some support for increased risk of UBC in association with low vitamin D status. However, the randomized placebo-controlled supplementation studies performed to date show no clear beneficial effect of vitamin D on incident cancer. In relation to vitamin D and skin cancer, the relationship is even more complex, because of the dual effects of UV light. This is discussed in more detail in the following section.

The special case of vitamin D and skin cancer

The relationship between UV, vitamin D and skin cancer is paradoxical. Evidence supports a cancer preventive role for vitamin D, and some studies have found that vitamin D can help to prevent UV-induced DNA damage and cell death, and to have anti-proliferative effects (Nair-Shalliker et al., 2012a and b; Dixon et al., 2013). In vitro studies and animal studies have provided evidence that vitamin D exerts cancer protective effects in skin, inhibiting keratinocytes proliferation and promoting differentiation (Tang et al., 2012). However, UV-induced mutation is the main causal factor for skin cancer (Wong et al., 2011). UVA light creates oxidation-induced lesions in DNA, and UVB is absorbed directly by DNA to form cyclopyrimidine dimers (CPDs) that are highly mutagenic (Wong et al., 2011). Therefore, the overall role of vitamin D in skin cancer is difficult to determine because of its dual effect, one damaging and one potentially protective.

There are different types of skin cancer. The two most common are basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). Melanoma is less common but is highly malignant (Leiter and Garbe, 2008). In an American study of non-melanoma skin cancers, 3,223 healthy Caucasian subjects were recruited and their serum 25(OH)D concentrations were measured (Eide et al., 2011). During follow-up (between 1997 and 2009), there were 240 cases of incident non-melanoma skin cancer (NMSC), 49 SCC, 163 with BCC, and 28 with both. In relation to vitamin D, higher NMSC incidence was associated with higher serum 25(OH)D level: the OR (95%CIs) was 1.6 (1.1, 2.3) in the highest quartile of 25(OH)D level (≥77.5 nmol/l) compared with the lowest quartile (25(OH)D level <47.5 nmol/l). On a binary divide of 25(OH)D at a cutoff of 37.5 nmol/l, those with higher 25(OH)D had higher risk of NMSC; OR (95%CIs) was 1.7 (1.04, 2.7). However, the association of higher vitamin D with increased risk of NMSC was not seen in cases of NMSC that developed in areas of low exposure to UV, implying that the apparent association with vitamin D is mediated through UV exposure, although it is noted that the number of NMSC cases in less UV exposed sites was low and there may not have been enough power to reveal an effect of vitamin D (Eide et al., 2011).

From this study, it is clear that exposure to UV light plays an important role in driving the apparent relationship of vitamin D and skin cancer. It is likely that high vitamin D status in skin cancer patients is a separate "by-product" effect of the main cause of skin cancer, that is, high UV exposure that induced mutagenic changes to DNA. The reported relationship between high dietary intake of vitamin D and lower risk of melanoma provides some supporting evidence for this. In the case-control study of Millen et al., (2004), there was a significant inverse trend of association between

melanoma and vitamin D intake from food and, separately for food and supplements and food ($p<0.05$). The study involved 738 melanoma cases and 1,030 controls. The OR (95%CIs) for melanoma in those in the highest quintile of vitamin D from food was 0.61 (0.40, 0.95). The effect appeared to be stronger in those with high vitamin D intake from food alone (Millen et al., 2004).

In summary, the dual effect of UV on skin makes it difficult to determine the role of vitamin D in modulating the risk of skin cancer. It is possible that increased dietary intake of vitamin D from food and/or supplements can help protect against UV-induced mutagenesis, but this is uncertain.

Vitamin D and neurological disorders

Vitamin D status has been reported to impact on various neurological disorders and conditions, including Alzheimer's disease (AD), Parkinson Disease (PD), multiple sclerosis and depression and mood (Ho et al., 2012; vinh quoc Luong and Thi Hoàng Nguyen, 2012 and 2013; Anglin et al., 2013; Wrzosek et al., 2013). However, the relationship between vitamin D and neurological disorders and cognitive function is unconfirmed (van der Schaft et al., 2013).

AD and PD

In an observational study conducted by Evatt et al., (2008), poorer vitamin D status was observed in PD patients, but this was not found for AD patients (Evatt et al., 2008). This study selected 100 age and sex matched, predominantly Caucasian subjects from each of three cohorts (a PD cohort, an AD cohort and a healthy control cohort) of the Clinical Research in Neurology database. Blood samples were available from all 100 PD

patients, from 97 of the AD patients and from 99 of the healthy controls. The vitamin D status of the PD patients ranged very widely, but on average was not low, however, it was significantly poorer than that of controls (p=0.01), with mean (SD) concentrations of plasma 25(OH)D of 79.8 (34.0) and 92.5 (38.5) nmol/l, respectively. No significant difference in vitamin D status was detected between AD patients and control subjects (Evatt et al., 2008). Interestingly, the PD group had highest prevalence even though there were more blood samples collected in summer and autumn in the PD group, at which times the vitamin D status is expected to be highest due to exposure to more sunlight.

A significant inverse association between vitamin D status and the risk of PD was found in a long-term study in which 3,173 subjects who were free of PD at baseline were selected as part of the mini-Finland Health Survey (Knekt et al., 2010). Serum samples were stored at -20 ^{o}C and the 25(OH)D concentrations were measured at the end of this study. During up to 29 years' follow up, 50 subjects were diagnosed with PD. The adjusted RR (95%CIs) of the highest serum 25(OH)D quartile compared with the lowest was 0.33 (0.14, 0.78), indicating a three-fold lower PD risk with high vitamin D status group, and those with 25(OH)D of 50 nmol/l of higher had 65% lower risk of PD compared to those with 25(OH)D of 25 nmol/l or less (Knekt et al., 2010).

In contrast to the study of Evatt et al., (2008), vitamin D status was found to be significantly associated with AD in a Japanese study (Sato et al., 2005). This case control study recruited 100 AD patients and 100 age-matched healthy controls. The illness severity was assessed, and 48/100 were defined as having mild AD and 58/100 had severe AD. Serum 25(OH)D concentrations were measured, and the mean value of

both AD patient groups (severe and mild) were significantly lower than the control group ($p<0.001$). However, the severe deficiency in the severely affected AD patients may be due to lack of exposure of debilitated patients to sunlight and to malnutrition (Sato et al., 2005).

Multiple sclerosis

The association between poorer vitamin D status and higher risk of multiple sclerosis is widely found in epidemiological studies (Pozuelo-Moyano et al., 2013). In an Indian study, serum 25(OH)D concentrations were significantly lower in multiple sclerosis patients compared with healthy controls (Pandit et al., 2013). In this case-control study, 110 multiple sclerosis patients and 108 healthy age and sex matched controls were recruited, and among the multiple sclerosis patients, 63 samples were collected during relapse in patients, and 77 were collected during period of remission. The median serum 25(OH)D levels were significantly lower in multiple sclerosis patients overall than the controls (39.0 vs. 46.5 nmol/l, $p=0.003$). Furthermore, the vitamin D status was significantly poorer in the patients in relapse compared with the patients in remission: median serum 25(OH)D levels were, respectively, 37.0 nmol/l and 46.0 nmol/l, $p=0.001$. The results from this study indicated that the vitamin D status is not just associated with the multiple sclerosis develop, as well as the severity of this disease (Pandit et al., 2013).

Depression

The link between vitamin D status and depression was widely found in both epidemiological studies and supplementation trials. A systematic review and meta-analysis found that low vitamin D status was associated with higher risk of depression

(Anglin et al., 2013). Nine cross-sectional studies and three prospective studies were involved. From the cross-sectional studies, higher risk of depression was seen in those with lower vitamin D status, but this did not reach statistical significance: the OR (95% CIs) of the risk of depression compared with the lowest to highest vitamin D status category was 1.31 (1.00, 1.71), but it is noted that the cut-off points for vitamin D status definition used in each study were different. From the prospective studies, those in the lowest baseline vitamin D category had ~two-folds higher risk in developing depression compared with those in the highest category: HR (95% CIs) was 2.21(1.40, 3.49) (Anglin et al., 2013).

In a supplementation trial in Iran, it was found that vitamin D supplement improved depression (Mozaffari-Khosravi et al., 2013). The 109 subjects who completed this study were all vitamin D deficient at entry (defined as serum 25(OH)D <40 nmol/l), they were randomly allocated to three treatments: 300,000 IU vitamin D intramuscular injection (n=39), 150,000 IU vitamin D intramuscular injection (n=36) or no treatment (n=34). The depression status was determined by the Beck Depression Inventory II test, normal was considered as test scores of 1~10, while very severe depression was over 40. The median serum 25(OH)D was around 17 nmol/l overall before intervention and there was no significant difference between groups. Blood samples were collected after 3 months' intervention. The median serum 25(OH)D were significantly increased to 42.0, 41.0 and 19.4 nmol/l respectively. The depression test scores were significantly decreased by 9.3, 6.8 and 2.1, respectively. It is noted that the scores decreased more in higher dosage group than the lower one while the serum 25(OH)D concentration did not differ too much (Mozaffari-Khosravi et al., 2013).

In summary, these studies indicated that the association between vitamin D status and neurological disorders is commonly found, but the evidence was not strong except for depression. The high vitamin D status appears to be beneficial in relation to both lower risk of and lower severity of neurological disorders, although the mechanisms are not known, and well designed supplementation trials on the effects of vitamin D on AD, PD and multiple sclelosis are lacking.

Vitamin D and diabetes

There is a strong link between vitamin D and diabetes. Several recent systematic reviews and meta-analyses have shown an inverse association between vitamin D status and diabetes risk and severity (Afzal et al., 2013a; Khan et al., 2013; Song et al., 2013). However, the evidence from supplementation trials is found but insufficient. Vitamin D supplementation was associated with slightly lower fasting plasma glucose and insulin resistance in diabetic patients and those with impaired fasting glucose, but not in healthy people (George et al., 2012).

In an Australian observational study, lower serum 25(OH)D concentrations were found in children and adolescents with type 1 diabetes compared with healthy controls (Greer et al., 2013). In this case control study, 42 children with established type 1 diabetes, 14 with newly diagnosed type 1 diabetes, 46 controls and 12 non-diabetic siblings of diabetes patients were recruited. Serum 25(OH)D concentration of the 56 patients were significantly lower than the controls (78.7 vs. 91.1 nmol/l, $p = 0.01$) (Greer et al., 2013). However, it is noted that all subjects had relatively high vitamin D status.

In a Finnish observational study, vitamin D was found to be inversely associated with type 2 diabetes incidence in men but not in women. A total of 7,503 subjects from two cohorts (the Finnish Mobile Clinic Health Examination Survey and Mini-Finland Health Survey) who were free of diabetes at entry were recruited. During the up to 22 years of follow up, a total of 412 subjects (188 male and 224 female) developed type 2diabetes. Plasma samples at baseline from the cases and 986 matched controls that had been stored at -20 ^{o}C were measured for 25(OH)D concentration. The adjusted ORs (95%CIs) of the highest vitamin D quartile compared to the lowest one were 0.28 (0.10, 0.81) in men and 1.14 (0.60, 2.17) in women (Knekt et al., 2008). The reason for the gender difference is not clear.

Vitamin D has been found to be related to insulin sensitivity and β cell function in observational studies. In a study of 126 healthy subjects with normal glucose tolerance, oral glucose tolerance test (OGTT), insulin sensitivity and β cell function assessments were investigated in US (Chiu et al., 2004). The insulin sensitivity index was positively associated with higher plasma 25(OH)D levels ($r=0.4600$, $p<0.0001$). β cell function was evaluated by OGTT and first and second phase insulin response. A significant inverse association between plasma 25(OH)D concentration and 60, 90 and 120 min post-challenge plasma glucose concentrations ($p<0.05$) were seen, and inverse associations were found also between both first and second phase insulin responses ($r=-0.2513$, $p<0.05$ and $r=-0.3487$, $p<0.001$, respectively), indicating that lower vitamin D is a predictor of lower insulin sensitivity and poorer β cell function (Chiu et al., 2004).

Overall, epidemiological and prospective trials have produced strong and robust evidence for the association between poor vitamin D and higher diabetes risk and insulin sensitivity and β cell function. This is supported by supplementation trials, some are presented in the following section.

The observational evidence is supported by some experimental evidence. In a supplementation study performed in New Zealand, 81 women with low D (defined as serum 25(OH)D <50 nmol/l) were randomly allocated to two treatments: 4000 IU vitamin D per day (n=42) or placebo (n=39) for 6 months (von Hurst et al., 2010). Vitamin D status, insulin resistance and β cell function (evaluated by homeostasis model assessment (HOMA) model) were measured at baseline and post intervention. Median serum 25(OH)D concentrations increased significantly in both groups, from 21 to 80 nmol/l in the supplementation group and from 19 to 23 nmol/l in placebo group (this was likely due to seasonal difference). The insulin sensitivity, insulin resistance and fasting serum insulin were significantly improved in vitamin D group ($p<0.05$), while no significant difference was detected in the placebo group. However, there was no significant difference found in fasting serum glucose and β cell function (von Hurst et al., 2010).

In the US, 314 healthy subjects completed a three years' supplementation study. Subjects were defined as normal fasting glucose (NFG, fasting plasma glucose <5.6 mmol/l, n=222) and impaired fasting glucose (IFG, fasting plasma glucose 5.6-6.9 mmol/l, n=92). They were randomly allocated to take 700 IU vitamin D_3 and 500 mg calcium (n=114 in NFG and n=47 in IFG) or take placebo (n=108 in NFG and n=45 in IFG). For the IFG subjects, the increases of both fasting plasma glucose and insulin

resistance (measured by HOMA-IR) in the supplemented group were smaller than those in the placebo group (both $p<0.05$), whole no significant difference in the post-pre change was seen in the NFG subjects. This study showed that vitamin D supplement may be more beneficial in people with high risk of diabetes than normal subjects, in the case of glycaemic control (Pittas et al., 2007)

In summary, the association between vitamin D and various types of diabetes is convincing as found in observational studies. Poor vitamin D status is associated with higher risk of diabetes, lower insulin resistance and poorer β cell function. Some supplementation studies also showed a similar relationship.

Conclusion

The association between poor vitamin D status and chronic diseases were commonly investigated in epidemiological studies and some supplementation trials. However, the mechanism by which vitamin D influence health status is still unknown. Some studies showed that poorer vitamin D status is associated with higher DNA damage, lower DNA repair, higher oxidative stress and inflammation and endothelial dysfunction, the 'common soil' of chronic diseases. This will be discussed in the next section.

VITAMIN D AND THE "COMMON SOIL" OF NCDS: THE CURRENT EVIDENCE

The relationship between vitamin D and key elements or components of the "common soil" (such as poor glycaemic control, inflammation, oxidative stress, DNA damage and

repair, and endothelial dysfunction) have been investigated, however data from published studies are not consistent or clear. A summary of the findings of some recent epidemiological studies and supplementation trials that have looked at vitamin D in relation to the various components of the common soil are presented in Table 1.4.

Data shown in Table 1.4 indicate that vitamin D is linked to the "common soil" of the development of NCDs. However, the outcomes from different studies are not yet substantial enough to confirm the link. For glycaemic control, although the link between vitamin D status and the risk of diabetes and poor glycaemic control was commonly found in observational studies (Afzal et al., 2013a; Khan et al., 2013; Song et al., 2013), the direct improvement driven by vitamin D supplement is not confirmed. In supplementation trials, one meta-analysis suggested that vitamin D supplementation was linked to small but significant decrease in fasting plasma glucose and insulin resistance in diabetic patients or those with impaired fasting glucose at baseline but not with normal subjects; while in another meta-analysis, no significant effect of vitamin D supplementation on insulin resistance, insulin secretion or HbA1c was seen (George et al., 2013; Seida et al., 2014). For oxidative stress and antioxidant status, the link with vitamin D status or supplementation is not yet confirmed. SOD activity was found to be associated with higher vitamin D levels in epidemiological study and vitamin D supplement increased SOD activity in one study but had opposite effect in another (Javanbakht et al., 2010; Zinnuroglu et al., 2012). In non-diabetic subjects, no significant association was found between vitamin D status and advanced oxidation protein products (Krivošíková et al., 2015). Significant improvement was found in total antioxidant capacity (TAC) in elderly women and pregnant women, but not in gestational diabetic patients (de Medeiros Cavalcante et al., 2015; Asemi et al., 2015a).

For inflammation, lower IL-6 was consistently associated with higher vitamin D status in both epidemiological studies and supplementation studies (Assimon et al., 2012; Shab-Bidar et al., 2012); the association between hsCRP and vitamin D status was not commonly found in observational studies, though the vitamin D fortified drink decreased hsCRP in a supplementation study (Luo et al., 2009; Codoñer-Franch et al., 2012; Yadav et al., 2012); and the effect on TNF-α was not convincing (Codoñer-Franch et al., 2012; Shab-Bidar et al., 2012). For endothelial health, the relationship between vitamin D status and endothelial biomarkers were conflicting in epidemiological studies, but in supplementation trials, vitamin D supplement helps to improve endothelial health (Assimon et al., 2012; Haque et al., 2012). The investigations focusing on the relationship between vitamin D status and DNA damage and repair is few, only two supplementation trials were found, and the results were conflicting (Fedirko et al., 2010; Lan et al., 2014). Besides, there are lacks of well-rounded studies which detect the association between vitamin D and DNA damage/repair, oxidative stress, inflammation and endothelial health in a systematic and comprehensive manner.

Table 1.4. A Summary of Findings on the Relationship Between Vitamin D and Components of the 'Common Soil" of NCDs

A: Epidemiological Studies

Biomarker, measurement and method	Subjects	Result	Reference
Common soil element: Glycaemic control			
Fasting glucose	52 metabolic syndrome subjects and 58 apparently healthy subjects with fasting plasma glucose mean(SD) of 5.8(0.9) vs. 5.1(0.6) mmol/l, respectively	Plasma 25(OH)D significantly ($p<0.05$) lower in the metabolic syndrome group; median 29.5 vs. 43 nmol/l	Makariou et al., 2012
Common soil element: Oxidative stress & Antioxidant Status			
Plasma nitrotyrosine; malondialdehyde (MDA)	7~14 years old, severely obese, Caucasian children (n=66; 37 M; 29 F): 20 with plasma 25(OH)D <50nmol and 46 with 50 nmol/l or greater	Nitrotyrosine significantly higher in low 25(OH)D group; median 0.65 vs. 0.42 µmol/l; p=0.037. MDA significantly higher in low 25(OH)D group; median 0.91 vs. 0.50 µmol/l; p=0.015.	Codoñer-Franch et al., 2012

advanced oxidation protein products	411 non-diabetic subjects aged 19 to 80 years	No significant association was found between vitamin D status and the advanced oxidation protein products ($p>0.05$)	Krivošíková et al., 2015
Common soil element: Inflammation			
hsCRP	7~14 years old, severely obese, Caucasian children (n=66; 37 M; 29 F): 20 with plasma 25(OH)D <50nmol and 46 with 50 nmol/l or greater	No significant difference between the groups; median 2.24 vs. 3.0 µg/l; p=0.667	Codoñer-Franch et al., 2012
hsCRP	110 apparently healthy subjects, 52 of whom were found to have metabolic syndrome	Plasma 25(OH)D (log adjusted) and hsCRP showed no significant correlation in 52 Met Syndrome subjects (r=-0.096; p = 0.562)	Makariou et al., 2012
hsCRP	101 subjects with chronic kidney disease (CKD) and 40 healthy controls	Mean serum 25(OH)D significantly lower in CKD group; 44.7 vs. 67.2 nmol/l ($p<0.001$). Serum 25(OH)D correlated inversely with hsCRP (r=-0.2,	Yadav et al., 2012

		p=0.047) in CKD patients	
hsCRP	109 Chinese type 2 diabetes patients replete (n=70) or deficient (n=39) in vitamin D (threshold 50 nmol/l)	No significant difference across the groups (p=0.9); no change in hsCRP in those deficient subjects who were given 3m of 2000 IU/d vitamin D supplementation (n=21); no data shown	Luo et al., 2009
IL-6; TNF- α	7~14 years old, severely obese, Caucasian children (n=66; 37 M; 29 F): 20 with plasma 25(OH)D <50nmol and 46 with 50 nmol/l or greater	IL-6 significantly higher in vitamin D deficient group; median 18 vs. 13.7 ng/l; p=0.036; TNF-α showed no significant difference between groups; p=0.30	Codoñer-Franch et al., 2012
Common soil element: Endothelial Health/Dysfunction			
sICAM-1	186 rheumatoid arthritis patients aged 45-84 years	Plasma 25(OH)D showed inverse correlation with sICAM-1; p=0.020)	Haque et al., 2012
sICAM-1	7~14 years old, severely obese, Caucasian children	sICAM-1 showed no significant difference between the groups: p=0.983	Codoñer-Franch et al., 2012

sVCAM-1	(n=66; 37 M; 29 F): 20 with plasma 25(OH)D <50nmol and 46 with 50 nmol/l or greater	sVCAM-1 significantly lower in vitamin D sufficient group; 412 mg/l vs. 372 mg/l; p=0.040	Codoñer-Franch et al., 2012
E selectin		E selectin showed no significant difference between the groups: p=0.275	Codoñer-Franch et al., 2012
Common soil element: DNA damage and repair: No published studies found			

B: Supplementation trials

Biomarker	Subjects	Intervention Dose and Duration	Result	Reference
Common soil element: Poor glycaemic control				
Fasting plasma glucose; Insulin resistance; HbA1c	Meta-analysis of 15 studies	Varies from studies involved	For people with diabetes or impaired fasting glucose, significant lower fasting glucose and insulin resistance was seen in the vitamin D group: mean(95% CIs) values were -0.32 mmol/l (-0.57, -0.07) and -0.25(-0.48, -0.03), respectively. no significant change was seen in HbA1c; No significant improvement in any of these biomarkers seen in normal subjects.	George et al., 2012
Fasting plasma glucose;	Meta-analysis of 35 studies	Varies from studies involved	No significant difference between vitamin D group and placebo group was	Seida et al., 2014

Insulin resistance and secretion; HbA1c			seen in Fasting plasma glucose; insulin resistance and secretion or HbA1c	
Common soil element: Oxidative stress and antioxidant status				
Erythrocyte SOD activity	52 atopic dermatitis patients	Group A: vitamin D and E placebos (n=11); group B: 1600IU (40 µg) vitamin D_3 and vitamin E placebo (n=12); group C: taking 600IU (400 mg) synthetic all-rac-α tocopherol and vitamin D placebo (n=11); group D: 1600IU (40 µg) vitamin D_3 and 600 IU (400 mg) synthetic all-rac-α tocopherol (n=11); the supplementation lasted for 60 days	Baseline erythrocyte SOD activity showed a significant correlation with serum 25(OH)D; r=0.378, p=0.01; n=52; Erythrocyte SOD activity increased significantly in group B, C and D after 60 days' supplementation; (p=0.002, p=0.016, p=0.015, respectively)	Javanbakht et al., 2010

SOD activity; GPx activity; Serum MDA	23 postmenopausal patients (no vitamin D level before and after provided)	1,200 mg calcium, 800 IU (20 µg) vitamin D and 5 mg risedronate per day for 6 months	SOD decreased significantly after treatment; median 7.1 U/ml vs. 3.2 U/ml; p=0.006; GPx increased significantly after treatment; median 135 U/ml vs. 158 U/ml; p<0.05; MDA increased significantly after treatment; median 1030 vs. 1900 nmol/l; p<0.05	Zinnuroglu et al., 2012
Common soil element: Inflammation				

hsCRP; IL-6 peripheral blood mononuclear cells culture media	Type 2 diabetic patients	Yoghurt supplement contained 170 mg calcium and no detectable vitamin D/250 ml (n=50); vitamin D supplement contained 170 mg calcium and 500 IU (12.5 µg) vitamin D/250 ml, 500 ml per day (FD group, n=50) for 12 weeks	hsCRP increased significantly in yoghurt supplement group while decreased in FD group.(Yoghurt group: 1.56 mg/l vs. 2.50 mg/l; $p<0.001$; FD group: 2.04 mg/l vs. 1.60 mg/l; $p<0.01$); the treatment effect in hsCRP significantly lower in FD groups; mean +0.8 mg/l vs. -0.39 mg/l; $p<0.001$; IL-6 showed significant increase in PD group; mean 633.6 ng/l vs. 690.7 ng/l; $p<0.001$ but no significant difference in FD group (631.9 ng/l vs. 632.7 ng/l; $p=0.638$); The treatment effect in IL-6 significantly lower in FD group; +62.0 ng/l vs. +6.3 ng/l; $p=0.002$.	Shab-Bidar et al., 2012

IL-6	Haemodialysis patients	Case group: received vitamin D_2 for around 2 months (no dosage provided, n=20); control group: no special supplement (n=20)	No significant difference between groups; mean 4.19 ng/l vs. 4.92 ng/l; p=0.52, however, the vitamin D level of control group was as high as mean=62.2 nmol/l	Assimon et al., 2012
TNF-α peripheral blood mononuclear cells culture media	100 type II diabetic patients	Yoghurt supplement contained 170 mg calcium and no detectable vitamin D/250 ml (n=50); vitamin D supplement contained 170 mg calcium and 500 IU (12.5 µg) vitamin D/250 ml, 500 ml per day (n=50) for 12 weeks	TNF-α level showed no significant differences in both yoghurt supplement group (mean 108.6 ng/l vs. 204.0 ng/l; p=0.259) and FD group (mean 202.5 ng/l vs. 98.1 ng/l; p=0.122); The treatment effect in TNF had significant difference between PD group and FD group; mean +106.3 ng/l vs. -57.9 ng/l; p<0.05	Shab-Bidar et al., 2012

TNF-α	Haemodialysis patients	Case group: received vitamin D_2 for around 2 months (no dosage provided, n=20); control group: no special supplement (n=20)	No significant difference between groups; mean 12.0 ng/l vs. 11.1 ng/l; p=0.92	Assimon et al., 2012
Common soil element: Endothelial Health/Dysfunction				
sICAM-1; sVCAM-1; E-selectin	Haemodialysis patients	Case group: received vitamin D_2 for around 2 months (no dosage provided, n=20); control group: no special supplement (n=20)	sICAM-1 was significantly lower in case group; mean 235.5 mg/l vs. 176.0 mg/l; p<0.01; sVCAM-1 was significantly lower in case group; mean 2592.0 mg/l vs. 1933.4 mg/l; p<0.05; E-selectin showed no significant difference in groups; mean 90.7 mg/l vs. 85.6 mg/l; p=0.059; However, serum 25(OH)D had a significant correlation with E-selectin; r=-0.34, p<0.05	Assimon et al., 2012

Common soil element: DNA damage				
8-oxodG Labelling in rectal biopsy samples	Previous colorectal adenoma patients	800 IU (20 µg)/d vitamin D_3 (n=23) or 800 IU (20 µg)/d vitamin D_3 plus 2.0 g calcium (n=23) or matching placebo (n=23) for 6 months prior to biopsy	No significant difference in 8-oxodG in vitamin D group and vitamin D plus calcium group compared with placebo group, all $p>0.05$	Fedirko et al., 2010
DNA damage score in alkaline comet assay	Asthma patients	300,000 IU vitamin D was given to 8 subjects at day 1 and day 4 (n=8), or matching placebo (n=8) Both groups received 80 mg/day of methylprednisolone for 7 days	Significantly higher DNA damage score was seen in the placebo group compared with the vitamin D group on day 7. The DNA damage score estimated from image was ~80% and ~10%, respectively	Lan et al., 2014

SUMMARY AND FOCUS OF THE STUDY

Vitamin D is less a true vitamin than a pre-hormone, and it plays a vital role in bone health. However, accumulating but as yet incomplete evidence shows that vitamin D is important for overall health protection and promotion far beyond the skeletal system. Vitamin D can be obtained from food, however there are only a few foods that are good natural sources of vitamin D. Some countries have mandatory food fortification programmes, but there is no such requirement in Hong Kong. The main source of vitamin D is cutaneous synthesis, and this requires exposure to UVB. Generally, a healthy person can synthesize enough vitamin D to meet their daily requirements with only 10~15 min of sun exposure daily. Still, vitamin D inadequacy is very common in many parts of the world. There are many factors that can lead to vitamin D deficiency, such as ageing, obesity, vegetarian diet and diseases, but the most important factor is the lack of exposure to sunshine. The modern lifestyle is largely an indoor lifestyle for both work and leisure activities, and that lack of vitamin D is highly prevalent, and this has important implications for the long-term health of young people. However, data on young adults and children in Hong Kong are lacking. This is an important knowledge gap because of the postulated influence of vitamin D on human health. Vitamin D deficiency has been reported to associate with increased risk of various NCDs. The mechanisms are unclear, but vitamin D deficiency has also been linked to biological features including increased DNA damage, poor glycaemic control, dyslipidaemia, hypertension, inflammation, oxidative stress, to poorer DNA repair and to endothelial dysfunction, all of which underlie the development of chronic disease, forming the so-called “common soil”. To date, no comprehensive biomarker study has been performed

looking at the underlying biological impact of vitamin D deficiency on these various features, nor has there been any study of how improvement of vitamin D status affects these various health-related features. This is the focus of this study, which is represented in Figure 1.8. If vitamin D deficiency is found to be associated with the elements of the 'common soil' in early adults life (which can be detected by using sensitive biomarkers), the impact of these "hidden" but important changes that link to NCDs development on their long-term health is profound.

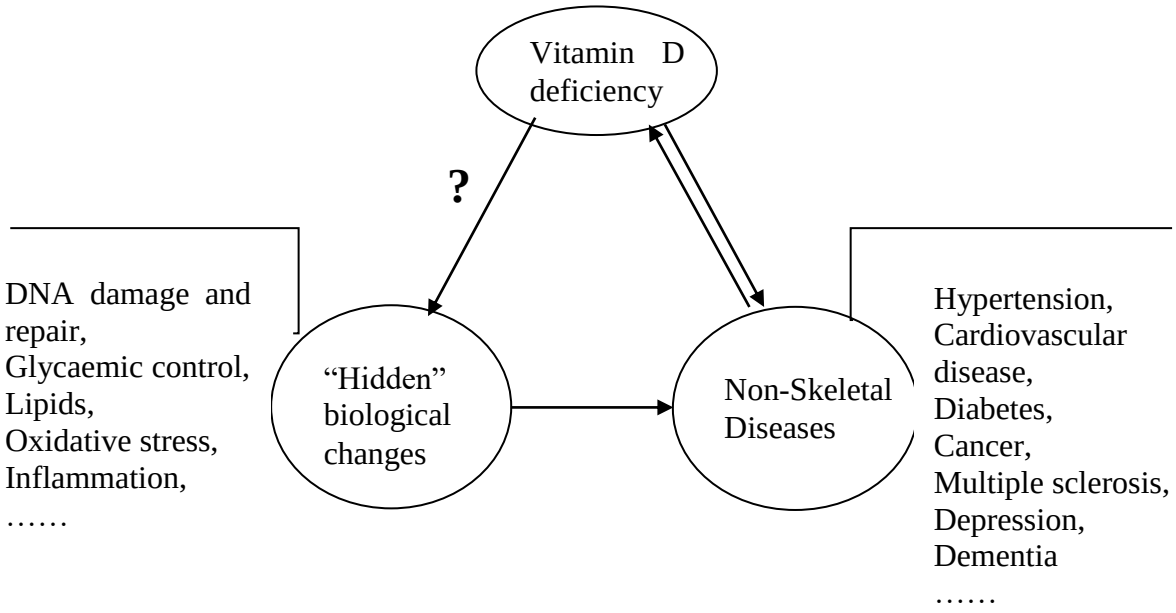


Figure 1.8. Possible Influence of Vitamin D on Non-Skeletal Disease and Potential Mechanisms

CHAPTER 2

AIMS AND STUDY DESIGN

MAIN AIMS

1. To determine the vitamin D status (plasma 25(OH)D concentration) of a group of young, apparently healthy adults in Hong Kong;
2. To investigate the inter-relationship between vitamin D status and selected biomarkers of interest (cardiometabolic risk factors, DNA damage, and antioxidant status and oxidative stress, Table 2.1);
3. To explore the response of the biomarkers to 12 weeks of vitamin D supplementation in a sub-group of subjects found to have low vitamin D status.

SECONDARY AIM

4. To investigate the cardiometabolic risk factors profile (blood pressure, fasting plasma glucose, haemoglobin A1c (HbA1c), total cholesterol, high density lipoprotein cholesterol (HDL-C), TC/HDL-C ratio, low density lipoprotein cholesterol (LDL-C), triglycerides (Tg), Log(Tg/HDL-C), high sensitivity C reactive protein (hsCRP) and uric acid) in a group of young, apparently healthy adults in Hong Kong.

In this study, the biomarker focus was on:

- Vitamin D status (assessed by plasma 25(OH)D concentration);
- Cardiometabolic risk factors (assessed by blood pressure, fasting plasma glucose, HbA1c, TC, HDL-C, TC/HDL-C ratio, LDL-C, Tg, Log(Tg/HDL-C), hsCRP and uric acid in plasma);
- DNA damage in peripheral lymphocytes (assessed using the formamidopyrimidine-DNA glycosylase (Fpg)-assisted comet assay);
- Antioxidant status and oxidative stress (assessed by measuring plasma Ferric Reducing Antioxidant Power (FRAP) value (total and corrected for urate), ascorbic acid, allantoin, and urine 8-oxo-2'deoxyguanosine (8-oxodG)).

POWER CALCULATION

The power calculation was performed using GPower 3.1. The previous work of our group was used to estimate the SD and effect size for calculating the effect size for this study (Ma et al., 2005; Choy et al., 2005; Han et al., 2011). In the comet assay, the mean(SD) of the %DNA in the comet tail (our key biomarker in this study) of healthy subjects is generally around 15(3.5). For the observational study, with 120 subjects (providing 40 subjects in each tertile of vitamin D concentration), a true difference of +/- 2.0% in the DNA damage score can be detected (using ANOVA) with 0.8 power and a significance level of 0.05. For fasting plasma glucose, which was found to have potential association with vitamin D status in previous studies (George et al., 2012), SD value used in the calculation was 0.4 mmol/l, based on the previous healthy people in this age range data (Simon et al., 1992). With 200 subjects (providing 50 subjects in

each quartile of vitamin D concentration), a true difference of +/- 0.1 mmol/l in fasting plasma glucose can be detected (using ANOVA) with 0.92 power and a significance level of 0.05.

In this study, the target sample size in the observational arm was 200, and 204 subjects were recruited, but only 196 samples were available for analysis.

EXPERIMENTAL DESIGN

This study was approved by the Human Subjects Ethics Sub-committee of the Hong Kong Polytechnic University, and all volunteers gave their written informed consent. All procedures involving human subjects complied with the Declaration of Helsinki.

There were two parts in this study: A) an observational study (the main part), and B) a pilot supplementation study with vitamin D.

A. Observational study

A total of 204 subjects (with only 196 viable samples available), all in self-reported good general health and aged 18-26, were recruited during January 2014 to March 2016 with their written informed consent. A fasting venous blood sample (20 ml) and a fasting mid-stream urine sample were collected from each subject (see Chapter 3 for detailed procedures of sample collection and processing). Plasma 25(OH)D concentration was used as the vitamin D status indicator, and measured by LC-MS/MS. The various biomarkers were measured using validated techniques and according to

published protocols and our established procedures, or following manufacturers' instructions (for commercial kits). All methods used are given in detail in Chapter 3.

B. Supplementation trial-a pilot study

A sub-group of subjects from part 1 who were found to have low vitamin D status (plasma 25(OH)D <40 nmol/l) were recruited into a placebo-controlled, double-blinded supplementation study with their written informed consent. The volunteers in the supplementation trial took 2,400 IU per day of vitamin D (2 tablets of 1,200 IU) for 12 weeks, or a matching placebo. Fasting blood and urine samples were collected at baseline and the end of the 12 weeks' supplementation period.

Table 2.1. Biomarkers of Interest in Parts A and B of the Study, and the Corresponding Method Used

Detailed protocols are given in Chapter 3; all samples were fasting samples; plasma used was heparinized except for glucose, in which case fluoride samples were used.

Biomarker	Sample	Method
25(OH)D	plasma	LC-MS/MS
Glucose^	plasma	Commercial kit
HbA1c^	whole blood	Commercial kit
Total cholesterol^	plasma	Commercial kit
HDL-C^	plasma	Commercial kit
LDL-C^	plasma	by Friedewald calculation
Triglycerides^	plasma	Commercial kit
Uric acid ^	plasma	Commercial kit
hsCRP^	plasma	Commercial kit
DNA damage^	lymphocytes	Fpg-assisted comet assay
FRAP value^	plasma	FRAP assay
Ascorbic acid^	plasma	FRASC assay
allantoin	plasma	LC-MS/MS
8-oxodG	urine	LC-MS/MS
creatinine ^	urine	Commercial kit

This PhD student investigator was directly involved in subject recruitment, sample collection and processing, and in measuring the biomarkers marked^. The biomarkers that were measured by LC-MS/MS were analyzed by an experienced member of the

Chief Supervisor's research team, and the data were provided to this investigator for data analysis, which was also performed by this investigator.

CHAPTER 3

MATERIALS & METHODS

All subject criteria and measurement methods used are consolidated in this chapter, in order to focus the subsequent chapters on specific aspects of the study being addressed, and the data collected and analysed.

This study received the ethical approval from the Human Subjects Ethics Sub-committee of The Hong Kong Polytechnic University (Appendix I).

SAMPLE COLLECTION

Inclusion criteria

A group of apparently healthy young subjects were recruited, all with their written informed consent. The subjects met the recruitment requirements as follows:

- Aged between 18 to 26 years
- In self-reported good general health
- Were not suffering from any infectious or chronic disease
- Had not been hospitalized within the previous 12 months
- Had not required medical treatment in the previous 6 months
- Did not smoke
- Were not overweight or obese (body mass index <24 kg/m^2 for females and <25 kg/m^2 for males)
- Did not take any regular medication
- Did not take any regular supplements of vitamins or minerals or Chinese medicines.

Sampling procedures

After the volunteer read the information sheet and signed the informed consent forms, fasting blood samples were collected by an experienced phlebotomist, and a mid-stream urine sample was collected into a clean container with no preservative, either immediately prior to or after blood collection. Samples were coded. A total of 20 ml of venous blood was collected from each volunteer; 16 ml was assigned to heparin blood collection tubes, 2 ml to an EDTA blood collection tube and 2 ml to a sodium fluoride blood collection tube. Heparin blood and sodium fluoride blood samples were centrifuged at 2000 rpm (~1000 g) for 8 min using a Jouan SA centrifuge (Jouan SA, Saint-Herblain, France), and plasma was separated immediately. Fluoride plasma was harvested and used for glucose measurement on the sampling day. Heparinized plasma from each volunteer was aliquoted and stored at -80^{o}C until use except for the Ferric Reducing Antioxidant Power and ascorbic acid. EDTA blood was stored at 4 ^{o}C, and used for haemoglobin A1c (HbA1c) measurement within one week of collection. Lymphocytes from heparinized blood were harvested within 1h of collection and used in the comet assay.

Blood pressure (systolic and diastolic blood pressure) and body weight and height were measured in all subjects after fasting blood and urine sample collection, within one week of the sampling. Body mass index (BMI) was calculated as BMI=weight/height^{2}, the unit for weight is kilogram (kg), and the unit for height is meter (m). Blood pressure and BMI were measured in the University Health Centre.

MEASUREMENT METHODS

Plasma 25(OH)D by LC-MS/MS

This measurement method mainly followed the protocol of Ong et al., for sample pre-preparation (Ong et al., 2012), with some modification.

Mobile phase

Two mobile phases were used in the plasma 25(OH)D measurement, they were prepared as follows: mobile phase A was 70.5% v/v methanol and 29.5% v/v aqueous ammonium acetate (4.0 mmol/l); mobile phase B was 95% v/v methanol and 5% v/v aqueous ammonium acetate (23.6 mmol/l), both mobile phase A and B were adjusted to a final pH of 5.25 with acetic acid. The mobile phases were filtered with 0.22-µm Millipore filter before use. Water used in the mobile phase was MilliQ water (18.2 MΩ. cm); ammonium acetate (A.S.C. reagent, ≥97.0%) was purchased from Sigma (Sigma-Aldrich Inc., St. Louis, MO); methanol (HPLC grade) was from Duksan (Duksan Pure Chemicals Co., Ltd., Korea).

Internal standard, calibrators and controls

3-epi-25-Hydroxyvitamin D_3 was used as internal standard. The stock internal standard was 500 nmol/l internal standard dissolved in methanol. Before use, the stock internal standard was diluted by water to 250 nmol/l as routine use internal standard. 3-epi-25-Hydroxyvitamin D_3 was purchased from Sigma.

Commercial calibrators and controls were used in the test. 3PLUS® Multilevel serum calibrators set 25-OH-Vitamin D3/D2 was used as calibrator; Lyophill, for 25-OH-Vitamin D3/D2, Level I&II were used as controls. Calibrator was purchased from Chromosystems (Chromosystems, Gräfelfing, Germany), and controls were purchased from Recipe (Recipe, München, Germany).

Sample pre-preparation procedures (Ong et al., 2012)

Plasma samples from subjects and calibrators and controls were thawed at room temperature, and 100 µl of a 250 nmol/l of working internal standard was spiked into 300 µl of thawed plasma samples (test samples, plasma-based controls and calibrators), incubated in a 37ºC water bath for 1 hour after mixing well. After incubation, the plasma was mixed with 30 µl 1 mol/l aqueous sodium hydroxide, vortexed for 10 s, then 200 µl 0.6 mol/l aqueous zinc sulphate was added, followed by 1 min vortex for deproteinization. 600 µl methanol was added into the tube, followed by vortex mixing, then 615 µl of the mixture was transferred to a clean tube. Hexane was used to extract the 25(OH)D and internal standard from the deproteinized plasma: 1 ml hexane was added to both tubes, and mixed with the plasma by vortexing for 10 min. Hexane was separated by centrifugation at 3,000 g for 2 min, the supernatant from two tubes (but with the same sample) was removed to a clean glass tube. To totally extract 25(OH)D, the remaining liquid was pooled in one tube, vortexed for 5 s followed by centrifugation at 3,000 g for 1 min. As much hexane as possible was removed to the glass tube, and another 200 µl hexane was added to the mixtures, with vortexing for 1 min, followed by centrifugation at 3,000g for 1 min. The supernate hexane from all steps was transferred to the glass tube, and evaporated under nitrogen gas at 45^{o}C. The dried 25(OH)D extract was re-dissolved in 50 µl of mobile phase B and 20 µl mobile phase A, the re-dissolved

25(OH)D was removed to another clean tube and centrifuged at 15,000 g for3 min. forty microliters of this mixture was injected into the insert, all the samples were kept in the autosampler (4 ^{o}C) before injection. A typical extraction requires 4~5 hours and a maximum of 20 samples can be extracted in one batch. The extraction/sample pre-preparation procedure is summarized in Figure 3.1.

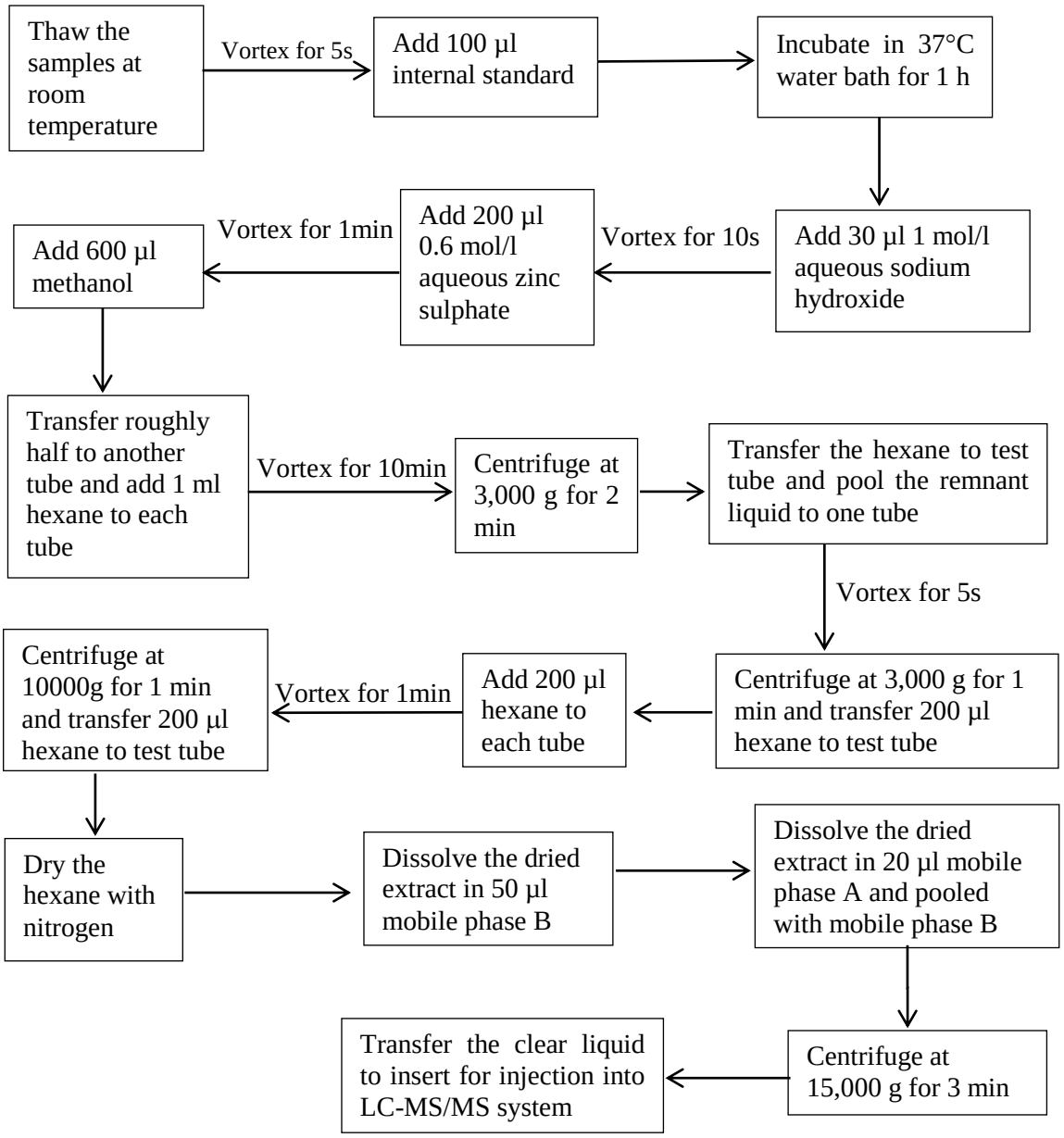


Figure 3.1. The Flow of Sample Preparation for 25(OH)D Measurement

LC-MS/MS settings:

An Agilent Zorbax StableBond (SB-CN) column (80Å, 5μm, 4.6mm ID x 250mm L) and Agilent Zorbax StableBond guard cartridge (5μm, 4.6mm ID x 12.5mm L) were linked and used for 25(OH)D separation. The HPLC system was from Agilent (Agilent 1100 Series, quaternary pump, G1311A and microvacuum degasser, G1379A, Agilent Technologies Inc., Waldbronn, Germany). A non-thermostatted well-plate autosampler (G1313A) was used. The mass spectrometer linked to the HPLC system was a 3200 QTRAP mass spectrometer from Applied Biosystems/MDS Sciex (MDS Inc., Concord, ON, Canada).

The "plasma measuring" sample run time for plasma 25(OH)D measurement was 30 min per sample, with a constant flow rate of 1.0 ml/min. The program used was a gradient elution. The mobile phase allocation was as follows: 1-17 min, 100% mobile phase A; 17.1-25 min, 100% mobile phase B; 25.1-30 min, 100% mobile phase A. The HPLC eluate at 12.5-18.5 min was sent to the mass spectrometer for analysis, and the rest went to waste.

Tandem mass spectrometry settings

The MS/MS analysis was performed in positive ion mode with a TurboIonSpray® source (Sciex, Framingham, United States). The source parameters were set as follows: curtain gas (nitrogen), 14.0 psi; collision gas (nitrogen), medium, ionspray voltage, 5500 V; temperature, 350 °C; ion source gas 1 (air), 11.0psi; ion source gas 2 (air), 30.0 psi; and interface heater was on. All MS data were obtained by multiple reaction monitoring (MRM) acquisition: for 25(OH)D_3, quantifier precursor ion ([M+H]+ at m/z

401.5amu, Q1), product ion (at m/z 383.5amu, Q3); qualifier at 401.5/365.0; for 25(OH)D_2, quantifier at 413.4/395.4; qualifier at 413.4/337.5; IS, quantifier at 404.4/386.5; qualifier at 404.4/368.5. All the product ions were quantified using dwell time 500ms/channel with unit mass resolution for Q1 and Q3. The parameters for the optimized compound were: for 25(OH)D_3, declustering potential (DP), 66.0V, entrance potential (EP), 4.0V, collision entrance potential (CEP), 20.0V, collision energy (CE), 15.0eV, and, collision cell exit potential (CXP), 6.0V; for 25(OH)D_2, DP, 69.0V, EP, 4.0V, CEP, 15.0V, CE, 14.0eV, CXP, 6.0V; for IS, DP, 49.0V, EP, 1.0V, CEP, 15.0V, CE, 13.0eV, CXP, 3.0V.

Quantification of LC-MS/MS results

All data were analyzed by Analyst® Software 1.4.2 (Sciex) with ".wiff" files. After selecting the target sample from the wiff files, 25(OH)D_3, 25(OH)D_2 and internal standard can be distinguished by the retention time as: 25(OH)D_3 was 14.3 min; 25(OH)D_2 was 15.6 min; and IS was 14.2 min. Manual analysis was used in this method with "manual Integration mode". After expanding the chromatogram by clicking "Expand Active Graph", the peak area was determined by dragging from top moving downwards in the baseline, and accepted by clicking "Accept". Next sample was shown in "Show All Graph", the steps before were repeated until all samples were finished. Peak area was used for analysis in this study.

Plasma Glucose and Urine Creatinine by automatic biochemical analyzer

The glucose (in sodium fluoride plasma) and creatinine (in urine) were measured by the Hitachi 902 Chemistry Analyzer (Roche Diagnose, Basel, Switzerland) or the AU 480 Chemistry System (Beckman Coulter, California, United States) using, respectively,

glucose oxidase linked spectrophotometric method and the Jaffe method. Glucose and creatinine were measured on the same day as sample collection. Urine was pre-diluted into 1/5, 1/10 and/or 1/20 as appropriate for Hitachi 902 but not for AU480, as the AU480 has urine mode, while Hitachi 902 does not.

Basic principles

Glucose:

$$\text{glucose + ATP} \xrightarrow{\text{Hexokinase, } Mg^{2+}} \text{glucose-6-phosphate + ADP}$$

$$\text{glucose-6-phosphate + } NAD^{+} \xrightarrow[\text{dehydrogenase}]{\text{Glucose-6-phosphate}} \text{6-phosphogluconate + NADH + } H^{+}$$

For a clear appearance in principle, abbreviations are used:

ATP: adenosine triphosphate

ADP: adenosine diphosphate

NAD^{+}: nicotinamide adenine dinucleotide

NADH: nicotinamide adenine dinucleotide

The change in absorbance at 340/380 nm is proportional to the amount of glucose present in the sample.

Creatinine:

$$\text{creatinine + alkaline picrate} \longrightarrow \text{yellow-orange complex}$$

The rate of change in absorbance at 520/800nm is proportional to the creatinine concentration.

Calibrator and controls

For Hitachi 902, Calibrator for automated system (c.f.a.s) was used as calibrator and for AU 480, Chemistry calibrator was used. Lyphochek® Assayed Chemistry Control (levels I and II) were used as controls. The c.f.a.s was purchased from Cobas® (Cobas® Roche, Rotkreuz, Switzerland), Chemistry calibrator was purchased from Beckman Coulter, and the controls were purchased from Bio-Rad (Bio-Rad laboratories, Hercules, California, USA).

Reagents

Glucose was measured by Gluco-quant for Roche/Hitachi, and creatinine was measured by CREA for Roche/Hitachi. The reagents were ordered from Cobas®. The reagents for AU480 were all purchased from Beckman Coulter, OSR6121 for glucose and OSR6178 for creatinine.

Procedures (manufacturer instructions were followed)

Hitachi 902:

Calibrators and controls were loaded in the rack, and the test was selected from the pre-programmed test menu. After calibration, samples were tested through selecting Glu and Crea from the menu.

AU 480:

Calibrators and controls were loaded in the rack, and the test was selected from the menu. After calibration, samples were tested through selecting tests from the programmed test menu.

HbA1c by ion exchange chromatograph

EDTA whole blood was used for HbA1c, and the test was done within one week from the sampling day, with samples stored at 4^{o}C until tested. The HbA1c was measured with commercial kits using Hemoglobin A1c, a column chromatographic – spectrophotometric, ion exchange method, purchased from BioSystems (Barcelona, Spain).

Controls

Glycated Hb Control (Normal) and Glycated Hb Control (Elevated) were used as controls in this test. They were purchased from BioSystems.

Procedures

Fifty microliters of EDTA whole blood or control was mixed with 200 µl reagent 1 for lysis, with thorough mixing, and these were left to stand at room temperature for 10 to 15 min. Then 50 µl of the haemolysate was added into the drained HbA1c column, and 200 µl reagent 2 was added after the haemolysate had been absorbed onto the column. A further 2 ml reagent 2 was added to each column, and drained to waste. The column was then transferred to a clean test tube and 4.0 ml reagent 3 was added in. The eluate of reagent 3 was collected and the absorbance at 415 nm was read within 40 min. A_{HbA1c} was used to represent the reading of the reagent 3 eluate against distilled water.

Fifty microliters of haemolysate was added to 12.0 ml reagent 3 and the absorbance at 415 nm wavelength was read within 40 min. $A_{HbTOTAL}$ was used to represent the reading of this mixture against distilled water. The HbA1c relative concentration was calculated as follows:

$\%HbA1c = (A_{HbA1c/} A_{HbTOTAL})*(100/3)$

(note: %HbA1c was used in this project, not mmol/mol as we are more familiar with this unit (Little and Sacks, 2009))

Plasma lipid profiles, uric acid and hsCRP by automatic biochemical analyzer and immunoassay

The measurements were performed by AU480 Chemistry Systems from Beckman Coulter. Total cholesterol, triglycerides, uric acid were measured by enzyme linked spectrophotometric methods; HDL-C was measured by enzymatic spectrophotometric method, with other lipoprotein blocked by anti human-β-lipoprotein antibody; and hsCRP was measured by a latex immunoassay method.

Basic principles

Total cholesterol:

$$\text{2 cholesterol esters} + 2H_2O \xrightarrow{\text{cholesterol esterase}} \text{2 cholesterol} + \text{2 fatty acid}$$

$$\text{2 cholesterol} + 2\ O_2 \xrightarrow{\text{cholesterol oxidase}} \text{2 cholestene-3-one} + 2\ H_2O_2$$

$$2\ H_2O_2 + \text{4-aminoantipyrine} + \text{Phenol} \xrightarrow{\text{peroxidase}} \text{quinoneimine} + 4\ H_2O$$

The absorbance of the red-coloured quinoneimine was measured by spectrophotometer at 540/600 nm.

HDL-C:

$$HDL\text{-}C + H_2O + O_2 \xrightarrow{\text{cholesterol esterase and oxidase}} \text{cholest-4-en-3-one} + H_2O_2$$

$$H_2O_2 + \text{4-aminoantipyrine} + DSBmT \xrightarrow{\text{peroxidase}} \text{blue colour complex}$$

The absorbance of the blue colour complex was measured by spectrophotometer at 600/700 nm.

Triglycerides:

$$\text{triglycerides} + 3\ H_2O \xrightarrow{\text{lipase}} \text{glycerol} + 3\ \text{fatty acids}$$

$$\text{glycerol} + ATP \xrightarrow{\text{glycerol kinase, } Mg^{2+}} \text{glycerol-3-phosphate} + ADP$$

$$\text{glycerol-3-phosphate} + O_2 \xrightarrow[\text{oxidase}]{\text{glycerol phosphate}} H_2O_2 + \text{Dihydroxyacetone phosphate}$$

$$H_2O_2 + \text{4-aminoantipyrine} + MADB \xrightarrow{\text{peroxidase}} \text{blue Dye} + OH^- + H_2O$$

For a clear appearance in principle, abbreviations are used:

MADB: 4-aminophenazone and N,N-bis(4-sulfobutyl)-3,5-dimethylaniline, disodium salt

The absorbance of the blue colour complex was measured by spectrophotometer at 660/800 nm.

Uric acid:

$$\text{Uric Acid} + O_2 + H_2O \xrightarrow{\text{uricase}} \text{allantoin} + CO_2 + H_2O_2$$

$$H_2O_2 + \text{4-Aminoantipyrine} + \text{MADB} \xrightarrow{\text{peroxidase}} \text{Blue Dye} + OH^- + H_2O$$

The absorbance of the blue colour complex was measured by spectrophotometer at 660/800 nm.

hsCRP:

Latex particles coated by rabbit anti-CRP antibodies were used in this method. The particles in solution scattered light in proportion to size, shape and concentration. In this method, the reaction between CRP in plasma samples reacted with the antibodies, and this reaction leads to a decrease in light intensity reaching the detector due to turbidity, and the rate of decrease in absorbance was used to calculate the concentration of CRP in plasma samples according to the absorbance of calibrator curve.

Calibrators

Chemistry calibrator was used for total cholesterol, triglycerides and uric acid; HDL cholesterol calibrator was used for high density lipoprotein (HDL-C); CRP LATEX calibrator [highly sensitive (hs) set] was used for high sensitive C reactive protein (hsCRP). All the calibrators were purchased from Beckman Coulter.

Controls

Lyphochek® Assayed Chemistry Control (levels I and II) from Bio-Rad were used as controls for total cholesterol, triglycerides, HDL-c and uric acid; Precicontrol ClinChem Multi 1 from Cobas® was used as control for hsCRP.

Reagents

The reagents were all purchased from Beckman Coulter (OSR6116 for total cholesterol, OSR6287 for HDL-C, OSR60118 for triglycerides, OSR6098 for uric acid and OSR6199 for hsCRP).

LDL-C:

The LDL-C concentration was calculated by Friedewald equation (Friedewald et al., 1972): (note: all biomarkers involved were using mmol/l as units

LDL-C = TC - HDL-C - Tg/2.2

Procedures (manufacturer instructions were followed)

Calibrators and controls were loaded in the rack, and the test was selected from the menu. After calibration, samples were tested through selecting tests from the menu.

Fpg-assisted comet assay

The reagent preparation and procedure of the comet assay followed the protocol established by this research team as well as the ComNet project (Wong et al., 2005; Collins et al., 2014).

Working Stock Formamidopyrimidine DNA glycosylase (Fpg) Solution

Fpg was a commercial enzyme purchased from Trevigen with 500 units in each aliquot. The enzyme was diluted in Fpg diluting solution in 1:1 ratio. This 'working stock' enzyme solution was aliquoted into Eppendorf tubes with 7 µl of the enzyme solution added to each tube. These were placed immediately at -80^{o}C and stored frozen until used. The Fpg diluting solution was 2X Fpg reaction buffer and glycerol in 1:1 ratio.

Working Fpg solution

One aliquot of working stock Fpg solution was thawed on ice within 3min before use by adding a total amount of 693 µl of cold 1X Fpg reaction buffer to the Eppendorf tube.

10X Fpg reaction buffer

10X Fpg reaction buffer solution was made of 0.4M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1M potassium chloride (KCl), 5mmol/l Na_2EDTA and 2mg/ml Bovine Serum Albumin (BSA). The solution was adjusted to pH 8.0 using concentrated potassium hydroxide (KOH) solution and pellets. The 10X Fpg reaction buffer was aliquoted and stored at -80^{o}C. Before use, one aliquot was thawed and diluted to 1/10 and 1/5 with MilliQ water to give, respectively, 1X and 2X Fpg working reaction buffer, stored at 4^{o}C for less than 1 week.

Phosphate buffered saline (PBS)

Commercial PBS was purchased from Sigma (cat no. p5368, pH 7.4, BioPerformance Certified grade), one bag of PBS powder was dissolved in 1 litre MilliQ water, stored at room temperature.

1% (w/v) standard agarose for coating microscope slides

Typically, 0.5 g standard agarose (Ameresco, Solon, OH, USA) was dissolved in 50 ml distilled water, and heated in a microwave oven until dissolved. The clean microscope slides (Sailing boat, Jiangsu, China) were coated in agarose by dipping the slides into the agarose solution before solidification. One side of each slide was wiped clean of agarose, and the slides were laid flat on this side for the agarose to dry. The slides were

then collected in a paper box with desiccant and stored at a dry place at room temperature.

1% (w/v) standard agarose in PBS

Typically, 0.05 g standard agarose was dissolved in 5 ml PBS, heated in a microwave oven avoiding boiling. The standard agarose was purchased from Ameresco (Solon, OH, USA).

1% (w/v) low melting point agarose in PBS

Typically, 0.05 g standard agarose was dissolved in 5 ml PBS, heated in microwave oven avoiding boiling, and then placed in 37^{o}C water bath for a minimum 5 min before use. The low melting point agarose was purchased from Sigma.

Lysis solution

Lysis solution was made of 0.1M ethylenediaminetetraacetic acid (Na_2EDTA; 32.22g/l), 2.5 mol/l sodium chloride (NaCl), and 10 mmol/l Tris in MilliQ water: it is noted that the chemicals were added into the MilliQ water one by one, with each totally dissolved before the addition of the next. The solution was adjusted to a final pH of 10.0 using concentrated sodium hydroxide (NaOH) solution and pellets. The lysis solution was stored at 4^{o}C until use. Just before use, Triton X-100 was added into the buffer to give 1% (v/v) Trixon-X, i.e. 400 μl Triton X-100 was added into 40 ml of lysis solution.

10X electrophoresis buffer

10X Electrophoresis buffer was made of 3 mol/l NaOH and 10 mmol/l Na_2EDTA, stored at 4°C. 1X working buffer was prepared by diluting the 10X electrophoresis buffer 1/10 in MilliQ before use.

Na_2EDTA, NaCl, NaOH, KCl, KOH, Triton X-100, and BSA were purchased from Sigma at molecular biology grade, HEPES was also purchased from Sigma at biotechnology grade, Tris was purchased from GE Healthcare (GE Healthcare, Uppsala, Sweden).

10X SYBR Green

SYBR Green was purchased from Life Technologies (Life Technologies, Carlsbad, CA, USA) as "10,000X" concentration in dimethylsulfoxide (DMSO) (this concentration was provided by the manufacture). The SYBR Green was diluted to 100X in DMSO as a working stock solution, and stored at -20°C until use. The 100X SYBR Green was thawed before use and diluted with DMSO to 50X, and this was further diluted with MilliQ water to give 10X, and the 10X SYBR Green was used in the gel staining.

Lymphocyte harvesting from venous blood samples

After plasma was removed, the buffy coat in venous blood in a centrifuged heparin blood tube was transferred to a centrifuge tube using an autopipette, and the white cells were washed by adding 3 ml cold phosphate buffered saline (PBS) before underlying the cell suspension with ~3 ml Histopaque®-1077 using a disposable dropping pipette. Mononuclear cells (mainly lymphocytes and some monocytes, in this study,

'lymphocytes' is used to refer to the harvested cells) are isolated due to density differences, and lie between the PBS and Histopaque®-1077 layers after 20 min centrifugation at 2,000 rpm (~1,000 g, by Jouan SA centrifuge) at 20ºC (without brake on), and can be seen as a blurry layer between these layers. The lymphocytes were transferred to a clean centrifuge tube and washed with PBS by re-suspending into 3 ml cold PBS and then centrifuged at 1,200 rpm (~360 g) 4ºC for 5 mins. The PBS was discarded and the wash step was repeated. After removing the PBS, the cells were re-suspended in freezing medium containing 90% foetal bovine serum (FBS) and 10% DMSO and aliquoted into to 1.5 ml Eppendorf tubes. The cells were immediately stored in a thick walled polystyrene box and placed in the freezer for cooling to -80ºC slowly (about 1ºC per minute). Cells were stored at -80ºC and each aliquot was thawed once only for use.

The chemicals used for lymphocyte harvesting were purchased from Sigma, and were of molecular biology grade or equivalent.

Fpg comet assay procedures

Seventy microliters 1% (w/v) standard agarose in PBS was applied on two areas of a pre-coated slide, covered by cover slips (18X18 mm; from Deckgläser, Western Germany) and left to set at room temperature for at least 5 min, after which cover slips were removed. Thus, two gels were on one slide. An aliquot of cryopreserved lymphocytes was removed from the freezer and centrifuged at 1200 rpm (~125g, with Beckman Coulter microcentrifuge) for 5 min after thawed. Freezing medium was removed and the cell pellet was re-suspended in 1 ml cold PBS to wash away the freezing medium. The PBS was removed after centrifugation at 1200 rpm (~125g) for 5

min and the wash step was repeated once more. Then 800 µl warm 1% (w/v) low melting point agarose (in PBS, and taken from the 37^{o}C water bath as noted above) was added to the cell pellet in the Eppendorf tube, with gentle mixing, immediately after which, 70 µl agarose/cell mixture was applied onto each of the two standard agarose gels on two microscope slide, i.e. there were two slides (total 4 gels) for each sample. Cover slips were placed onto each gel and the slides were placed at room temperature for 8 min to solidify. The cover slips were removed after solidification. The slides were placed in a coplin jar containing lysis solution (with 1%v/v Triton X-100) at 4^{o}C for one hour. During the lysis time, cell membranes and nuclear envelopes and histones were removed. Slides were transferred to another clean coplin jar containing 1X enzyme reaction buffer and incubated for 5 min to wash away the lysis solution. This wash step was repeated three times. After washing, the slides were placed in a moisture chamber which was prepared by layering a box with wet tissue. One slide (with two gels) of each sample received enzyme reaction buffer treatment and the other slide (with two gels) received Fpg treatment. For these treatments 50 µl 1X enzyme reaction buffer or 50 µl of Fpg working solution was added directly onto each gel. The gels were covered by parafilm after the application of buffer or enzyme. The moisture chamber was incubated in a 37 ^{o}C incubator for 30 min, providing reaction time for the enzyme. Parafilm was removed, and slides were then moved to a coplin jar with 1X electrophoresis buffer, and incubated at 4^{o}C for 20 min. The electrophoresis buffer was discarded afterwards, and a fresh supply of 1X electrophoresis buffer was placed in the jar, and slides incubated for another 20 min. During this time the DNA unwinds at the high pH of the electrophoresis incubation. After DNA unwinding, slides were placed on a platform of an electrophoresis tank and immersed in cold 1X electrophoresis buffer. The electrophoresis tank was surrounded by ice in a tray. The “ice bath” provided a cold

environment during the electrophoresis time. Electrophoresis lasted for 30 min at voltage of 25V and current of ~300 mA, which was adjusted by the volume of electrophoresis buffer. Electrophoresis pulls relaxed loops of DNA containing single strand breaks toward the anode, forming the "tail" of "comet", which is visible after DNA staining under a fluorescent microscope. After electrophoresis, slides were incubated in PBS for 5 min, followed by incubation in distilled water for 5min twice (Figure 3.2).

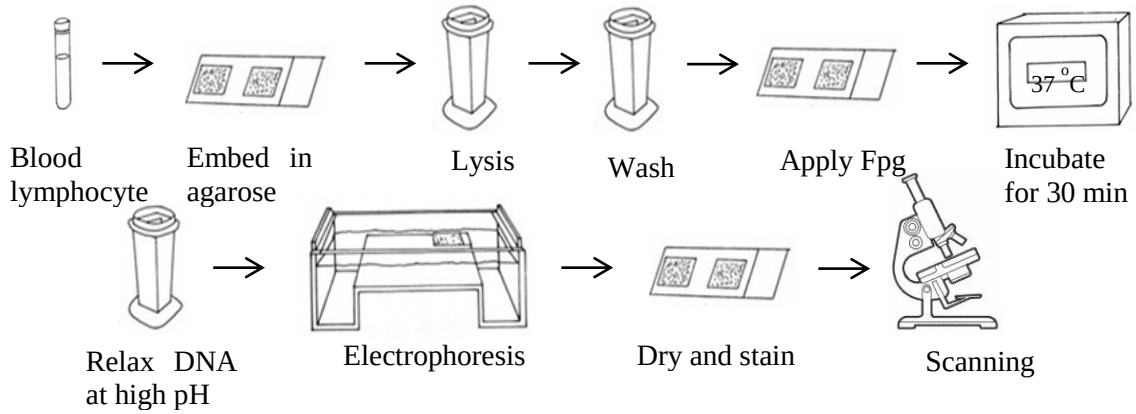


Figure 3.2. The Flow of Fpg-Assisted Comet Assay

Scanning of comet assay slides

Before scanning, the slides were dried at room temperature overnight. Twenty-five microliters of 10X SYBR Green was applied on each gel. The gel was covered by a cover slip and transferred to a dark chamber for a minimum of 5 min before scanning.

The fluorescent lamp, microscope (Leica CTR6500) and the software (LAS-AF) used for scanning were from Leica Microsystems (Leica Microsystems Ltd. Wetzlar, Germany).

LAS-AF settings

For scanning the comet slides, “DefaultDynamicWildfieldTree” was used as configuration and “Dynamic” as microscope (selected in the Leica Application Suite dialog box). After logging into the software, the “Black and White mode” was selected in the camera in the configuration menu and the “FLUO” was selected in the “Acquire” menu. In the “Acquisition” panel, “IFW” was used as the light mode, and green excitation under “IFW” was used for SYBR Green. "Wide field optimized" was selected in the Auto Focus Panel Settings, and the capture range was set to 40 microns. For precision of the auto focus, “6” steps was used.

Scanning

Stained slide was placed on slide holder and inserted on the microscope stage, focused under the microscope from eyepiece with the Z controller in the smartmove. In the LAS-AF, the live image was activated, and the exposure time and gain was adjusted to obtain satisfactory images. For “low damaged” slides (i.e. Buffer or PBS treated slides), gain was set to 1 and exposure time was set to 280 ms; while for the “high damaged” slides (i.e. Fpg or H_2O_2 treated slides), gain was set to 1 and exposure time was set to 380 ms. Images were captured with 20X objective lens. A desirable area on the gel was marked on the LAS-AF and the tile scan started afterwards. Images were merged to the whole area scanned and the merged image was exported as ‘tiff’ image files.

Scoring comet assay images through Comet Assay IV Lite

The Comet Assay IV Lite system for analyzing scanned images of comets (to measure %DNA in the comet tail) was purchased from Perceptive Instruments (Perceptive Instruments Ltd. Bury St Edmunds, UK). Images to be scored (converted to bmp form

at 8 bite) were loaded into the software. DNA% in tail was the outcome measured used for the comet assay. DNA% in tail was detected by the software by clicking on each nucleoid and encompassing the 'head' and the 'tail' of each comet separately, with 50 nucleoids scored on each gel and in total 100 nucleoids were scored on each slide. Therefore, 100 Fpg-treated and 100 Fpg buffer-treated nucleoids were scored per sample. Typical comet assay images of different levels of damage are shown in Figure 3.3.

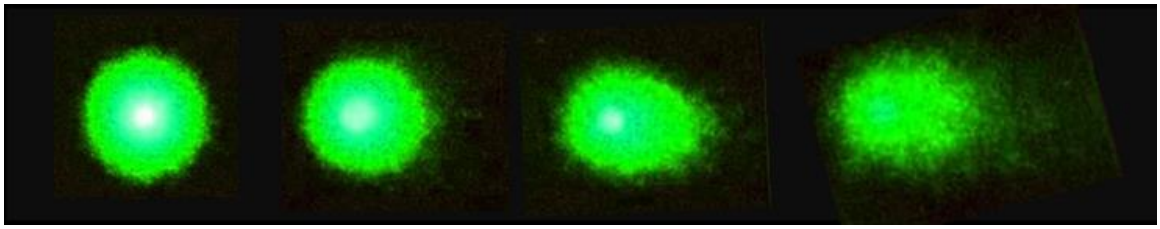


Figure 3.3. Gradient of DNA Damage Seen under Fluorescent Microscope (Low to High) in the Comet Assay

The Ferric Reducing Antioxidant Power (by FRAP assay) and Ascorbic Acid (by FRASC assay)

Basic principles:

When the Fe^{3+}-TPTZ (2,4,6-tripyridyl-s-triazine) compound is reduced to Fe^{2+}-TPTZ in low pH condition, the Fe^{2+}-TPTZ compound has a dark blue colour, which can be read at wavelength of 593 nm, and the reading is linearly associated with the amount of Fe^{2+}-TPTZ. In this method, high amount of Fe^{3+} ion is provided, and mixed with TPTZ solution under low pH condition. The absorbance at 593 nm is recorded after the Fe^{3+}-

TPTZ was reduced to Fe^{2+}-TPTZ. By comparison against the 1000 µmol/l Fe^{2+} calibrator, the total amount of the non-enzymatic antioxidant capacity of the plasma samples can be calculated.

$$Fe^{3+}\text{-TPTZ} + e^- \longrightarrow Fe^{2+}\text{-TPTZ}$$

Reagent preparation and procedures followed well established protocols (Benzie and Strain, 1996b; Chung et al., 2001).

300 mmol/l acetate buffer

The acetate buffer was made by dissolving 3.1 g sodium acetate trihydrate ($CH_3COONa \cdot 3H_2O$) in 800 ml distilled water, with addition of 16 ml of glacial acetic acid. The pH was adjusted to 3.6 by glacial acetic acid, and the volume was finally adjusted to 1 litre. The acetate buffer was stored at room temperature and used within 6 months.

20 mmol/l Iron (III) chloride solution

The iron (III) chloride solution was made by dissolving 270mg iron (III) chloride ($FeCl_3 \cdot 6H_2O$) in 50 ml MilliQ water. The iron (III) chloride solution was stored at 4^oC and used within one month.

36 mmol/l hydrochloride (HCl) solution

The HCl solution was prepared by dissolving 3 ml concentrated HCl (12 mol/l) solution into 997 ml MilliQ water.

10 mmol/l 2,4,6-tripyridyl-s-triazine (TPTZ) solution

The TPTZ solution was made by dissolving 78 mg 2,4,6-tripyridyl-s-triazine (TPTZ) in 25 ml 36 mmol/l hydrochloride (HCl) solution. The TPTZ solution was stored at 4^{o}C and used within one month unless observed colour change was seen. If any turbidity or blue colour was seen the reagent was discarded.

1000 µM Iron (II) calibrator

The calibrator used in the FRAP assay was made by dissolving 27.8 mg iron (II) sulphate ($FeSO_4 \bullet 7H_2O$) in exactly 100 ml MilliQ water. The iron (II) calibrator was stored at 4^{o}C and used within one month unless observed colour change or precipitation was seen. This solution could also be used as calibrator for the FRASC assay for ascorbic acid, but in this case its value was equivalent to 500 µmol/l ascorbic acid (Chung et al., 2001).

Ascorbic acid solutions

Pure ascorbic acid solution was made freshly before each run by dissolving 17.6 mg ascorbic acid dissolved in 10.0 ml MilliQ water to generate 10 mmol/l stock standard. The working standards (100, 250, 500, 1000 µmol/l M ascorbic acid solution) were prepared immediately from the freshly prepared 10 mmol/l standard.

Sodium acetate trihydrate, glacial acetic acid, $FeCl_3 \bullet 6H_2O$, TPTZ, $FeCl_2 \bullet 7H_2O$, and extra pure ascorbic acid crystals were purchased from Sigma.

FRAP and FRASC procedures (Benzie and Strain, 1996b; Benzie and Strain, 1999; Chung et al., 2001)

The FRAP values and ascorbic acid concentration of the plasma samples were and measured as soon as possible after separation of plasma and on the day of sampling, with plasma stored at 4^{o}C after harvesting. Working FRAP assay solution was made by mixing 300 mmol/l acetate buffer, iron (III) chloride solution and TPTZ solution in the ratio of 10:1:1. MilliQ water was used as sample blank. For the FRASC assay, 100 µl of MilliQ water, iron (II) calibrator, ascorbic acid standards and subjects' samples were mixed with 40 µl MilliQ water or ascorbate oxidase in paired FARA cups. The test was operated followed by established method generated in our lab (Benzie and Strain, 1996b; Chung et al., 2001). The test was performed on a Cobas FARA biochemical analyser (Roche Diagnostics, Indianapolis, US). Working solution and prepared samples were inserted in the corresponding racks and loaded on the FARA. 300 µl working solution was added into each reaction cup and mixed with 10 µl sample. Absorbance at 593 nm wavelength was read at 1min (for ascorbic acid) and 4 min (for FRAP value).

Results calculation

The MilliQ water provided background absorbance of the test, all the samples (calibrator, ascorbic acid standard and test samples) were blanked with it.

ΔA593 = A593 of sample -A593 of MilliQ water

FRAP value = [(ΔA593 of test sample at 4 minutes / (ΔA593 of calibrator at 4 min)] X 1000 µmol/l (iron(II) calibrator)

Ascorbic acid = [(ΔA593 of test sample without ascorbate oxidase at 1 min - ΔA593 of test sample with ascorbate oxidase at 1 min)/ (ΔA593 of calibrator at 1 min without ascorbate oxidase)] x 1000 µmol/l (iron(II) calibrator)/2 (or the value of ascorbic acid solutions if these were used for calibration, however, the pure ascorbic acid solutions were generally used to validate the performance of the test overall as quality control samples).

Allantoin by LC-MS/MS

The plasma allantoin was measured using LC-MS/MS, with protocol established by this research team (Chung and Benzie, 2013). Water used in this session means MilliQ water.

Mobile phase

The mobile phase was a mixture with 90%v/v of acetonitrile and 10 3.6%v/v water. The mobile phase was filtered through a 0.22- µm Millipore filter before use.

Calibrator

The calibrator was made by diluting concentrated stock aqueous 1mmol/l (158 ng/ml) allantoin to 10 µmol/l with water, and further diluted with water to 0, 0.625, 1.25, 2.5, 5 and 10 µmol/l as routine use calibrators.

Quality control

The quality control was made by spiking concentrated aqueous 1mmol/l allantoin to pool plasma aliquots to three final concentrations of 1, 2.5 and 5 µmol/l, and the

mixtures were vortexed vigorously for well mix. The aliquoted quality control samples (100 µl each) were stored at -80 ^{o}C and thawed with other plasma samples before use.

Internal standard (IS)

The isotopic internal standard used in this method was allantoin-$^{13}C_2$,$^{15}N_4$, which is unstable in water, thus the routine used IS must be diluted freshly before use. The IS was prepared by dissolving 0.1 mg allantoin-$^{13}C_2$,$^{15}N_4$ with 100 µl water to 6.1 mmol/l and stored at -80 ^{o}C for less than 1 week, and the daily used IS was prepared by diluting the 6.1 mmol/l IS solution with water to 5 µmol/l before use.

Diluent

The diluent was made by 95% v/v acetonitrile and 5% v/v water, with final pH adjusted to 6.0 with acetic acid.

The allantoin and acetic acid were purchased from Sigma, allantoin-$^{13}C_2$,$^{15}N_4$ was from Santa Cruz (Santa Cruz Biotechnology Inc., Santa Cruz, CA), and the acetonitrile (LC-MS/MS grade) was from BDH (VWR International Ltd., Poole, UK).

Sample pre-preparation procedures

The samples (plasma samples from subjects, pooled plasma for calibrator and control samples) were thawed at room temperature. After vigorous vortex for 5 s, all the samples were centrifuged at 3,000 g for 3 min, and transfer 10 µl supernatant to a clean Eppendorf tube. For plasma samples from subjects and control samples, 10 µl water, 10 µl daily used IS and 570 µl diluent were added into each tube, and for calibrators, 10 µl calibrator (0, 0.625, 1.25, 2.5, 5 or 10 µmol/l), 10 µl daily used IS and 570 µl diluent

were added into the pooled plasma. All of the samples were vortexed for 5 s, and centrifuged at 15,000 g for 10 min at 4 ^{o}C. The clear supernatant (480 µl) were transferred to a clear vial, 10 µl of the supernatant was injected into the HPLC system, all the samples were kept in autosampler (4 ^{o}C) before injection.

LC-MS/MS settings

A Waters Atlantis Hilic Silica column (3 µm, 4.6mm ID x 50mm L) and Waters Atlantis Hilic Silica guard cartridge (3 µm, 3.9mm ID x 20mm L) were linked and used in this assay. The HPLC and Mass spectrometer were the one used for 25(OH)D measurement, they are: HPLC system from Agilent (Agilent 1100 Series, quaternary pump, G1311A and microvacuum degasser, G1379A, Agilent Technologies Inc., Waldbronn, Germany); non-thermostatted well-plate autosampler (G1313A); and the mass spectrometer linked to the HPLC system was a 3200 QTRAP mass spectrometer from Applied Biosystems/MDS Sciex (MDS Inc., Concord, ON, Canada).

The sample run time for allantoin measurement was 6.0 min per sample, with a constant flow rate of 0.5 ml/min of mobile phase. The HPLC eluate at 2.4-3.5 min was sent to mass spectrometer, and the rest was sent to waste.

Tandem mass spectrometry settings

The MS/MS system was the one also used for 25(OH)D measurement, the MS/MS analysis was performed in negative ion mode with a TurboIonSpray® source (Sciex, Framingham, United States). The source parameters were set as follows: curtain gas (nitrogen), 12.0psi; collision gas (nitrogen), medium, ionspray voltage, -4500V; temperature, 610°C; ion source gas 1 (air), 34.0psi; ion source gas 2 (air), 45.0psi; and

interface heater was on. All MS data were obtained by multiple reaction monitoring (MRM) acquisition; for allantoin, precursor ion ([M-H]- at m/z 157.0, Q1), product ion (at m/z 114.0, Q3); for IS, these parameters were 163.0, 118.0. All the product ions were quantified using dwell time 500ms/channel with unit mass resolution for Q1 and Q3. The parameter for the optimized compound were: allantoin, IS; declustering potential, -27.0V, -25.5V; entrance potential, -5.0V, -5.0V; collision cell entrance potential, -10.0V, -10.0V, collision energy, -18.5eV, -18.0eV; and collision cell exit potential, -1.0V, -2.0V.

Quantification of LC-MS/MS results

All data were analyzed by Analyst® Software 1.4.2 (Sciex) with ".wiff" files, with the same procedure of 25(OH)D analysis, please refer to that section for details.

8-oxodG by LC-MS/MS

The plasma 8-oxodG was measured using LC-MS/MS, with protocol established by this research team (Lee et al., 2010). Water used in this session means MilliQ water.

10 mmol/l ammonium acetate

The ammonium acetate solution was made by dissolving 77.8 mg ammonium acetate with 1L water, and the pH was further adjusted to 4.3.

Mobile phase

The mobile phase was a mixture with 96.4%v/v of 10 mmol/l ammonium acetate and 3.6%v/v acetonitrile. The mobile phase was filtered through a 0.22-μm Millipore filter before use.

Calibrator

The calibrator was made by dissolving concentrated aqueous 1.13 mmol/l (320 ng/ml) 8-oxodG with pooled urine to 0.0 (blank), 7.1, 14.1, 28.2, 56.5, 113.0 nmol/l.

The pure 8-oxodG and ammonium acetate (Sigma Ultra) were purchased from Sigma, and the acetonitrile (LC-MS/MS grade) was purchased from BDH.

Quality control

The quality control was made by spiking concentrated aqueous 320 ng/ml 8-oxodG to pool urine to three concentrations of 7.1, 14.1 and 56.5 nmol/l, and the mixture was vortexed vigorously for well mix. The aliquoted quality control samples were stored at -80 ^{o}C and thawed with urine samples before use.

Sample pre-preparation procedures

Urine samples and quality control samples were thawed at room temperature before use, after mixing well with sonicating for one minute, all of the samples were centrifuged at 13,000 g for 10 min. The clear supernatant was injected into clear HPLC inserts, and 25 µl of the supernatant was injected into the HPLC system, all the samples were kept in autosampler (4 ^{o}C) before injection.

LC-MS/MS settings

A Merck Chromolith Performance RP-18e column (4.6 mm ID x 100 mm L, particle size: 2 µm x 13 nm pore) and a Phenomenex guard cartridge (C18, 3 mm ID x 4 mm L) were linked and used in this assay. The HPLC and Mass spectrometer were the one used for 25(OH)D measurement, they are: HPLC system from Agilent (Agilent 1100 Series,

quaternary pump, G1311A and microvacuum degasser, G1379A, Agilent Technologies Inc., Waldbronn, Germany); non-thermostatted well-plate autosampler (G1313A); and the mass spectrometer linked to the HPLC system was a 3200 QTRAP mass spectrometer from Applied Biosystems/MDS Sciex (MDS Inc., Concord, ON, Canada).

The sample run time for 8-oxodG measurement was 10.5 min per sample, with a constant flow rate of 0.5 ml/min of mobile phase. The HPLC eluate at 0-7.5 min was sent to waste and the eluate at 7.5-10.5 min was sent to mass spectrometer. A 6 mim wash period with mobile phase was performed between samples, with a constant flow rate of 2.0 ml/min.

The tandem mass spectrometer settings

The MS/MS system was the one also used for 25(OH)D measurement, the MS/MS analysis was performed in positive ion mode with a TurboIonSpray® source (Sciex, Framingham, United States). The source parameters were set as follows: curtain gas (nitrogen), 12.0psi; collision gas (nitrogen), medium, ionspray voltage, 5500V; temperature, 375°C; ion source gas 1 (air), 11.0psi; ion source gas 2 (air), 32.0psi; and interface heater was on. All MS data were obtained by multiple reaction monitoring (MRM) acquisition, precursor ion at m/z 284, Q1, and product ion at m/z 168, Q3. All the product ions were quantified using dwell time 500ms/channel with unit mass resolution for Q1 and Q3. The parameter for the optimized compound were: declustering potential, 26.0 V; entrance potential 3.5 V; collision energy, 19.0 eV; and, collision cell exit potential, 3.9 V.

Quantification of LC-MS/MS results

All data were analyzed by Analyst® Software 1.4.2 (Sciex) with ".wiff" files, with the same procedure of 25(OH)D analysis, please refer to that section for details. As the urine samples were spot urine samples, the concentration differed greatly, thus, all of the 8-oxodG results were normalized with creatinine concentration as 8-oxodG (nmol/l)/creatinine (mmol/l), because creatinine excretion is relatively stable in healthy people through the day and day by day (Lee et al., 2010).

STATISTICAL ANALYSIS

Results are presented as mean and standard deviation (SD). For normally distributed interval or ratio data, parametric statistical methods were used, or non-parametric methods were performed on skewed data. As the statistical analysis method used in each chapter differs, based on the data presented in each chapter, for avoiding confusion, specific methods are described in the *Materials and Methods* sections in each chapter.

Chapter 4-8 are designed to be read as complete scientific articles.

CHAPTER 4

VITAMIN D STATUS IN YOUNG APPARENTLY HEALTHY ADULTS IN HONG KONG

INTRODUCTION

Vitamin D plays a crucial role in the musculoskeletal system, regulating the absorption and utilization of calcium and phosphate, but there is increasing evidence showing that vitamin D is associated with various non-skeletal diseases and that adequate vitamin D is important for maintenance of more than musculoskeletal health (Holick, 2007; Spedding et al., 2013). Low vitamin D status is reported to be an independent risk factor for various common non-communicable diseases (NCDs), including cancer, cardiovascular disease (CVD), diabetes, as well as multiple sclerosis, dementia and depression (Vuolo et al., 2012; Afzal et al., 2013a; Anglin et al., 2013; Khan et al., 2013; van der Schaft et al., 2013; Feldman et al., 2014; Pliz et al., 2016). Therefore, low vitamin D status is increasingly regarded as a public health problem (Holick, 2007; Hilger et al., 2014).

The indicator, or biomarker, used in the assessment of vitamin D status is the concentration of 25-hydroxyvitamin D (25(OH)D) in plasma (or serum). Plasma 25(OH)D <25 nmol/l is an undisputed sign of severe deficiency, and concentrations

≥25<50 nmol/l are generally agreed to reflect inadequate vitamin D (Holick et al., 2011; Ross et al., 2011). However, there is no confirmed 'optimal' level of 25(OH)D. The US Institute of Medicine (IOM) suggested that plasma 25(OH)D ≥50 nmol/l is sufficient to maintain the needs of 97.5% of the population in relation to bone health (Ross et al., 2011), while the US Endocrine Society regards 75 nmol/l as the threshold of vitamin D sufficiency, based on the relationship between vitamin D and PTH together with bone health and calcium absorption (Holick et al., 2011). Interestingly, what is an 'optimal' Vitamin D status may be disease-specific. Based on reviews of non-skeletal diseases, minimum effective doses of 75, 80, 95 and 100 nmol/l were recommended for prevention of, respectively, premature mortality, CVD and diabetes, respiratory infections and depression, and cancer (Spedding et al., 2013). The different levels of vitamin D status used in this current study are defined in Table 4.1. However, due to the controversy of what thresholds should be used for severe deficiency (<25 or <30 nmol/l), deficiency (<50 or <75 nmol/l), data are also presented with different cut-off points in this chapter.

Table 4.1. The Definition of Vitamin D Status Levels used in This Study

Status	Defined as plasma 25(OH)D of
Severely deficient	<25 nmol/l
Deficient	≥25<50 nmol/l
Insufficient	≥50<75 nmol/l
Sufficient	≥75 nmol/l

Some foods contain vitamin D, but diet is a minor contributor to vitamin D status (Cashman and Kiely, 2015). Most vitamin D is synthesized in sun-exposed skin, and for

most healthy adults, 10-15 min of direct exposure to sunshine is sufficient to prevent vitamin D deficiency (Holick, 2007). Still, given the modern, mainly indoor lifestyle, the well publicized links between sunshine and skin ageing as well as skin cancer, and certain cultural factors (Bonevski et al., 2013), many people have low exposure to sunshine and consequently have low vitamin D status. Reports from various parts of the world show that vitamin D deficiency is common, even in sunny countries. A study in Qatar showed that 62% of children aged 11-16 years had serum vitamin D <50 nmol/l (Bener et al., 2009), and an Iranian study of 216 girls aged 14-17 years revealed mean(SD) serum 25(OH)D of 18.1(7.0) nmol/l, with 96% of participants having 25(OH)D <50 nmol/l (Rafraf et al., 2014). A systematic review that involved over 168,000 subjects from 44 countries showed that 88% of the studied subjects had plasma or serum 25(OH)D concentration <75 nmol/l, and in 37% of subjects it was <50 nmol/l (Hilger et al., 2014).

Hong Kong is located at latitude 22^{o} North and enjoys abundant sunshine most of the year. Still, poor vitamin D status has been reported in local adults. Using an immunoassay method, Woo et al. found that 18% (40/221) of non-pregnant women (aged 20-35) studied had serum 25(OH)D ≤25 nmol/l and 92% (203/221) had serum 25(OH)D ≤50 nmol/l (Woo et al., 2008). As previously noted, vitamin D deficiency is increasingly recognized as a risk factor for various age-related NCDs. The underlying biological changes that result in these disorders take many years to develop into overt disease. If people in their young age and early adult life have low vitamin D status, and if this poor status is maintained, their long-term health may be affected, and early biomarker signs of the changes that eventually lead to NCDs may exist even in an apparently healthy person. Furthermore, vitamin D deficiency may have an important

effect earlier in life in the case of pregnancy and its outcome. Pregnant women who develop gestational diabetes were reported to have lower vitamin D status, and vitamin D deficiency is reported to increase risk of pre-eclampsia, and to be associated with lower birth weight and lower bone mass of the baby (Moon et al., 2015). Deficiency of vitamin D is also reported to increase risk of multiple sclerosis, and may play a role in determining risk of multiple sclerosis in the offspring of women with vitamin D deficiency during gestation, although reports are not consistent (Mirzaei et al., 2011). Therefore, chronic deficiency of vitamin D could have profound effects on health not only in elderly people, but also in young women during pregnancy and on the health of their children.

The aim of this part of the study was to investigate the vitamin D status of a group of apparently healthy young adults in order to assess the prevalence of severe deficiency, deficiency, and insufficiency in this group, and to create a database to investigate the association between vitamin D status and biomarkers that are linked to risk of NCDs, particularly cardiovascular disease, Type 2 diabetes and cancer. Gender differences in vitamin D status and seasonal influence were also explored.

MATERIALS AND METHODS (PLEASE ALSO REFER TO CHAPTER 3 FOR DETAILED DESCRIPTIONS)

Fasting venous blood samples were collected, from 196 subjects (63 males, 133 females) aged 18-26 years, with their written informed consent. Blood samples were collected into heparinized blood collection tubes and centrifuged within 1 hour of

collection. Plasma was separated, aliquoted and stored at -80^{o}C until thawed, once only, for 25(OH)D measurement (by LC-MS/MS). Detailed procedures of blood collection and plasma 25(OH)D measurement are given in Chapter 3.

Data handling and statistical analysis: Results are presented as mean and standard deviation (SD), median and range (low to high). Each value was assigned to a category of vitamin D status (as defined in Table 4.1). Gender difference was explored using the unpaired t-test, and seasonal effects were investigated using Kruskal-Wallis test with Dunn's post hoc test. The difference in the frequency of each vitamin D status categories between genders was investigated by Chi-square test. The difference of 25(OH)D concentration according to their lifestyle record was detected by one way ANOVA with Newman-Keuls post hoc test or Kruskal-Wallis test and the Dunns post hoc test, as appropriate for distribution. A p value <0.05 was considered statistically significant.

RESULTS

The plasma 25(OH)D concentrations of the 196 subjects studied are shown in Tables 4.2 and 4.3 and Figure 4.1. It can be seen that males had significantly higher plasma 25(OH)D (by ~10%; $p<0.05$) than females, but there is no significant difference in the distribution of different vitamin D status categories between gender. Figure 4.1 presents the distribution of values, and Table 4.3A shows the prevalence in each of the Vitamin D status groups based on the categories we defined in Table 4.1. Additional distribution data of vitamin D status with different cut-off points are given in Table 4.3B. Results

show that 13/196 of the apparently healthy young subjects had plasma 25(OH)D <25 nmol/l. Most of these severely deficient subjects were women. The majority of the subjects were found to be vitamin D deficient, as defined in Table 4.1, with similar prevalence of deficiency seen in males and females. Only two subjects (males) among the 196 studied was found to have plasma 25(OH)D ≥75 nmol/l.

Table 4.2. Descriptive Results of Plasma 25(OH)D Concentration (nmol/l) in the 196 Apparently Healthy Young Subjects Studied

	All (n=196)	Male (n=63)	Female (n=133)
Mean(SD)	42.1(13.0)	45.3(14.4)	40.6(12.1)*
Median	40.4	44.2	38.5
Range	15.7-86.8	17.4-86.8	15.7-67.4

*p<0.05 compared to value in males

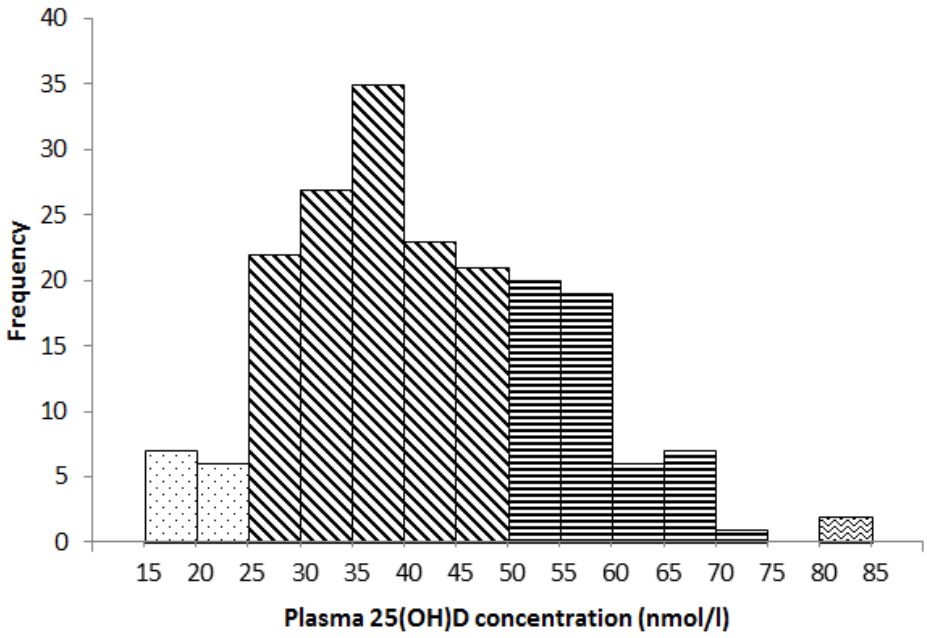


Figure 4.1. The Distribution of Vitamin D Status in the 196 Subjects Tested

dotted bars= severely deficient zone; diagonal bars = deficit zone; horizontal bars= insufficient zone; wavey line = sufficient zone

Table 4.3A. Vitamin D Status in the 196 Subjects Studied

Vitamin D status	Total (n=196)	Male (n=63)	Female (n=133)
Vitamin D severely deficient (plasma 25(OH)D <25 nmol/l)	13 (6.63%)	2 (3.17%)	11 (8.27%)
Vitamin D deficient (plasma 25(OH)D ≥25<50 nmol/l)	128 (65.31%)	38 (60.32%)	90 (67.67%)
Vitamin D insufficient (plasma 25(OH)D ≥50<75 nmol/l)	53 (27.04%)	21 (33.33%)	32 (24.06%)
Vitamin D sufficient (plasma 25(OH)D ≥75 nmol/l)	2 (1.02%)	2 (3.17%)	0 (0%)

Table 4.3B. The Distribution of Vitamin D Status using Different Cut-off Points

Vitamin D status	Total (n=196)	Male (n=63)	Female (n=133)
plasma 25(OH)D <25 nmol/l	13 (6.63%)	2 (3.17%)	11 (8.27%)
plasma 25(OH)D <30 nmol/l	33 (16.84%)	8 (12.70%)	25 (18.80%)
(plasma 25(OH)D <40 nmol/l	96 (48.98%)	25 (39.68%)	71 (53.38%)
(plasma 25(OH)D <50 nmol/l	141 (71.94%)	40 (63.49%)	101 (75.94%)
(plasma 25(OH)D <60 nmol/l	180 (91.84%)	55 (87.30%)	125 (93.98%)
(plasma 25(OH)D <75 nmol/l	194 (98.98%)	61 (96.83%)	133 (100%)

In regard to seasonal differences, results are presented in Figure 4.2. It is noted that only eight samples were collected in summer. Results in samples collected in winter were lowest, but no statistically significant difference across the seasons was detected; mean(SD) plasma 25(OH)D (nmol/l) values were: spring, 43.8(14.0), n=98; summer, 47.1(16.4), n=8; autumn 44.0(12.0), n=29; winter, 37.9(10.3) n=61; p=0.0324, no significant differences were found between groups after Dunn's multiple comparison correction.

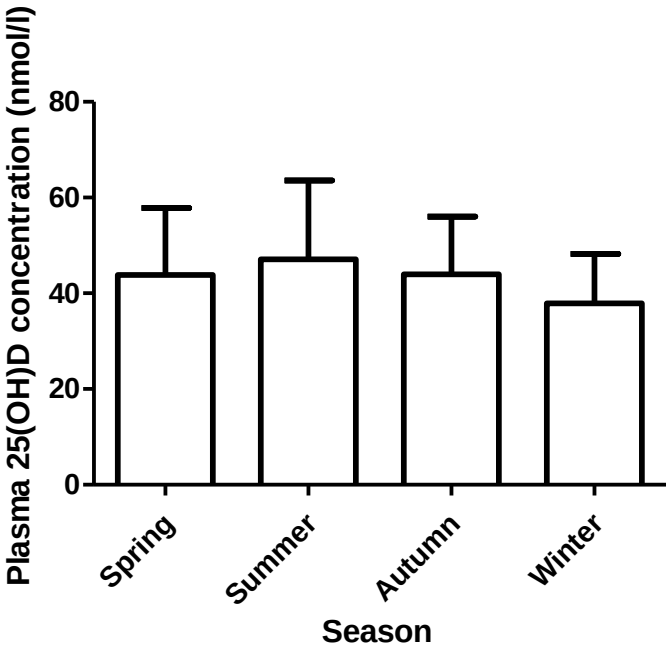


Figure 4.2. Plasma 25(OH)D in Samples Collected at Different Times of the Year

Data are Mean+SD in: spring n=98 (35 males, 63 females); summer n=8 (4 males, 4 females); autumn n=29 (3 males, 26 females); winter n=61 (21 males, 40 females).

DISCUSSION

In this part of study, the vitamin D status of 196 young (18-26 years), apparently healthy adults in Hong Kong was assessed using plasma 25(OH)D concentration measured by LC-MS/MS. Results showed that the prevalence of vitamin D deficiency and insufficiency was very high, with 98.9% of the subjects (indeed, all but two subjects) having a sub-optimal plasma 25(OH)D concentration (<75 nmol/l). These new data agree with the high prevalence of vitamin D deficiency shown in a previous study in Hong Kong that involved women aged 20-35 years (Woo et al., 2008). In contrast, a study of community living elderly men in Hong Kong (Chan et al., 2011) showed that 53% of the subjects had serum 25(OH)D concentration ≥75 nmol/l, and that only 6% had serum 25(OH)D <50 nmol/l (which was defined as deficiency by those authors). The markedly higher vitamin D status seen in the elderly men was suggested to be due to high sunshine exposure among the elderly, as outdoor physical activities are common in this group (Population Health Survey Hong Kong, 2004; Chan et al., 2011).

There have been other studies of vitamin D status in Hong Kong (Table 4.4). Especially poor vitamin D status has been found in institutionalized patients and in young women (Wong et al., 2006; Woo et al., 2008). The Hong Kong study of Zhao et al., (2014) focused mainly on the vitamin D status of patients with psoriasis and sub-clinical myocardial infarction, but included a control group of 53 middle-aged men and women without inflammatory disease. Their serum 25(OH)D averaged 64.5 nmol/l, which is ~50% higher than that was found in this current study of 196 young, healthy adults. Other Hong Kong studies of vitamin D status (Table 4.4) have involved chronic

hepatitis B carriers, patients undergoing thyroid surgery, those with elevated CVD risk, subjects with mental handicap and on anticonvulsant therapy, and patients with end-stage renal failure (Wong et al., 2006; Wang et al, 2008; Lang et al., 2013; Chan et al., 2015; Mok et al., 2015; Wong et al, 2015). Mean values of 60-75 nmol/l were found in some studies (Chan et al., 2015; Wong et al., 2015), with lower values found in others, and particularly low values (mean(SD) 14.4(7.9) nmol/l) were found in long-term institutionalized subjects (Wong et al., 2006).

A recent study in Hong Kong reported on determinants of serum 25(OH)D in 2,694 subjects aged from 6 to over 65 years (Xu et al., 2015). Limited data on 25(OH)D were shown, but overall mean values varied somewhat, though not greatly, across the seasons, ranging from a low of ~42 nmol/l (in spring) to a high of ~53 nmol/l (in autumn). In this current study, we noted a similar lack of marked seasonal differences. Overall, results suggest that if someone is found to have vitamin D deficiency in winter or spring in Hong Kong, their status may improve in summer or autumn, but the improvement is generally limited, and is unlikely to correct a deficiency of vitamin D. Therefore, season is not the main determinant of vitamin D status in Hong Kong. The main determinants, as found by Xu et al. (Xu et al., 2015), are sunshine exposure time and vitamin D rich food/supplementation intake.

Table 4.4. Summary of Results of Vitamin D Related Studies in Hong Kong

Study	Sample size	Age in years	Males/ Female	Subject characteristics	Plasma/serum 25(OH)D concentration (nmol/l)
This study	196	Mean(SD) 20.8(1.6)	63/133	Young apparently healthy men and women aged 18-26 years	Mean(SD) 42.1(13.0) Median(range) 40.6(15.7-86.8)*
Woo et al., 2008	221	Mean(SD) 27.9(0.3)	0/221	Non-pregnant women aged 20-35 years	Mean(95%CIs) 34(33,36)
Chan et al., 2011	939	Mean (SD) 72.8(20.5)	939/0	Community living men aged ≥65 years	Mean(SD) 77.9(20.5)
Xu et al., 2015	1,165	6-17	Not stated	Children accepted for influenza vaccine trial	Mean †39-53
	933	18-44	"	Their family members	Mean †42-57
	544	45-64	"		Mean †47-63
	51	65+	"		Mean †41-56
Zhao et al., 2014	53	Mean (SD) 45(9)	32/21	Adults with no inflammatory	Mean(SD) 64.5(16.2)

				(control group in a psoriasis study)	
	74	Mean (SD) 47(9)	52/22	Patients with severe psoriasis	Mean(SD) 62.2(19.2)
Wong et al, 2015	426	Mean(SD) 41(13)	Mixed	Chronic hepatitis B patients	Mean(SD) 60.7 (23.5)
Chan et al., 2015	114	Mean(SD) 66.1(11.4)	81/33	High CVD risk patients without statin use	Mean(SD) 67.0(21.2)
	443	Mean(SD) 68.1(9.8)	258/71	High risk CVD patients on statin therapy	Mean(SD) 75.5(32.0)
Mok et al., 2015	290	Mean(SD) 38.9(13.1)	16/274	Systemic lupus erythematosus patients	Mean(SD) 47.7(15.5)
Lang et al., 2013	281	Median (range) 53.2 (22.9-87.4)	34/247	Patients undergoing total thyroidectomy	Median(range) 32.3(7.5-82.8)
Wang et al, 2008	230	Mean(SD) 52(12)	118/ 112	Chronic peritoneal dialysis patients	Median(inter-quartile range) 45.7(35.7-60.7)
Wong et al., 2006	122	Mean (range)	23/99	Long-stay patients in mental	Mean(SD) 14.4(7.9)

		31.3(17-51)		handicap unit on anticonvulsants	

*by LC-MS/MS; all other studies presented here used immunoassay.

†The mean serum 25(OH)D values were calculated for each season of sample collection; no overall mean values or ranges for 25(OH)D in these groups were provided.

There are various reports of low vitamin D status in young adults in different parts of the world (Table 4.5). A multi-centre (Athens (Greece), Dortmund (Germany), Ghent (Belgium), Heraklion (Greece), Lille (France), Pecs (Hungary), Rome (Italy), Stockholm (Sweden), Vienna (Austria) and Zaragoza (Spain)) European study (the HELENA study) with 1,006 adolescent subjects aged 12.5-17.5 years found that 15% of the subjects had plasma 25(OH)D <27.5 nmol/l (the threshold used to define severe deficiency in that study), 27 % had plasma 25(OH)D ≥27.5<50 nmol/l (defined as deficiency) and 39% had plasma 25(OH)D ≥50<75 nmol/l (defined as insufficiency) (González-Gross et al., 2012). The mean plasma 25(OH)D of these European adolescents [58.8(23.1) nmol/l] was higher than that of the young adults studied [41.8(12.9) nmol/l] in this current study.

A large study conducted in South Korea measured serum 25(OH)D by immunoassay in 3,450 healthy young university students, with blood samples collected in spring (Joh et al., 2015). The mean serum 25(OH)D concentration overall was 27.8 nmol/l, compared to 41.8 nmol/l in this current study of young adults in Hong Kong. Similar with this current study, the South Korean males had significantly ($p<0.01$) higher 25(OH)D than

the females (also by ~10%) (Joh et al., 2015). The authors claimed that lifestyle and dietary habits were the major reasons for the poor vitamin D status among these young Koreans, and that increased physical activity, exposure to sunshine, taking regular breakfasts and dairy products, fatty fish and vitamin D supplementation were significantly associated with higher vitamin D status (Joh et al., 2015). These are common observations. It is noted that South Korea, unlike Hong Kong, has long, cold winters, and that the samples were collected in spring, when serum 25(OH)D concentration tend to be lowest (Webb and Engelsen, 2006). It is further noted that the samples in this current study were collected all through the year, and that in Hong Kong there is no dramatic seasonal effect on 25(OH)D (Xu et al, 2015). Therefore, the lower average 25(OH)D value obtained in the South Korean study is likely due to the time of sampling (spring). Samples were also collected in spring in a Mongolian study of 420 women of child bearing age. The serum 25(OH)D, measured using LC-MS, was 19.0 nmol/l, 79% of the subjects were severely deficient (<25 nmol/l), 99% (415/420) of the women had serum 25(OH)D concentration <50 nmol/l, and none reached the sufficiency threshold of 75 nmol/l (Ganmaa et al., 2014).

Studies in regions of lower latitude have also shown very low vitamin D status, especially in women. In India and Saudi Arabia, mean values for serum 25(OH)D concentrations were reported as 32.4 and 18.3 nmol/l, respectively (Marwaha et al., 2011; Al-Mogbel, 2012). The Indian study (Marwaha et al., 2011) involved 186 healthy women aged 18-21 years in New Delhi, and 25(OH)D was measured by immunoassay. All the participants were college students, and 90 of them were college sportswomen, with the rest (the control group) doing <150 min of physical activity (including walking) per week. Sun exposure was reported to be 2h/day for the sportswomen and

0.5h/d for the others. The sportswomen had mean 25(OH)D of 53 nmol/l, with values ranging from 19-100 nmol/l, while the control group had mean 25(OH)D of 12.9 nmol/l, with values ranging from 0.7-38 nmol/l. This study focused on differences in bone mineral density (BMD) of the two groups, and found significantly ($p<0.01$) higher BMD in the athletes. Plasma PTH was significantly ($p<0.001$) lower in the sportswomen, no differences between the groups were seen in plasma calcium or phosphate, though alkaline phosphatase was slightly though statistically significantly ($p<0.001$) lower in the sportswomen. The huge variation seen (<1 to 100 nmol/l) in serum 25(OH)D concentrations in this group of young Indian college students highlights the influence of lifestyle factors on vitamin D status. No other biomarkers were measured in that study, so it is not possible to assess the impact of the very low status seen in many of these apparently healthy young women in regard to factors that modulate risk of NCDs. The Saudi Arabian study (Al-Mogbel, 2012) recruited 465 women aged 19-40 years who visited primary health centres during winter and spring. Serum 25(OH)D and PTH concentrations were measured by immunoassay. Almost 80% of the women (368/465) had serum 25(OH)D <25 nmol/l, and over half of the women studied had elevated PTH levels, highlighting the lack of a simple linear relationship between 25(OH)D and PTH (Ross et al., 2011). The prevalence of severe deficiency (79%) of vitamin D in the Arabian women was considerably higher than that found in this current study of young women in Hong Kong (~10%), but, as in this current study, none of the women studied in Saudia Arabia had serum 25(OH)D concentration ≥75 nmol/l (Al-Mogbel, 2012). Saudi Arabia is located at similar latitude to Hong Kong, and has a great abundance of sunshine. The very low vitamin D status in Saudi Arabian women is likely due to avoidance of sun and high temperatures and to cultural factors that determine the way of dressing for outdoors, which generally involves covering the

whole body so that no skin is exposed. Another study conducted in India had a larger sample size (n=1,137) and involved approximately equal numbers of healthy men and women aged 25-35 years in Maharashtra, which is in the south-western part of India (Shivane et al., 2011). The overall mean serum 25(OH)D concentration was 43.5 nmol/l, and, as with this current study, men were found to have significantly ($p<0.05$) higher mean concentration (45 nmol/l) than women (40 nmol/l). The prevalence of vitamin D deficiency was high overall, with 70% of the men and women having serum 25(OH)D concentrations <50 nmol/l (Shivane et al., 2011), although these young Indian women had slightly higher mean 25(OH)D than the young women in New Delhi (Marwaha et al., 2011), which is 10^{o} North of Maharashtra.

A study of 65 young (18-21 years) healthy men was performed in Finland, with samples collected in winter (Välimäki et al., 2007). Median serum 25(OH)D, measured by LC-MS, was 33.5 nmol/l (range 13.0–68.0 nmol/l). These generally low levels reflect the lack of solar radiation at higher latitudes during winter (Välimäki et al., 2007). An Australian study collected samples throughout an entire calendar year and found relatively high vitamin D overall status in 946 healthy subjects (466 men, 480 women) aged 18-22 years (Yazar et al., 2014). In contrast to the findings in most other studies, the men had slightly but significantly ($p<0.05$) lower vitamin D status than the women: mean(SD) values of serum 25(OH)D concentration were 70.9 and 71.7 nmol/l, respectively. This study involved 798 subjects of European ancestry and 60 of East Asian ancestry (the ancestry of the 88 others was not defined). Those of European ancestry had significantly higher vitamin D status than those of East Asian ancestry: mean serum 25(OH) values were, respectively, 73 and 55 nmol/l ($p<0.001$). The primary outcome of this study was the association between vitamin D status and the risk

of myopia, and it was found that an increase of 10 nmol/l in serum 25(OH)D was associated with a 12% lower risk of myopia ($p<0.001$) (Yazar et al., 2014). In a Canadian study (Sham et al., 2015), 310 people aged 18-35 years were recruited in Toronto during autumn, at which time of year the vitamin D status of those in higher northern latitudes is expected to be highest (Webb, 2006). Subjects of European (n=111), East Asian (n=104) and South Asian (n=95) ancestries were studied (Sham et al., 2015). Serum 25(OH)D concentrations were measured by LC-MS. Sunshine exposure time and exposed skin areas, as well as vitamin D intake were recorded. The median 25(OH)D concentration was 49.7 nmol/l. As in the Australian study (Yazar et al., 2014), those of Asian ancestry had lower vitamin D status than those of European ancestry who lived in the same geographical area. Prevalence of vitamin D deficiency (defined by the authors as 25(OH)D <30 nmol/l) was 12% for the East Asian subjects and 36% for the South Asian subjects. No subjects of European ancestry were found to be vitamin D deficient, although 55% had insufficient vitamin D (defined as serum 25(OH)D <75 nmol/l) (Sham et al., 2015). The insufficiency figures for the East and South Asian subjects were, respectively, 91% and 97%. This is similar to the findings in this current study in the East Asian city of Hong Kong, in which 99% of the young subjects had insufficient vitamin D. Similar to other studies, the Canadian study found that vitamin D status was highly associated with sunshine exposure habit and vitamin D intake (Sham et al., 2015). The differences seen in prevalence of vitamin D deficiency across different ancestral groups (Yazar et al., 2014; Sham et al., 2015) living in the same area is likely due to cultural and dietary differences, and to the degree of skin pigmentation, which affects cutaneous synthesis of vitamin D in response to exposure to sunshine (Nair and Maseeh, 2012).

In China, a large cross-centre study was performed in 2,173 healthy subjects from Urumqi (located 43^{o}N; n=402), Beijing (40^{o}N; n=415), Dalian (39^{o}N; n=480), Hangzhou (30^{o}N; n=437) and Guangzhou (23^{o}N; n=469) (Yu et al., 2015). The majority of the subjects were aged 18-65 years, though a few (no exact number provided) were >65 years. The overall mean serum 25(OH)D concentration was 48.5 nmol/l. The prevalence of the vitamin D deficiency was high. Overall, only 117/2,173 (5.4%) subjects were found to have serum 25(OH)D >75 nmol/l, and over half (1,215/2,173) had serum 25(OH)D <50 nmol/l (Yu et al., 2015). Statistically significant ($p<0.001$) higher vitamin D status was found in Dalian and Guangzhou compared to the other three cities. Ranking of 25(OH)D concentration in the different cities was: Dalian (highest: mean 54.5 nmol/l) >Guangzhou (53.5 nmol/l) >Hangzhou (47.5 nmol/l) >Urumqi (45.7 nmol/l) >Beijing (40.5 nmol/l). The vitamin D status was not a simple reflection of latitude, indicating the influence of differences in lifestyle, and air pollution is also a factor, as this can block up to 60% of UVB light (Wacker and Holick, 2013). Overall, the vitamin D status in subjects from all the cities was poor. Status in males was significantly ($p<0.001$) higher than the females, with mean serum 25(OH)D values of 52.7 and 44.5 nmol/l, respectively. In those subjects aged 18-29 years (a similar age group to those in this current study in Hong Kong), the respective mean serum 25(OH)D values of males (n=289) and females (n=269) were 51.8 and 41.5 nmol/l (Yu et al., 2015). In comparison with the values obtained in this current study, the young women living in Mainland China had similar vitamin D status, but the corresponding values in young men were generally higher, even in the more Northerly cities. A summary of the studies described above is given in Table 4.5.

Table 4.5. Summary of Vitamin D Status in Young Healthy Adults in Other Regions

Study	Region	Sample size	Male/ Female	Age (years)	Plasma/Serum 25(OH)D concentration (nmol/l)
This study	Hong Kong	196	63/133	Range 18-26	Mean(SD) 42.1(13.0) Median(range) 40.6(15.7-86.8)*
Yu et al., 2015	Multi-centre China	558	289/ 269	Range 18-29	Mean(SD) Males: 51.8(14.8)* Females: 41.5(11.7)*
González-Gross et al., 2012	Multi-centre Europe	1,006	470/ 536	Range 12.5-17.5 Mean(SD) 14.7(1.2)	Mean(SD) 58.8(23.1)
Joh et al., 2015	Seoul South Korea	3,450	1,768/ 1,682	Range 18-29 Mean(SD) 22.5(2.9)	Mean(SD) 27.77(10.22)

Sham et al., 2015	Toronto, Canada	310	102/ 208	Range 18-35 Mean(SD) 21.1(3.0)	Mean(IQR) 49.7(36.7-70.3)*
Yazar et al., 2014	Australia no specific place	946	466/ 480	Range 18-22 Mean(SD) 20.0(0.4)	Mean(IQR) Males: 70.9(56.1-84.8) Females: 71.7(58.6-85.2)
Ganmaa et al, 2014	Mongolia	420	0/420	Range 18-40	Mean(SD) 19.0(10.0)*
Al-Mogbel, 2012	Riyadh, Saudi Arabia	465	0/465	Range 19-40 Mean(SD) 28.62(6.57)	Mean(SD) 18.34(8.2)
Marwaha et al., 2011	New Delhi India	186	0/186	Range 18-21 Mean(SD) 18.6(1.3)	Mean(SD) 32.4(24.6)
		90 sports women	0/90	Range 18-21 Mean(SD) 18.7(1.2)	Mean(SD) 53.0(18.9)

		96 control 'no-exercise' women	0/96	-	Mean(SD) 12.9(7.7)
Shivane et al., 2011	Maharashtra, India	1,137	558/ 579	Range 25-35	Mean(SD) 43.5(22.75)
Välimäki et al., 2007	Finland	65	65/0	Median (range) 23.1 (22.1-24.0)	Median (range) 33.5 (13.0–68.0)*

*by LC-MS/MS; all other studies presented here used an immunoassay method to measure plasma or serum 25(OH)D.

The studies listed in Table 4.5 show that vitamin D deficiency in young adults is highly prevalent, and as shown by the new data from this current study, young men and women in the affluent and modern city of Hong Kong are no exception. Low vitamin D status is common also in older age groups around the world (Hilger et al., 2014), though older men in Hong Kong may be an exception (Chan et al., 2011). The major reason for poor vitamin D status is the lack of skin exposure to sunshine, but the reasons behind this vary across the world and between cultures. For people living in high latitude regions like North Europe and Canada, the solar energy they receive is lower even in summer compared to those living at lower latitudes (Webb, 2006). Moreover, for most of the winter in very high latitude regions, there is no solar radiation of sufficient energy for 25(OH)D synthesis, removing the major source of vitamin D (Engelsen et al., 2005; Kimlin, 2008). However, people living in sunny, low latitude regions also have

inadequate exposure to sunshine, thereby compromising their vitamin D status. The high temperatures make direct exposure to sunshine uncomfortable, traditional or cultural modes of dress mean that skin is rarely exposed, and darker skin pigmentation requires a longer period of sun exposure for adequate 25(OH)D synthesis (Bassir et al., 2001; Armas et al.,2007). Furthermore, there is increasing awareness of the skin ageing effects of UV light, and its skin cancer causing effects and this leads to the use of powerful UB blocking sun screens, and to avoidance of sunshine (Holick, 2001). Also, in modern, affluent and technologically advanced cities like Hong Kong, the lifestyle is mainly an indoors one for both working and social activities, especially for young people.

To address the problem of poor vitamin D status public health measures in the way of mandatory food fortification with vitamin D have been introduced in some countries like US and Canada (Cashman, 2015), though as yet not in Hong Kong. Increased intake of vitamin D fortified food can help improve vitamin D status, but is usually not effective in attaining optimal levels (Cashman, 2015). In the Finnish study of Välimäki et al. (2007), blood samples and information on milk consumption were collected from 65 subjects in January 2001 and again in January 2004. Serum 25(OH)D concentrations of both batches were measured using immunoassay. The vitamin D status in 2004 was slightly higher than in 2001 (mean values of 27 and 24 nmol/l respectively, $p<0.01$), but was still very low, despite the introduction of vitamin D fortification of milk in 2003 (Välimäki et al., 2007).

Vitamin D supplementation is another measure to increase vitamin D status, but the application is more complicated. Even though toxic dose (LD_{50}) of vitamin D is very

high, very unlikely to be obtained from dietary or supplemental sources (Ozkan, 2012), long-term, unregulated high intake of vitamin D increases risk of hypervitaminosis D, which may increase the risk of cancer or CVD (Tuohimaa et al., 2004). Besides, the role of vitamin D in non-skeletal health maintenance is still unclear, there is a lack of robust evidence confirming the benefit of achieving optimal vitamin D status in relation to risk of NCDs and promotion of healthy ageing. Therefore, the National Institute of Health (NIH) does not recommend vitamin D supplementation as a routine therapeutic measure (Taylor et al., 2015). More studies, especially clinical trials investigating the effect vitamin D on risk of NCDs, and the underlying mechanisms are needed. If it can be confirmed that those with vitamin D deficiency in early adult life have some indication of early changes associated with increased risk of NCDs, then proactive public health measures to improve vitamin D status will be supported.

This current study investigated the vitamin D status in a group of apparently healthy young adults, enriching the database of vitamin D status in people living in Hong Kong, a subtropical city located 22^{o}N with an average duration of sunshine of 1,835.6 hours (42%) per year) (Hong Kong Observatory, monthly solar radiation record from 1981 to 2010, data accessed in March, 2016). Despite abundant sunshine, the prevalence of vitamin D deficiency is high in the young people studied. It is noted that, although approximately equal numbers of men and women were sought, there were almost twice the number of women recruited than men in this study. Men were found to have plasma 25(OH)D ~10% higher than women, similar to the findings of others (Yu et al., 2015). This is likely due to the larger amount of adipose tissue in the female body (Lagunova et al., 2009). Vitamin D is lipophilic, and the additional adipose tissue likely sequesters more 25(OH)D in women. Therefore, the overall vitamin D status of the group of young

people may be slightly underestimated. Still, it is noted that the vitamin D status in the 63 young men studied was poor, averaging 45 nmol/l.

To conclude, prevalence of vitamin D deficiency in the 196 young, apparently healthy adults studied was found to be very high. The new data provide a database for the study of inter-relationships between vitamin D status and biomarkers associated with risk of NCDs, particularly cardiometabolic risk factors, DNA damage and oxidative stress. These form the focal points of Chapters 5, 6 and 7 of this thesis.

Some of the information presented in this chapter was presented as:

1. *Poster presentation in the World Congress on Public Health and Nutrition, March 2016, Madrid*
2. *Abstract accepted by 4th Asia-pacific Global Summit & Expo on Healthcare, 2016*

CHAPTER 5

CARDIOMETABOLIC RISK FACTORS IN APPARENTLY HEALTHY YOUNG ADULTS AND RELATIONSHIP WITH VITAMIN D STATUS

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death globally, and is estimated to cause death of over 17.5 million people each year, which is 31% of all annual deaths (Deaton et al., 2011; WHO, CVD fact sheet, data accessed in May, 2016). Like most of the other non-communicable diseases, CVD is strongly associated with lifestyle and behavioral factors. Smoking, unhealthy diet, obesity, physical inactivity and alcohol over-consumption increase risk of CVD via deleterious effects on blood pressure, lipids, inflammation, glucose control and uric acid, among others (Gordon et al., 1977; Perk et al., 2012). CVD is generally the end result of many years of accumulated underlying changes that are 'hidden' from view and begin in early adult life (Raitakari et al., 2003; Perk et al., 2012). Therefore, even apparently healthy young adults may have early biomarker signs of elevated risk of CVD occurring in a later stage of life. These biomarkers include blood pressure, fasting glucose, HbA1c, lipids, uric acid and hsCRP.

Apart from the well established 'traditional' CVD risk factors, and others mentioned above, other underlying and perhaps yet-to-be-discovered factors most likely play a role in overall CVD risk, because CVD can develop in those who do not seem to be at high risk (Helfand et al., 2009). In this regard, vitamin D deficiency is a potential candidate as an emerging risk factor (Norman and Powell, 2014). A meta-analysis of 19 prospective studies involving a total of 6,123 CVD cases in a total population of 65,994 men and women aged >18 years showed that the RR for incident CVD increased by 18% with every 25 nmol/l decrease in plasma 25(OH)D concentration (Wang et al., 2012). From the data shown, there is an exponential increase in RR for CVD as plasma 25(OH)D decreases below 60 nmol/l (Wang et al., 2012). A prospective study in US involving 41,504 middle aged (mean age 55 years) men and women compared incident hypertension, hyperlipidaemia, type 2 diabetes and peripheral vascular disease in follow-up (average 1.3 years, maximum 9 years) in those with baseline serum 25(OH)D concentration ≤37.5 nmol/l compared to those with 25(OH)D >75 nmol/l at baseline (Anderson et al., 2010). Significant increases (all $p<0.05$) in Hazard Ratio for all four endpoints were found. Furthermore, in those subjects aged >50 years (n=27,686), low vitamin D status (25(OH)D <37.5 nmol/l) was associated with higher ($p<0.05$) prevalence of CHD, heart failure, atrial fibrillation, ventricular tachycardia, peripheral vascular disease, and previous myocardial infarction, stroke, or transient ischaemic attack, compared to those with 25(OD)D >75 nmol/l (Anderson et al., 2010). Further evidence that vitamin D may play a role in cardiometabolic health was presented recently in a cross-sectional study of 411 non-diabetic subjects aged 18-81 years (Krivošíková et al., 2015). It was found that the plasma 25(OH)D concentration decreased as the number of cardiometabolic risk factors (including central obesity, elevated BP, increased atherogenic index (based on lipids) and insulin resistance)

(Krivošíková et al., 2015). However, it is noted that the age range was very wide (18-81 years), and data were pooled, with no separate data shown for younger subjects.

In this part of the study, the association between vitamin D status (as plasma 25(OH)D concentration) and various biomarkers of CVD risk (blood pressure, fasting plasma glucose, HbA1c, lipids, uric acid and hsCRP) was investigated in apparently healthy young adults. The primary aim was to determine possible impact of vitamin D deficiency on cardiometabolic health and CVD risk factors in early adult life. In addition, the profile of CVD risk factors in young apparently healthy men and women studied was investigated.

MATERIALS AND METHODS (PLEASE ALSO REFER TO CHAPTER 3 FOR DETAILED DESCRIPTIONS)

Fasting venous blood samples were collected, with their written informed consent, from 196 subjects (63 males, 133 females) aged 18-26 years. Blood samples were collected into fluoride oxalate blood collection tubes (for fasting plasma glucose), EDTA blood collection tubes (for HbA1c) and heparinized blood collection tubes (for the other biomarkers). Samples were centrifuged within 1 hour of collection. Fluoride plasma was separated and fasting plasma glucose was measured on the same day by commercial kit. Heparinized plasma was separated, aliquoted and stored at -80^{o}C until thawed, once only, for 25(OH)D, total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), total triglycerides (Tg), hsCRP and uric acid measurement by commercial kits. Low density lipoprotein cholesterol (LDL-C) concentration was calculated by Friedewald

equation: LDL-C = TC - HDL-C - Tg/2.2, with unit of mmol/l of all biomarkers involved (Friedewald et al., 1972). EDTA whole blood was stored at 4^{o}C, and HbA1c was measured by column chromatography method within one week. Blood pressure (systolic and diastolic) was measured on the same day or another day within one week of the sampling day at the University Health Centre.

Data handling and statistical analysis: Results are presented as mean and standard deviation (SD), median and range (low to high). The association between CVD related biomarkers and vitamin D status was analyzed by Pearson's correlation or Spearman's correlation, as appropriate. The difference of CVD biomarkers across the quartiles of plasma 25(OH)D concentration and three categories of plasma 25(OH)D concentrations (<25 nmol/l, ≥25<50 nmol/l and ≥50 nmol/l) was analyzed by one-way ANOVA with Newman-Keuls post hoc test or Kruskal-Wallis test with Dunns post hoc test, as appropriate.

Each of the biomarker results of interest was assigned to "normal" or "increased cardiometabolic risk" categories using the threshold values listed in Table 5.1, and the number of "increased cardiometabolic risk" biomarkers for each subject was counted. The number of risk factors was scored for each subject, and subjects were assigned to different categories based on how many risk factor numbers were present (0, 1, 2, 3 or more). It is noted that:

- If total cholesterol (TC) and LDL-cholesterol (LDL-C) were both high, the risk number was counted as one, because the TC was probably elevated by LDL-C;
- If HDL-C and LDL-C were both in the increased risk range, these were both counted, as they are independent risk factors for CVD;

- If fasting plasma glucose and HbA1c were both high, this was counted as one risk, i.e. of impaired glycaemic control;
- If HbA1c was high but fasting plasma glucose was low, high HbA1c was still regarded as one risk factor (of impaired glucose tolerance).
- If TC/HDL-C ratio was high while none of the TC, HDL-C and LDL-C concentrations was in the high risk range, then the high ratio was considered as one risk factor.
- If Log(Tg/HDL-C) was high while none of the Tg, TC, HDL-C and LDL-C concentrations was in the high risk range, then the high ratio was considered as one risk factor for athrogenesis.

It is noted that some subjects may not have been truly fasting when blood was collected. For example, if a subject had a normal HbA1c (<5.7%) level but had elevated fasting plasma glucose (≥5.6 mmol/l) or Tg (>2.0 mmol/l), this subject was defined as "likely non-fasting subject", and his/her fasting plasma glucose, LDL-C and Tg values were removed and the risk factor number presented for these subjects was re-calculated.

The plasma 25(OH)D concentrations across the different risk factor groups, and the number of risk factors across three plasma 25(OH)D concentration groups (<25 nmol/l, ≥25<50 nmol/l and ≥50 nmol/l) were compared using one-way ANOVA with Newman-Keuls post hoc test or Kruskal-Wallis test with Dunns post hoc test, as appropriate. Male-female differences in cardiometabolic risk factors were also explored using the unpaired t-test or Mann-Whitney test, as appropriate. A p value <0.05 was considered statistically significant.

Table 5.1. The Thresholds Used to Assign Biomarker Results to Increased Cardiometabolic Risk Factor Category

Biomarker	Threshold for risk	Rationale and Reference
fasting glucose	≥5.6 mmol/l for impaired fasting glycaemia	American Diabetes Association 2015
HbA1c	≥6.5% for Diabetes mellitus; ≥5.7%, <6.5% for impaired glucose tolerance	
TC	>5.0 mmol/l	European Guidelines on cardiovascular disease prevention in clinical practice (version 2012)
HDL-C	<1.0 mmol/l in men <1.2 mmol/l in women	
LDL-C	>3.0 mmol/l	
Tg	>1.7 mmol/l	
BP	SYS BP: >130 mmHg DIA BP: >85 mmHg	
TC/HDL-C ratio	>4	
Log(Tg/HDL-C)	>0.11	Dobiásová and Frohlich, 2001
hsCRP	>3.0 mg/l	Ridker, Circulation, Clin Biochem, 2003
Uric acid	>360 µmol/l in women >420 µmol/l in men	Feig et al., 2008

RESULTS

The values of the cardiometabolic biomarkers of the 196 subjects studied are shown in Table 5.2. It can be seen that the overall mean value for each of the cardiometabolic biomarkers measured was within the reference/desirable range (as described in Table 5.1). However, some of the values from the young apparently healthy subjects were in the increased risk category for every measured parameter, except for diastolic blood pressure.

Focusing on the main aim of this part of the study, the possible impact of vitamin D deficiency on the selected cardiometabolic risk factors measured was examined and results are shown in Table 5.3. Plasma 25(OH)D concentration was found to be significantly and inversely associated with fasting plasma glucose: $r=-0.18$; $p<0.05$; $n=196$, and to be significantly and directly associated with plasma uric acid ($r=0.17$; $p<0.05$). There was no significant difference found across the quartiles of plasma 25(OH)D in terms of the levels of individual cardiometabolic risk factors (Table 5.4). However, as shown in Table 5.5, significantly higher ($p<0.05$) HbA1c and TC/HDL-C ratio and lower HDL-C were seen in those with plasma 25(OH)D <25 nmol/l, compare with those with 25(OH)D ≥25<50 nmol/l and those with 25(OH)D ≥50 nmol/l. Log(Tg/HDL-C) was lower in subjects with 25(OH)D <25 nmol/l compared with results in the other two groups, but this did not quite reach statistical significance ($p=0.051$).

Table 5.2. Descriptive Results of CVD Related Biomarkers in 196 Apparently Healthy Adults Aged 18-26 Years

Biomarker	Total (n=196)			Male (n=63)			Female (n=133)			Threshold value used for increased CVD risk
	Mean(SD)	Median	Range	Mean(SD)	Median	Range	Mean(SD)	Median	Range	
SYS BP (mmHg)	112.8(10.1)	112.0	88.0-146.0	119.4(10.0)	120.0	93.0-140.0	109.6(8.6)*	109.0	88.0-146.0	≥130 mmHg
DIA BP (mmHg)	64.0(7.7)	64.0	42.0-81.0	66.1(8.2)	67.0	45.0-81.0	63.0(7.3)*	63.0	42.0-81.0	≥85 mmHg
Fasting glucose (mmol/l)	5.22(0.45)	5.20	4.50-8.40	5.29(0.58)	5.30	4.50-8.40	5.18(0.36)	5.20	4.50-6.70	≥5.6 mmol/l
HbA1c (%)	5.29(0.51)	5.30	3.80-6.70	5.31(0.54)	5.40	3.80-6.30	5.29(0.49)	5.20	4.10-6.70	≥5.7%
TC (mmol/l)	4.60(0.78)	4.52	2.69-7.24	4.61(0.89)	4.52	2.69-7.24	4.59(0.72)	4.53	2.76-6.63	>5.0 mmol/l
HDL-C (mmol/l)	1.51(0.30)	1.49	0.88-2.54	1.43(0.28)	1.46	0.88-2.03	1.54(0.31) *	1.51	0.90-2.54	<1.0 mmol/l in men <1.2 mmol/l in women
LDL-C (mmol/l)	2.72(0.63)	2.67	1.37-4.61	2.79(0.72)	2.79	1.37-4.61	2.69(0.59)	2.65	1.46-4.32	>3.0 mmol/l
TC/HDL-C ratio	3.12(0.58)	3.04	2.04-5.68	3.29(0.68)	3.1	2.16-5.68	3.03(0.50) *	2.93	2.04-4.81	>4
Tg (mmol/l)#	0.8(0.33)	0.73	0.34-2.61	0.87(0.36)	0.77	0.43-2.61	0.79(0.31)	0.72	0.34-2.31	>1.7 mmol/l
Log(Tg/HDL-C)#	-0.29(0.19)	-0.31	-0.37-0.37	-0.23(0.19)	-0.27	-0.57-0.26	-0.31(0.19)*	-0.33	-0.75-0.37	>0.11
Uric acid (µmol/l)	322.0(73.6)	313.5	174.0-611.1	376.9(82.4)	374.0	179.6-611.1	296.0(52.0) *	293.8	174.0-430.6	>360 µmol/l in women >420 µmol/l in men
hsCRP (mg/l)#	0.50(0.84)	0.25	0.06-5.89	0.66(1.08)	0.30	0.07-5.33	0.42(0.70)	0.22	0.06-5.89	>3.0 mg/l

*p<0.05 compared with the figures in male subjects #Because these results have a skewed distribution, Mann-Whitney U test was performed

Table 5.3. Correlation Table of Association between Plasma 25(OH)D Concentration and Individual Cardiometabolic Risk Factors in the 196 Subjects Studied

Biomarker	Pearson's r	p value
SYS BP (mmHg)	0.011	0.873
DIA BP (mmHg)	0.024	0.736
Fasting glucose (mmol/l)	-0.180	0.012
HbA1c (%)	-0.003	0.963
TC (mmol/l)	0.118	0.101
HDL-C (mmol/l)	0.066	0.273
LDL-C (mmol/l)	0.118	0.075
TC/HDL-C ratio	0.004	0.933
Tg (mmol/l)#	0.046	0.524
Log(Tg/HDL-C)#	0.007	0.926
Uric acid (μmol/l)	0.232	0.001
hsCRP (mg/l)#	0.000	0.999

Spearman's correlation was performed for skewed data, Pearson's correlation was performed for the others

Table 5.4. CVD Related Biomarkers across Quartiles (Q) Based on Plasma 25(OH)D Concentration

All results shown are mean(SD)

Biomarker	Q1	Q2	Q3	Q4	p*
25(OH)D (nmol/l)	27.0(4.7)	36.5(2.0)	45.5(3.3)	59.7(7.2)	<0.001
SYS BP (mmHg)	114.3(9.5)	111.0(11.2)	111.9(10.4)	113.8(9.0)	0.3090
DIA BP (mmHg)	64.3(6.3)	63.6(8.7)	63.2(8.8)	64.8(7.0)	0.7341
Fasting glucose (mmol/l)	5.29(0.44)	5.25(0.58)	5.21(0.30)	5.11(0.42)	0.2473
HbA1c (%)	5.32(0.58)	5.24(0.49)	5.28(0.46)	5.34(0.50)	0.8006
TC (mmol/l)	4.54(0.80)	4.64(0.72)	4.48(0.61)	4.73(0.94)	0.3990
HDL-C (mmol/l)	1.48(0.32)	1.54(0.33)	1.51(0.28)	1.50(0.28)	0.7649
LDL-C (mmol/l)	2.70(0.63)	2.74(0.56)	2.59(0.54)	2.85(0.76)	0.2482
TC/HDL-C ratio	3.15(0.62)	3.09(0.54)	3.05(0.61)	3.18(0.54)	0.6394
Tg (mmol/l)#	0.80(0.35)	0.80(0.33)	0.82(0.27)	0.84(0.36)	0.5443
Log(Tg/HDL-C)#	-0.29(0.20)	-0.31(0.21)	-0.28(0.19)	-0.27(0.19)	0.7998
Uric acid (μmol/l)	305.5(66.4)	307.4(62.8)	333.2(84.0)	342.0(74.6)	0.0258
hsCRP (mg/l)#	0.49(0.85)	0.49(0.92)	0.54(0.68)	0.48(0.93)	0.1747

*p value for differences across quartiles by ANOVA or #Kruskal-Wallis test for skewed data

Table 5.5. CVD Related Biomarkers across Vitamin D Status Categories

All results shown are mean(SD)

Plasma 25(OH)D concentration (nmol/l)	<25 nmol/l n=13 11F/2M	≥25 <50 nmol/l n=128 90F/38M	≥50 nmol/l n=55 32F/23M	p
25(OH)D (nmol/l)	*20.2(2.7)	37.2(6.5)	58.8(7.3)	<0.001
SYS BP (mmHg)	113.8(13.2)	111.9(10.1)	114.5(9.1)	0.2533
DIA BP (mmHg)	61.9(7.5)	63.7(8.0)	65.2(7.1)	0.2708
Fasting glucose (mmol/l)	5.26(0.42)	5.25(0.46)	5.12(0.41)	0.1657
HbA1c (%)	*5.69(0.52)	5.25(0.50)	5.30(0.49)	0.0129
TC (mmol/l)	4.45(0.82)	4.54(0.70)	4.76(0.91)	0.1697
HDL-C (mmol/l)	*1.30(0.23)	1.53(0.31)	1.50(0.29)	0.0265
LDL-C (mmol/l)	2.72(0.68)	2.65(0.56)	2.87(0.75)	0.0932
TC/HDL-C ratio	*3.50(0.79)	3.03(0.52)	3.23(0.59)	0.0048
Tg (mmol/l)#	0.95(0.46)	0.78(0.29)	0.86(0.36)	0.1945
Log(Tg/HDL-C)#	-0.17(0.22)	-0.31(0.19	-0.26(0.19)	0.0512
Uric acid (μmol/l)	301.9(66.1)	316.8(70.2)	338.8(80.9)	0.1068
hsCRP (mg/l)#	0.37(0.50)	0.52(0.86)	0.47(0.88)	0.8705

*p<0.05 compared to the value in the other two categories of vitamin D status

#Because these biomarkers have a skewed distribution, Kruskal–Wallis test was performed

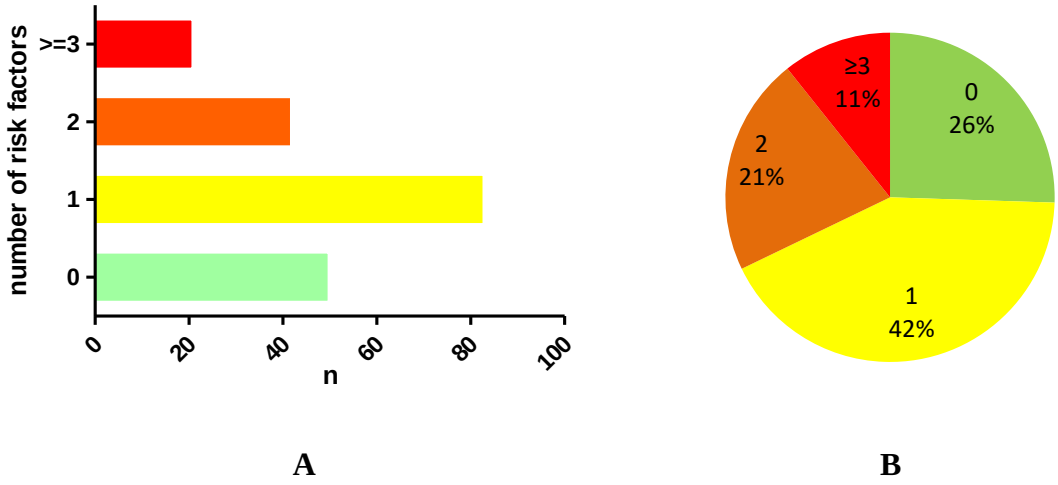
In regard to the number of volunteers who scored 0, 1, 2, or ≥3 biomarkers in the increased risk category, results are shown in Table 5.6 and Figure 5.1. It can be seen that, in the 196 young healthy subjects studied, only 50 (26%) had no biomarkers in the

elevated CVD risk category, and 21 (11%) had 3 or more biomarkers in the elevated CVD risk category. The distribution and ranking of increased cardiometabolic risk factors are given in Table 5.7. Elevated LDL-C was the most commonly observed elevated risk factor; 62/196 (31.6%; 24 men and 38 women) subjects had LDL-C >3.0 mmol/l. The second were elevated TC and uric acid, which was found in 56 subjects (28.6%; 20 men and 36 women; 37 men and 19 women, respectively); HbA1c was the third most commonly elevated risk factor, and was elevated in 50 subjects (25.5%, 20 men and 30 women). It is noted that not all cases of elevated TC were accompanied by high LDL-C. Some subjects with high TC had normal LDL-C concentration but high HDL-C, which is beneficial for cardiovascular health (Gordon et al., 1977). In addition, not all subjects with normal TC had normal LDL-C concentration. There were some cases where there was low HDL-C but elevated LDL-C, giving an apparently 'normal' TC level (<5.0 mmol/l). Therefore, the elevated TC/HDL-C ration (>4.0) was used in this current study as a risk factor, but, as noted earlier, the ratio was only counted separately as a risk factor in those cases where TC, HDL-C and LDL-C concentration were not in the elevated risk category.

In regard to the gender difference, men had a significantly larger number of cardiometabolic risk factors compared to women, the mean risk factor number in men (n=63) was 1.73 vs. 0.94 in women (n=133), $p<0.001$. The most common elevated CVD risk factor in men was uric acid (37/63), which was the fifth most common risk factor present in women (19/133). Elevated LDL-C was found in around one third of both males and females in this study: 38.1% of men and 28.6% of women had LDL-C >3.0 mmol/l.

Table 5.6. The Prevalence of Increased CVD Risk Factors in the Apparently Healthy Young Adults Studied (n=196)

Number of increased CVD risk factors present *(please refer to table 5.1 for thresholds)*	0	1	2	≥3
n	50	83	42	21



A **B**

Figure 5.1. The Distribution of Different Numbers of Increased CVD Risk Factors Present in Apparently Healthy Young Adults (n=196)

(A. bar chart showing absolute numbers for the 196 subjects studied; B. pie chart showing relative numbers (%))

Table 5.7. The Distribution n (%) of Increased Cardiometabolic Risk Factors in 196 Apparently Healthy Young Adults; [prevalence ranking]

Biomarker	Total (n=196)	Male (n=63)	Female (n=133)
LDL-C	62(31.6%) [1]	24(38.1%) [2]	38(28.6%) [1]
Uric acid	56(28.6%) [2]	37(58.7%) [1]	19(14.3%) [5]
TC	56(28.6) [2]	20(31.7%) [3]	36(27.1%) [2]
HbA1c	50(25.5%) [3]	20(31.7%) [3]	30(22.6%) [3]
Fasting glucose	31(15.8%) [4]	11(17.5%) [4]	20(15.0%) [4]
HDL-C	17(8.7%) [5]	4(6.3%) [7]	13(9.8%) [6]
TC/HDL-C ratio	13(6.6%) [6]	8(12.7%) [5]	5(3.8%) [7]
SYS BP	10(5.1%) [7]	8(12.7%) [5]	2(1.5%) [9]
Log(Tg/HDL-C)	7(3.6%) [8]	5(7.9%) [6]	2(1.5%) [9]
Tg	5(2.6%) [9]	2(3.2%) [8]	3(2.3%) [8]
hsCRP	5(2.6%) [9]	4(6.3%) [7]	1(0.8%) [10]
DIA BP	0	0	0

Turning to the study of association between the number of cardiometabolic risk factors of interest that were present and vitamin D status, there was no significant difference (p=0.64) observed in the plasma 25(OH)D concentration in relation to the presence, absence or number of elevated risk factors found (Table 5.8 and Figure 5.2). Furthermore, when the number of elevated risk factors was studied according to the different vitamin D status categories, no significant difference was seen (Table 5.9 and Figure 5.3).

Table 5.8. Plasma 25(OH)D Concentration in Relation to the Number of CVD Risk Factors Present

All data shown are mean(SD)

Number of CVD risk factors present	0	1	2	≥3
n	50	83	42	21
Mean(SD) of plasma 25(OH)D concentration (nmol/l)	42.8(11.4)	41.7(12.6)	43.3(15.0)	40.0(14.8)

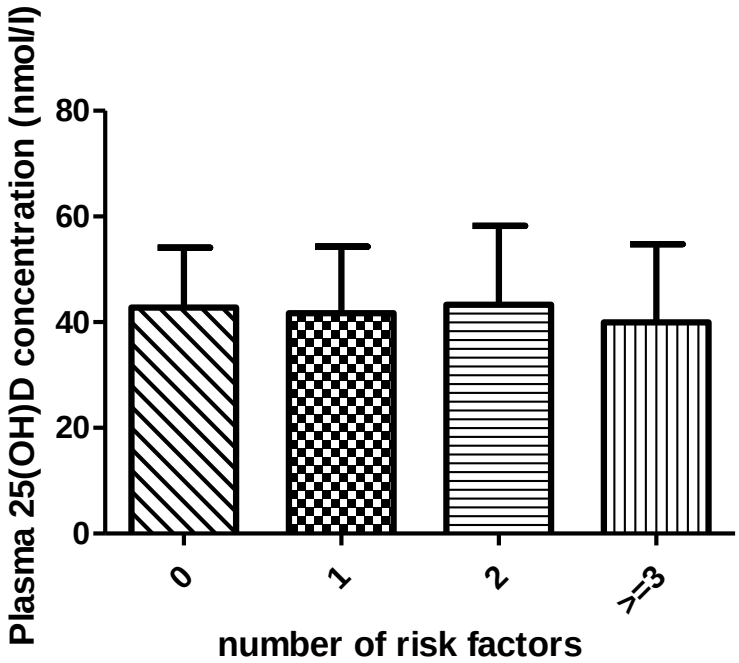


Figure 5.2. Plasma 25(OH)D Concentration in Relation to the Number of CVD Risk Factors Present

Results shown are mean+SD

Table 5.9. The Number of Risk Factors in Subjects in the Different Vitamin D Status Categories

Plasma 25(OH)D concentration (nmol/l)	<25 nmol/l n=13 11F/2M	≥25 <50 nmol/l n=128 90F/38M	≥50 nmol/l n=55 32F/23M
Mean(SD) value of number of risk factors	1.77(1.01)	1.12(0.97)	1.24(0.98)
The distribution of number of risk factors [n] (%)	0 [1] (7.7%) 1 [5] (38.5%) 2 [3] (23.1%) ≥3 [4] (30.8%)	0 [36] (28.1%) 1 [55] (43.0%) 2 [26] (20.3%) ≥3 [11] (9.6%)	0 [13] (23.6%) 1 [23] (41.8%) 2 [13] (23.6%) ≥3 [6] (10.9%)

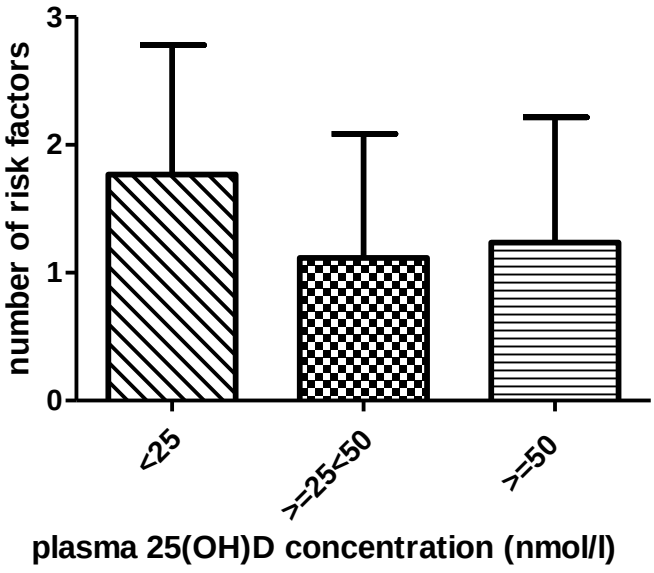


Figure 5.3. The Number of Risk Factors in Different Categories of Vitamin D Status

Results shown are mean+SD

The database was screened for possible non-fasting samples, and six subjects were identified with possible non-fasting status. Their fasting glucose, LDL-C and Tg results were removed from the dataset (Table 5.10). Non-fasting samples removed – data are presented in the following figures and tables; however, it is noted that the removal of these made no significant difference of the findings overall.

Table 5.10. Fasting glucose, HbA1c and Tg Concentrations of Possible Non-fasting Status Subjects

Subject code	Fasting glucose (mmol/l)	HbA1c (%)	Tg (mmol/l)
008	6.4	5.4	1.16
013	5.9	5.5	1.85
017	5.8	4.8	1.50
019	5.8	4.4	1.29
025	8.4	5.9	1.24
176	5.6	4.6	2.30

After the possible non-fasting data were removed, the number of subjects who had no detectable elevation of CVD risk based on their biomarker profile was increased by three, to 53 (27%), but 19/196 subjects studied still had 3 or more elevated risk factors (Table 5.9 and Figure 5.4). In terms of the other analyses as presented above, the removal of possible non-fasting data made no difference to the findings (Tables 5.10 and 5.11 and Figures 5.5 and 5.6).

Table 5.11. Table of Association between Plasma 25(OH)D Concentration and Individual Cardiometabolic Risk Factors in the 196 Subjects Studied after Removal of Possible Non-fasting Data

Biomarker	Pearson's r	p value
SYS BP (mmHg)	0.011	0.873
DIA BP (mmHg)	0.024	0.736
Fasting glucose (mmol/l)	-0.182	0.012
HbA1c (%)	-0.003	0.963
TC (mmol/l)	0.118	0.101
HDL-C (mmol/l)	0.066	0.273
LDL-C (mmol/l)	0.118	0.105
TC/HDL-C ratio	0.004	0.933
Tg (mmol/l)#	0.086	0.234
Log(Tg/HDL-C)#	0.007	0.926
Uric acid (μmol/l)	0.232	0.001
hsCRP (mg/l)#	0.000	0.999

#Because these biomarkers have a skewed distribution, Spearman's correlation was performed

Table 5.12. CVD-Related Biomarkers across Quartiles (Q) of Plasma 25(OH)D Concentration after Removal of Possible Non-fasting Data

All results shown are mean(SD)

Biomarker	Q1	Q2	Q3	Q4	p
25(OH)D (nmol/l)	26.7(4.6)	36.2(2.0)	45.7(3.4)	59.4(6.2)	<0.001
SYS BP (mmHg)	114.3(9.8)	111.8(11.1)	112.1(10.7)	113.2(8.4)	0.6231
DIA BP (mmHg)	64.3(6.4)	64.1(8.4)	63.3(9.1)	64.7(6.7)	0.8539
Fasting glucose (mmol/l)	5.27(0.43)	5.16(0.34)	5.21(0.30)	5.09(0.39)	0.1148
HbA1c (%)	5.30(0.44)	5.29(0.48)	5.28(0.48)	5.37(0.51)	0.8528
TC (mmol/l)	4.50(0.81)	4.65(0.59)	4.54(0.61)	4.75(0.97)	0.3898
HDL-C (mmol/l)	1.47(0.32)	1.53(0.31)	1.51(0.26)	1.50(0.29)	0.7882
LDL-C (mmol/l)	2.70(0.64)	2.75(0.56)	2.59(0.54)	2.85(0.77)	0.2301
TC/HDL-C ratio	3.15(0.63)	3.12(0.54)	3.09(0.59)	3.21(0.55)	0.8045
Tg (mmol/l)#	0.76(0.31)	0.74(0.22)	0.82(0.27)	0.83(0.36)	0.2747
Log(Tg/HDL-C)#	-0.29(0.20)	-0.31(0.21)	-0.28(0.19)	-0.27(0.19)	0.7998
Uric acid (μmol/l)	307.2(65.1)	308.7(64.8)	332.6(85.8)	339.8(73.5)	0.0709
hsCRP (mg/l)#	0.45(0.80)	0.46(0.70)	0.57(0.69)	0.50(0.97)	0.1024

#Because these biomarkers have a skewed distribution, Kruskal–Wallis test was performed

Table 5.13. CVD Related Biomarkers across Vitamin D Status Categories after Removal of Possible Non-fasting Data

All results shown are mean(SD)

Plasma 25(OH)D concentration (nmol/l)	<25 nmol/l n=13	≥25 <50 nmol/l n=128	≥50 nmol/l n=55	p
25(OH)D (nmol/l)	20.2(2.7)	37.1(6.7)	58.4(6.5)	<0.001
SYS BP (mmHg)	113.8(13.2)	112.3(10.2)	114.0(8.6)	0.5364
DIA BP (mmHg)	61.9(7.5)	63.9(8.0)	65.1(6.9)	0.3425
Fasting glucose (mmol/l)	5.21(0.39)	5.21(0.36)	5.09(0.37)	0.1307
HbA1c (%)	*5.69(0.52)	5.26(0.50)	5.32(0.50)	0.0159
TC (mmol/l)	4.45(0.82)	4.55(0.66)	4.79(0.93)	0.1322
HDL-C (mmol/l)	*1.30(0.23)	1.52(0.30)	1.50(0.29)	0.0299
LDL-C (mmol/l)	2.74(0.70)	2.65(0.56)	2.88(0.75)	0.0883
TC/HDL-C ratio	*3.50(0.79)	3.06(0.52)	3.25(0.61)	0.0088
Tg (mmol/l)#	0.88(0.39)	0.76(0.25)	0.85(0.36)	0.1711
Log(Tg/HDL-C)#	-0.17(0.22)	-0.31(0.19	-0.26(0.19)	0.0512
Uric acid (μmol/l)	301.9(66.1)	317.8(70.9)	336.6(80.4)	0.1860
hsCRP (mg/l)#	0.37(0.50)	0.51(0.76)	0.49(0.92)	0.7216

*p<0.05 compared to the value in the other two categories of vitamin D status

#Because these biomarkers have a skewed distribution, Kruskal–Wallis test was performed

Table 5.14. The Prevalence of Different Numbers of CVD Risk Factors Present in Apparently Healthy Young Adults after Removal of Possible Non-fasting Data

Number of CVD risk factors present	0	1	2	≥3
n	53	83	41	19

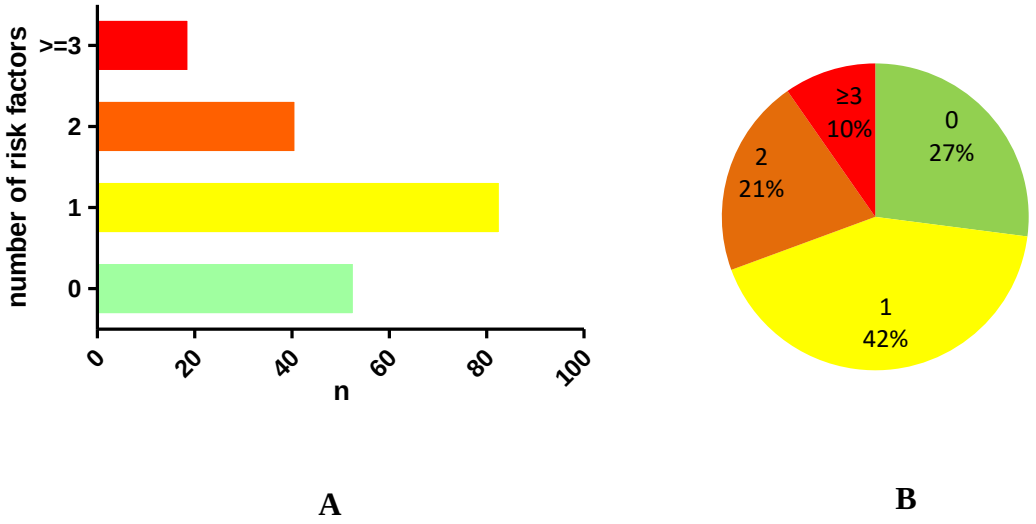


Figure 5.4. The Prevalence of Different Numbers of CVD Risk Factors Presented in Apparently Healthy Young Adults after Removal of Possible Non-fasting Data

(A. bar chart; B. pie chart)

Table 5.15. Plasma 25(OH)D Concentration According to the Presence of CVD Risk Factors after Removal of Possible Non-fasting Data

Number of CVD risk factors present	0	1	2	≥3
n	53	83	41	19
Mean(SD) of plasma 25(OH)D concentration	42.5(11.5)	41.4(12.6)	43.7(15.2)	41.3(14.9)

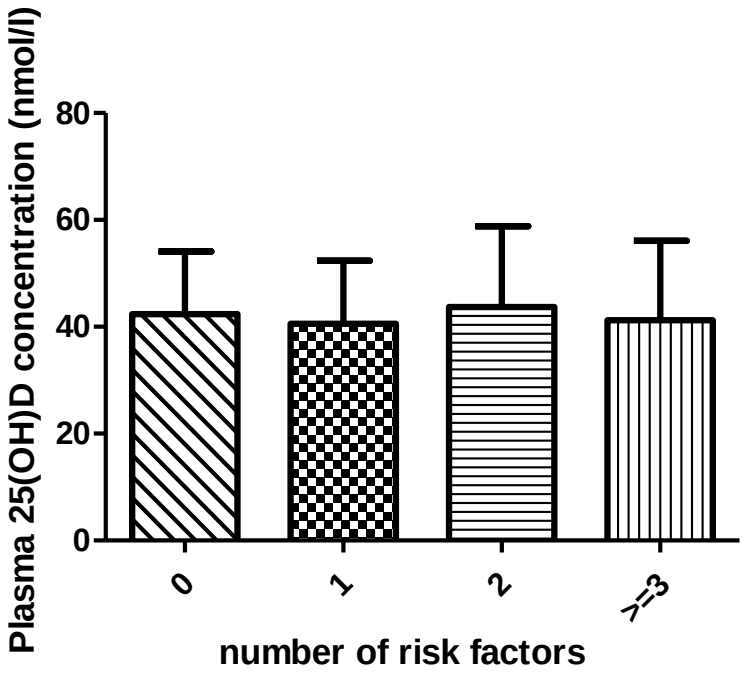


Figure 5.5. Plasma 25(OH)D Concentration According to the Presence of CVD Risk Factors after Removal of Possible Non-fasting Data

Results shown are mean+SD

Table 5.16. The Number of Risk Factors in Different Vitamin D Status Categories after Removal of Possible Non-fasting Data

Plasma 25(OH)D concentration (nmol/l)	<25 nmol/l	≥25 <50 nmol/l	≥50 nmol/l
n	13	128	55
Mean(SD) value of number of risk factors	1.54(1.05)	1.08(0.97)	1.26(1.02)
The distribution of number of risk factors	0 [2] (15.4%)	0 [37] (30.8%)	0 [14] (27.4%)
	1 [5] (38.5%)	1 [56] (46.7%)	1 [22] (43.1%)
	2 [3] (23.1%)	2 [25] (20.8%)	2 [13] (25.4%)
	≥3 [3] (23.1%)	≥3 [10] (8.3%)	≥3 [6] (11.8%)

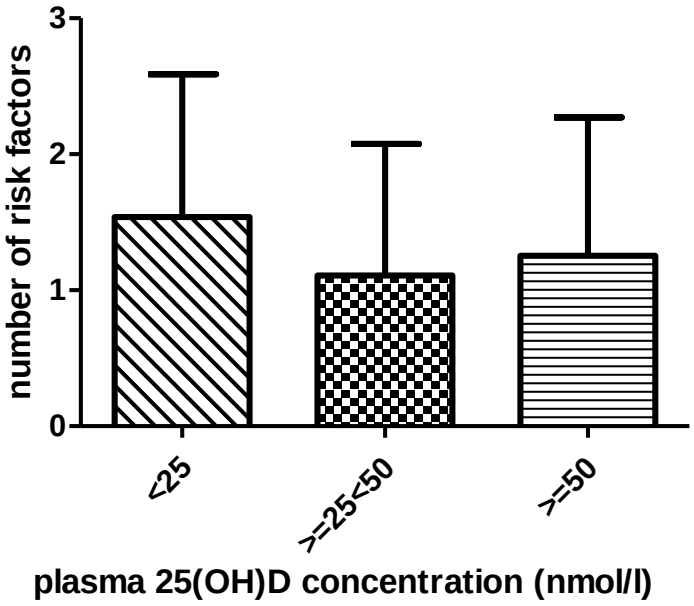


Figure 5.6. The Number of Risk Factors in Different Vitamin D Status Categories after Removal of Possible Non-fasting Data

Results shown are mean+SD

DISCUSSION

The aim of this part of the study was to investigate the association between vitamin D status and cardiometabolic risk in apparently healthy young adults using a variety of biomarkers. Results showed that higher vitamin D status is associated with lower fasting plasma glucose and higher uric acid, while very low vitamin D status (plasma 25(OH)D concentration <25 nmol/l) is linked to significantly higher HbA1c and TC/HDL-C ratio and lower HDL-C concentrations than those with higher vitamin D status. In addition, those with severe vitamin D deficiency had lower Log(Tg/HDL-C), but this did not quite reach statistical significance. No differences were seen in other biomarkers. Besides, the cardiometabolic risk profile of the young apparently healthy adults studied showed that ~75% had ≥1 CVD-related risk factors, including high blood pressure, impaired glucose control, dyslipidaemia, elevated uric acid and/or high hsCRP. It is noted that in this current study, higher uric acid was found to be weakly but significantly associated with higher plasma 25(OH)D concentration. This may due to the synthesis of both uric acid and 25(OH)D being modulated by the same genes (for example, rs2282679 and rs2231142) (Thakkinstian et al., 2015).

There are a few published studies on the association between vitamin D and CVD risk. A cross-sectional study in Singapore with 114 non-pregnant and healthy subjects (59 males and 55 females) aged 21-100 years, found that vitamin D status is associated with various cardiovascular risk factors, including body fat, insulin resistance (assessed by homeostasis model assessment of insulin resistance (HOMA-IR)), TC/HDL-C ratio, lean body mass and hand grip strength (Bi et al., 2016). The mean(SD) of plasma

25(OH)D of this group was 54.0(17.3) nmol/l, while males had significantly higher plasma 25(OH)D than females, the mean values were 58.3 and 49.5 nmol/l, respectively, $p<0.05$, which is higher than this current study. People with vitamin D deficiency (defined as 25(OH)D <50 nmol/l in this study) had significantly lower hand grip strength and higher body fat, lean body mass, fasting insulin, HOMA-IR, LDL-C, TC/HDL-C ratio and LDL/HDL ratio (Bi et al., 2016).

Another study in Slovakia was published recently, which also investigated the association between vitamin D status and the number of cardiometabolic risk factors. Poorer vitamin D status was found to be associated with larger number of cardiometabolic risk factors (Krivošíková et al., 2015). In this study, 411 healthy subjects (aged 18-81 years) were recruited, serum 25(OH)D concentration and four cardiometabolic risk factors (central obesity, elevated blood pressure, increased atherogenic risk and insulin resistance) were assessed. Generally, vitamin D status in this group was much higher than our subjects, the mean(SD) value of serum 25(OH)D concentration was 83.5(36.3) nmol/l, compared with 41.8(12.9) nmol/l in this current study (as presented in Chapter 4). In more detail, people who were cardiometabolic risk factor free had significantly higher serum 25(OH)D concentration compared with those with 1-2 or 3-4 risk factors, the mean(SD) values were 88.8(35.5), 81.5(35.8) and 77.5(37.8) nmol/l, respectively ($p<0.05$). However, it is noted that subjects in this study had larger age range, and age is an important factor that has impact on both vitamin D status and CVD. Besides, lipid profiles were also measured, but no association between lipids and vitamin D status was found (Krivošíková et al., 2015).

The association between vitamin D status and CVD risk factors was also found in a prospective study. A large prospective study with 1,739 middle-aged Framingham Offspring Study participants who were free of CVD at entry found that people with serum 25(OH)D concentration <37.5 nmol/l had significantly higher systolic BP, TC/HDL-C ratio and higher diabetes rate at baseline compared with those with baseline 25(OH)D concentration ≥37.5 nmol/l. During an average of 5.4 years follow-up, 120 cases of CVD developed, and people with lower baseline vitamin D status (25(OH)D <37.5 nmol/l) had 62% higher risk than those with higher vitamin D levels (95%CIs: 1.11, 2.36, p=0.01). Furthermore, it was also found that the lower 25(OH)D concentration levels were significantly associated with the CVD risk: compared with people with 25(OH)D ≥37.5 nmol/l, the HRs(95%CIs) of those with 25(OH)D ≥25 <37.5 nmol/l and with 25(OH)D <25 nmol/l were 1.53(1.00, 2.36) and 1.80(1.05, 3.08), respectively, p for trend <0.05, showing that the severity of vitamin D deficiency is linked to the higher risk of CVD (Wang et al., 2008a). Interestingly, similar with the meta-analysis of Wang et al. (2008), the association between vitamin D status and the risk of CVD was not linear. The increased hazard for CVD events was greater in those with baseline 25(OH)D concentration <50 nmol/l, when the baseline 25(OH)D concentration reached 60 nmol/l, the HR did not significantly decrease with the improvement of vitamin D status (Wang et al., 2008b).

The association of vitamin D status and higher CVD risk of various biomarkers was widely found in previous studies, and the results of this current study also support this finding. Compared with previous studies, the studied subjects in this current study had a narrower age range (18-26 years), and the subjects were all healthy and young people. The age and health status of subjects in this current study provides evidence that the

impact poor vitamin D status on CVD development may start at a very early stage of adulthood.

However, even though the association between vitamin D status and CVD was found in this current study and other observational studies, the relationship is not very strong and the effect of vitamin D supplementation has not shown robust evidence to be beneficial to cardiometabolic health (Hsia et al., 2007; George et al., 2012). A large supplementation study with primary outcome of fracture incidence found that supplementation of 200 IU vitamin D plus 500 mg calcium (n=18,176) for an average of seven years did not decrease the events of cardiovascular disease compared with placebo (n=18,106) in postmenopausal women aged at 50-79 years at entry (Hsia et al., 2007). During the follow-up, 499 cases of myocardial infarction or CHD death were found in the supplementation group, while 475 were confirmed in the placebo group, with HR of 1.04 (95%CIs: 0.92, 1.18). No difference was seen in the incidence of stroke, HR for stroke was 0.95 (95%CIs: 0.82, 1.10). However, it is noted that the vitamin D dose in this study was very low, and there is no data shown regarding whether the vitamin D status in the supplementation group was higher than the placebo group in the end of the study (Hsia et al., 2007).

A systematic review and meta-analysis with 21 studies (n=13,033) also found no significant difference on the incidence of cardiac failure, myocardial infarction nor stroke. Studies included in this systematic review had subjects of older people, with mean ages ranged 61 to 77 years. No significant reductions were seen in the risk of cardiac failure (HR: 0.82, credible intervals: 0.58, 1.10), myocardial infarction (HR: 0.96, credible intervals: 0.83, 1.10) nor stroke (HR: 1.07, credible intervals: 0.91, 1.29)

(Ford et al., 2014). Another systematic review found that vitamin D supplementation for CVD-free people does not have any significant effect on CVD risk compared with subjects in placebo group, relative risk was 0.90 (95%CIs: 0.77, 1.05) (Wang et al., 2010a).

For glycaemic control, while significant association with vitamin D was found in this current study, no significant improvement was seen in supplementation studies. In a systematic review with 15 trials (including 8 with fasting glucose, 4 with HbA1c and 9 with insulin resistance as biomarkers used for assessing glycaemic control), some but insufficient evidence was found to support that vitamin D supplementation improves the glycaemic control or insulin resistance (George et al., 2012). For fasting plasma, four studies were in people with normal fasting plasma glucose levels and the other four were with those with abnormal glucose levels. No significant change was found in the normal subjects and a statistically significant but slight decrease was found in those with impaired glycaemic control compared with placebo (-0.32 mmol/l, 95%CIs: -0.057, -0.07). For HbA1c, there was no significant improvement seen in the vitamin D supplemented group. For insulin resistance (measured by HOMA-IR or fasting insulin/C-peptide levels), there was no significant difference in the normal glycaemic control group, while a significant decrease was seen in the abnormal glucose tolerance group: -0.25 (95%CIs: -0.48, -0.03) (George et al., 2012). Overall, even though a significant improvement was seen on fasting glucose and insulin resistance in abnormal glucose tolerance people, because the changes were small, the author concluded that there was no sufficient evidence to prove the beneficial role of vitamin D supplementation on glycaemic control and insulin resistance (George et al., 2012).

However, the lack of strong evidence in clinical trials does not definitely mean vitamin D has no impact on the cardiometabolic system. As shown in this current study, the link between vitamin D deficiency and increased CVD risk can be detected in early adulthoods. Considering the low vitamin D status is very common (Hilger et al., 2014), this impact could lead to higher risk of suffering CVD and type 2 DM in the future. For most supplementation trials, the target subjects were those who already developed CVD or had impaired cardiometabolic symptoms, and it would be very difficult to reverse the long-term effect on the cardiovascular system by a relatively short duration of supplementation. Compared with intervention on those who already had pre-existing diseases, targeting at an earlier stage in life may be a more rewarding approach to improve the cardiometabolic health. It is noted that even though some evidence was found in this study, vitamin D status is not the only determinant of CVD and type 2 DM development. Genotypes and unhealthy lifestyles, like unhealthy dietary habit and physical inactivity, also play important roles in disease development. However, vitamin D status can be easily enhanced, making the improvement of vitamin D status a feasible measure to protect cardiometabolic health. As described in chapter 1, only 10-15 min of direct skin exposure to sunshine on arms and legs would be sufficient to maintain normal vitamin D status for most healthy people, and commercial vitamin D supplement is available to improve vitamin D status for those who are not able to receive sunshine exposure.

Besides, apart from the possible impact of vitamin D status on CVD and type 2 DM risk, it is noted that an alarming prevalence of high cardiometabolic risk factors is found in this study, 74% (146/196) of these young adults had at least one high CVD risk

factors, showing that the seed of the diseases begins to flourish at young age, this may cause various problems while their age increases, and is a public health concern.

This current study is a well designed study with a fairly homogenous group of young people, providing new evidence that vitamin D status is associated with the risk of cardiovascular disease in young people, but there are also some limitations because of the resources and the characteristics of this studied group. Some important biomarkers, for example, HOMA-IR, were not included into this study because of the limitation of resources. Moreover, only two of the subjects had plasma 25(OH)D ≥75 nmol/l, thus, whether reaching the optimal vitamin D status is associated with lower CVD risk cannot be probed.

In conclusion, this part of the study has shown that vitamin D status is associated with some underlying biochemical changes related to CVD and type 2 DM in a group of young apparently healthy adults, especially for the glycaemic control. This current study, as well as the other recent studies, indicates that vitamin D status plays a role in cardiometabolic health, and can be regarded as a potential, and importantly a modifiable, risk factor. This finding has an important implication for the promotion of public health policies to improve vitamin D status in young people living in Hong Kong and regard to risk of CVD and type 2 DM in later life.

Some of the information presented in this chapter was presented as:

3. *Poster presentation in the World Congress on Public Health and Nutrition, March 2016, Madrid*

4. *Presentation in the 4th International Vitamin Conference, Copenhagen, Denmark in May 2016*
5. *A research paper accepted by the Asia Pacific Journal of Clinical Nutrition*

CHAPTER 6

VITAMIN D STATUS AND DNA DAMAGE

INTRODUCTION

DNA damage is the major underlying cause of cancer and is associated with many other chronic, age-related diseases (Hoeijmakers, 2009). It has been estimated that up to 10^5 DNA lesions occur in each individual cell each day as a result of cellular metabolism (Cooke et al., 2003). There are various sources of DNA damage. Exogenous causes include radiation (UV light), cigarette smoke and air pollutants (Song et al., 2010; Duan et al., 2016). Endogenous causes include reactive oxygen species (ROS), nitrogen species, and alkylating species generated during aerobic metabolism (De Bont and van Larebeke., 2004). Oxidative stress-induced DNA damage is the majority of endogenous DNA damage (Swenberg et al., 2011), ROS are unavoidable, and regardless of the cause, oxidation-induced DNA damage can lead to cell dysfunction or death unless repaired. If repair is faulty or inadequate, mutations in the surviving cells may pass to the descendant cells, with deleterious consequences, such as apoptosis or malignant transformation (Hoeijmakers, 2009; Klaunig et al., 2011; Iyama and Wilson, 2013).

In epidemiological studies, low vitamin D status has been found to associate with increased risk of several types of cancer (Krishnan et al., 2010). In addition, in cell culture, animal and some human experimental studies, vitamin D has been reported to be beneficial to DNA damage resistance (Nair-Shalliker et al., 2012 a). For example, in

a cell culture study, $1,25(OH)_2D_3$ was found to be associated the resistance of various kinds of UV light induced DNA damage (Gordon-Thomson et al., 2012). In an animal study, $1,25(OH)_2D$ supplementation was also found to be beneficial to lower DNA damage induced by diabetes (Meerza et al., 2014). Human studies investigated the association between vitamin D and DNA damage are few. One was conducted in South Australia (Nair-Shalliker et al., 2012 b). In this cross-sectional study, no significant association was found between vitamin D status and DNA damage including chromosome breakage and loss, double-strand break, telomere end fusions and mitogenic response (Nair-Shalliker et al., 2012 b). A supplementation trial found that no significant difference was seen in people with vitamin D or calcium supplementation had lower oxidation induced DNA damage compared with the double placebo group (Fedirko et al., 2010).

It is noted that no published studies to date have investigated if vitamin D is associated with oxidation-induced DNA damage in apparently healthy people. As noted before, the oxidation-induced DNA damage is an important source of mutations and consequently causes cell dysfunction or even malignant transformation. If the association of vitamin D status and oxidation-induced DNA damage is found in apparently healthy people, evidence would be provided for the prevention and control of vitamin D related-cancer. This was the focus of this part of the study, in which the association between vitamin D status (as plasma 25(OH)D concentration) and the "global" oxidation induced DNA damage in lymphocytes (measured by Formamidopyrimidine DNA glycosylase (Fpg)-assisted comet assay) was investigated in a sub-group of young healthy subjects, aiming to determine the underlying impact of vitamin D status on oxidation-induced DNA damage levels in healthy people in early adulthood.

MATERIALS AND METHODS (PLEASE ALSO REFER TO CHAPTER 3 FOR DETAILED DESCRIPTIONS)

Fasting venous blood samples were collected from 196 subjects (63 males, 133 females) with their written informed consent. Blood samples were collected with heparinized blood collection tubes. After centrifugation and plasma collection, the buffy coat of each subject was transferred into another clean centrifuge tube for white blood cell (WBC) harvest. The WBC was stored with freezing medium (10% DMSO and 90% FBS) at -80 ^{o}C. Fpg-assisted comet assay was performed on 121 samples (44 males and 77 females). The samples were selected according to their collection date (after December 2014, as they are more fresh cells) (please refer to Chapter 3 for detailed procedure). DNA% in tail of 100 cells with Fpg buffer treatment represents the strand breaks of each sample (described as Buffer in this chapter) and DNA% in tail of 100 cells with Fpg treatment represents the stand breaks and the Fpg sensitive lesions of each sample (described as Fpg in this chapter). The difference between Fpg treatment and buffer treatment DNA damage (described as ΔFpg-buffer in this chapter), represents the Fpg sensitive DNA damage, i.e. the oxidation-induced lesions.

Data handling and statistical analysis: Results are presented as mean and standard deviation (SD), median and range (low to high). The association between DNA damages (DNA% in tail of buffer, Fpg and ΔFpg-buffer) and vitamin D status was analyzed by Pearson's correlation. The DNA damage across tertiles based on plasma 25(OH)D concentration was compared using one-way ANOVA. The difference of DNA

damage in subjects who had plasma 25(OH)D concentration <50 nmol/l or ≥50 nmol/l was analyzed with unpaired t test. A p value <0.05 was considered statistically significant.

RESULTS

The descriptive comet assay results of the 121 subjects (44 males, 77 females) are shown in Table 6.1. No significant gender difference was found. In relation to vitamin D status, no significant association was found between plasma 25(OH)D concentration and any of the buffer, Fpg and ΔFpg-buffer treated comet assay results (Figure 6.1). According to the tertiles of plasma 25(OH)D, there was no significant difference in the DNA damage score (DNA% in tail) of the Fpg-assisted comet assay (Table 6.2 and Figure 6.2). Besides, no significant difference of DNA damage score was seen between those with plasma 25(OH)D <50 or ≥50 nmol/l (Table 6.3 and Figure 6.3).

Table 6.1. Fpg-Assisted Comet Assay Results in 121 Apparently Healthy Adults Aged 18-26 Years: Descriptive Results of DNA% in Tail with 100 Nucleoids Scored per Sample in Each Treatment Mode

	Total (n=121)			Male (n=44)			Female (n=77)		
	Mean(SD)	Median	Range	Mean(SD)	Median	Range	Mean(SD)	Median	Range
25(OH)D (nmol/l)	44.68(13.03)	42.70	17.40-86.80	46.40(15.29)	45.30	17.40-86.80	43.70(11.54)	41.20	19.40-71.60
buffer	1.89(0.79)	1.73	0.55-3.92	1.94(0.81)	1.73	0.71-3.64	1.86(0.77)	1.73	0.55-3.92
Fpg	20.47(3.37)	20.18	11.66-32.57	20.72(3.25)	20.26	14.35-32.57	20.32(3.46)	20.14	11.66-27.46
ΔFpg-buffer	18.58(3.39)	18.50	9.77-30.01	18.77(3.34)	18.62	12.96-30.01	18.47(3.44)	18.34	9.77-26.75

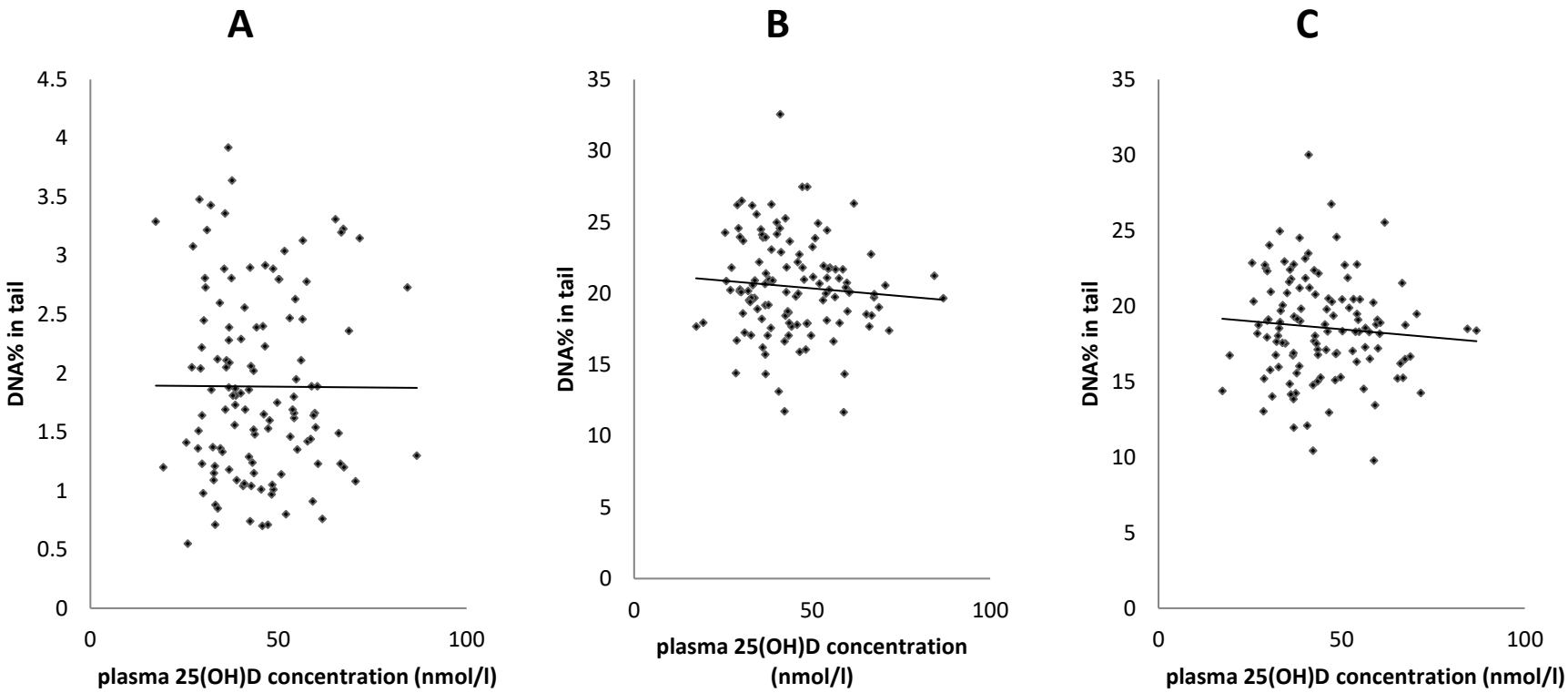


Figure 6.1. Scatter Plots of Fasting plasma 25(OH)D Concentration and %DNA in Comet Tail Results

A shows the buffer treated DNA% in 100 cells of each sample, r=-0.0045, p=0.9613;

B shows the Fpg treated DNA% in 100 cells of each sample, r=-0.0839, p=0.3602;

C shows the difference of Fpg and buffer treated DNA% in 100 cells of each sample, r=-0.0824, p=0.3690.

Table 6.2. The DNA Damage Scores (as DNA% in Tail) Across Tertiles (T) of Plasma 25(OH)D Concentration

All results shown are mean(SD)

	T1 (n=41)	T2 (n=40)	T3 (n=40)	p
25(OH)D (nmol/l)	31.6(4.4)	42.9(3.5)	59.9(8.5)	<0.0001
Buffer	1.98(0.88)	1.73(0.71)	1.95(0.76)	0.3089
Fpg	20.62(3.24)	20.63(4.09)	20.15(2.71)	0.7693
ΔFpg-buffer	18.64(3.30)	18.90(3.98)	18.19(2.84)	0.6478

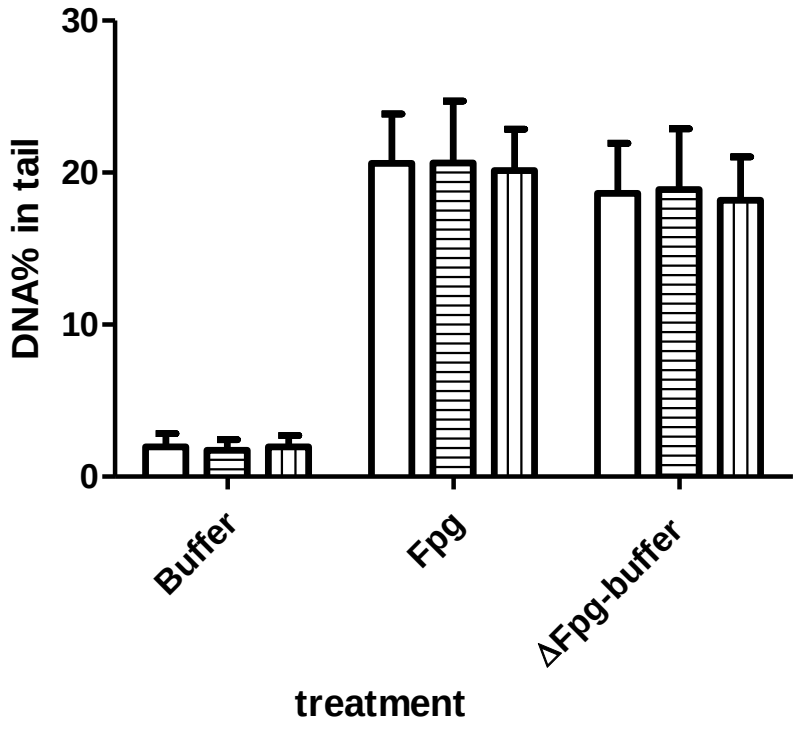


Figure 6.2. DNA% in Tail Across Tertiles according to Plasma 25(OH)D Concentration

Results shown are mean+SD**;**

T1 is in open bars, n=41; T2 is in horizontal bars, n=40; T3 is in vertical bars, n=40

Table 6.3. The DNA Damage Based on Categories of Plasma 25(OH)D Concentration

All results shown are mean(SD)

Plasma 25(OH)D concentration (nmol/l)	<50 nmol/l n=82	≥50 nmol/l n=39	p
25(OH)D (nmol/l)	37.3(7.0)	60.1(8.4)	<0.0001
Buffer	1.85(0.80)	1.96(0.77)	0.3089
Fpg	20.58(3.66)	20.23(2.70)	0.7693
ΔFpg-buffer	18.73(3.63)	18.27(2.84)	0.6478

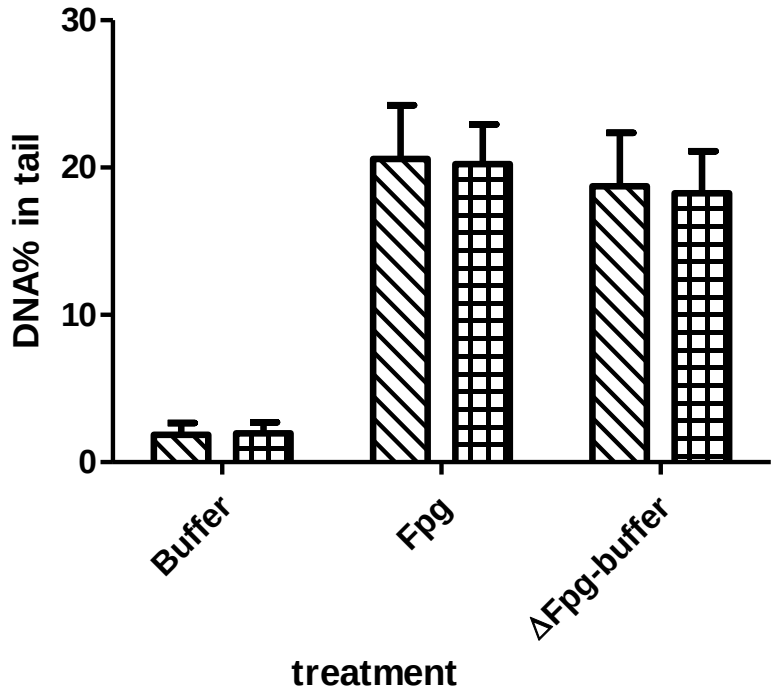


Figure 6.3. The DNA Damage Based on Categories of Plasma 25(OH)D concentration

Results shown are mean+SD**;**

plasma <50nmol/l is in diagonal bars, n=82; plasma ≥50 nmol/l is in grid bars, n=39

DISCUSSION

From previous studies on vitamin D and oxidation-induced DNA damage, the association was found in cell culture study (Gordon-Thomson et al., 2012), but no published study was aiming to investigate the "global" oxidation-induced DNA damage in healthy people. As links has been reported between vitamin D status and cancer, especially for colorectal cancer, the underlying mechanism of how vitamin D associates with DNA damage is of interest, which is the aim of this part of the study. However, no significant association was found between vitamin D status and DNA damage, and subjects with vitamin D deficiency (plasma 25(OH)D <50 nmol/l) did not have higher DNA damage compared with those with plasma 25(OH)D ≥50 nmol/l.

In a cell culture study, it was found that the active form of vitamin D played a role of protection skin cells from UV radiation in various kinds of DNA damage (Gordon-Thomson et al., 2012). Human skin cells (keratinocytes) received 1 nmol/l $1,25(OH)_2D_3$ treatment or 1% ethanol treatment as control and incubated at 37^{o}C after UV radiation. The comet assay, using three enzymes to recognize different lesions was used to assess the UV-induced DNA damage, measured as tail moment (tail length*percentage fluorescence in tail). The enzymes were, T4 endonuclease V (T4EV, specific for thymine-thymine dimers (a kind of cyclobutane pyrimidine dimer (CPD) as well as abasic sites), endonuclease IV (endo4, specific for abasic sites) and human 8-oxoguanine DNA glycosylase (hOGG1, specific for 8-oxodG, a kind of oxidation-induced damage). $1,25(OH)_2D_3$ treatment decreased the T4EV specific DNA damage score significantly with 0.5, 3 and 6 hours incubation, compared with the control cells

($p<0.001$). For endonuclease IV (endo4)-assisted comet assay, results at only one time-point (3 hours) was shown. The endo4 specific sites were also significantly lowered by $1,25(OH)_2D_3$ treatment ($p<0.05$). Besides, $1,25(OH)_2D_3$ treatment was also found to be protective against UV-induced oxidative damage. Tail moment of the hOGG1-assisted comet assay in $1,25(OH)_2D_3$ treated cells was significantly lower than the control cells. No significant difference in DNA damage was found between the irradiated cells and the $1,25(OH)_2D_3$ treated cells at all time points (0.5, 3 and 6 hours). Furthermore, in the nuclear staining of antibody to 8-oxodG sites found that the $1,25(OH)_2D_3$ also significantly ($p<0.05$) decreased the 8-oxodG in mouse skin after UV radiation (Gordon-Thomson et al., 2012). However, it is noted that the dose used in this study was far higher than the $1,25(OH)_2D$ concentration in normal physiological conditions, the mean values of circulating $1,25(OH)_2D$ concentration in human studies ranged from 70-160 pmol/l (Reinhardt et al., 1984; Hibler et al., 2014; Hussein et al., 2015).

In an animal study, vitamin D supplementation was found lowered diabetes-induced DNA damage. In this study, 24 initially normal mice were divided into control or alloxan-induced diabetes or diabetes with vitamin D supplementation groups. The mice in the diabetes and diabetes with vitamin D supplementation group both received alloxan injection until became diabetic, while the vitamin D supplementation group received 7 ng/gm/day $1,25(OH)_2D_3$ for 15 days after confirmed as diabetic. All mice were kept in a dark room to ensure no cutaneous synthesized vitamin D in these mice. The alkaline comet assay was used to measure the DNA damage in liver and pancreatic cells, and tail length was used as the biomarker to assess DNA damage in this study. Significant longer tail length (indicates more damaged) was found in the diabetic group, compared with the control mice, while the vitamin D supplemented diabetic mice had

significantly lower tail length in both liver cells and pancreatic cells compared with the diabetic group without vitamin D supplementation, showing that vitamin D supplementation is protective from diabetes-induced DNA damage (Meerza et al., 2014).

In human study, few published studies investigated the association between vitamin D and DNA damage. A cross-sectional study in South Australia included 207 subjects aged 25-60 years found no significant association between vitamin D and DNA damage. The cytokinesis-blok micronucleus cytome assay was used to assess a multiple set of DNA damage indicator in lymphocytes, including chromosome breakage and loss, double strand breaks, telomere end fusions and mitogenic response. However, no significant association were found between vitamin D status and any kinds of DNA damage in this study (Nair-Shalliker et al., 2012 b).

One supplementation study of a 2*2 factorial supplementation trial found that vitamin D supplementation had no effect on oxidative DNA damage (Fedirko et al., 2010). A total of 92 healthy subjects aged 25-60 years with history of colonic adenoma were allocated to four treatment: placebo (n=23), 2.0 g/day calcium (n=23), 800 IU/day vitamin D (n=23), and calcium plus vitamin D (n=23), all of the treatments lasted for 6 months. Biopsy specimens were taken from rectal mucosa from each subject and a labelled streptavidin-biotin method for 8-oxodG was used. The 8-oxodG labelling of the entire crypts in each group had no significant difference at baseline, and no significant improvement was seen in any group received vitamin D and/or calcium (Fedirko et al., 2010).

Compared with previous studies, this current study is the first human study investigated the link between vitamin D status and the "global" oxidation-induced DNA damage, using a validated, well-established method, the Fpg-assisted comet assay. However, it is noted that the subjects in this study had a relative low vitamin D status, mean plasma 25(OH)D concentration was 44.68 nmol/l, and only two persons reached the optimal level (plasma 25(OH)D concentration ≥75 nmol/l). A potential negative but not significant association was seen between the plasma 25(OH)D concentration and the DNA% in tails of Fpg and ΔFpg-buffer treated cells, but as lack of data shown in higher range of vitamin D status, whether this link can be strengthened when the plasma 25(OH)D concentration in a higher range cannot be probed in this current study. A supplementation study to enhance the vitamin D status in this population is needed for the overview of the association between vitamin D status and DNA damage for a wider range. Besides, DNA repair is another factor that should be taken into account. If the DNA repair systems work well, the damage also cannot be detected alone in the lymphocytes, thus, the association between vitamin D status and DNA repair should also be investigated in further study.

It is noted that the most well-known association was found in the colorectal cancer, the specific impact of vitamin D levels on the colorectal tissue is of interest. In the study conducted by Fedirko et al., biopsy specimen from rectal mucosa was used, though no evidence of the potential beneficial role of vitamin D on 8-oxodG sites was seen. In this study, the oxidative DNA damage was only detected in lymphocytes, because of the resource limitation and ethical problem. In further studies, to investigate the association of vitamin D status and the "global" DNA damage of colorectal cells may generate

more information of the underlying mechanism of how vitamin D lower the risk of colorectal cancer, providing new therapeutic regimens.

In conclusion, this current study is the first human study to investigate the association between vitamin D status and oxidation-induced DNA damage in lymphocytes in a group of young, healthy subjects. No significant association was found between vitamin D status and oxidation-induced lesions in a sub-group of study subjects (n=121), although a potential beneficial role of vitamin D on DNA damage was seen because a trend to lower oxidation-induced DNA damage in the highest tertile of 25(OH)D was observed, although this did not reach statistical significance. Further study, including the effect of supplementation with vitamin D on DNA damage, and investigation of DNA repair in relation to vitamin D status is warranted.

Some of the information presented in this chapter was presented as:

6. *Poster presentation in the World Congress on Public Health and Nutrition, March 2016, Madrid*
7. *Presentation in the 4th International Vitamin Conference, Copenhagen, Denmark in May 2016*
8. *A research article accepted by Mutagenesis, April 2016*

CHAPTER 7

VITAMIN D STATUS AND BIOMARKERS OF ANTIOXIDANT STATUS AND OXIDATIVE STRESS

INTRODUCTION

Oxidative stress is the imbalance of reactive oxygen species (ROS) and antioxidant defence, caused by diminished antioxidants and/or increased production of ROS (Benzie, 2000; Halliwell and Gutteridge, 2007). Oxidative stress leads to oxidation of some important biomolecules, such as protein, lipids and DNA (Halliwell and Grootveld, 1987; Lee et al., 2012). The attack to these biomolecules is linked to ageing and the development of chronic disease (Halliwell and Gutteridge, 2007). To measure ROS directly within the human body is very difficult, however, to measure biomarkers in plasma, urine or cells that reflect the antioxidant status or oxidative damage is feasible and is the usual approach. For example, the ferric reducing antioxidant power (FRAP) assay is a well-validated method to measure the 'total antioxidant power' (as the non-enzymatic reductive ability of redox-active compounds) in biological fluids (Benzie and Strain, 1996b); ascorbic acid is a key antioxidant in the human body and which in humans is obtained solely from diet, particularly vegetables and fruits, which are rich in this and other antioxidants (Carr and Frei, 1999; Chung et al., 2001; Benzie and Choi, 2014). For oxidative stress, plasma allantoin can be used as

an indicator of non-enzymatic oxidation of uric acid within the human body (Benzie et al., 1999; Chung and Benzie, 2013), and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) in urine is widely used as a biomarker of 'global' oxidative stress (Halliwell and Gutteridge, 2007; Lee et al., 2010). Plasma FRAP value, ascorbic acid and allantoin, and urine 8-oxodG were the biomarkers used in this part of the study. In addition, even though uric acid has antioxidant activity, contributing ~60% in the original FRAP value, because of its role in gout and as an independent risk factor for CHD, the FRAP value was also corrected for urate in this current study (Benzie and Strain, 1996a and b).

In relation to vitamin D status, some previous studies have indicated association with oxidative stress through various pathways. For example, vitamin D was reported to protect cultured human endothelial cells from oxidative stress induced by hydrogen peroxide, as evidenced by lower superoxide anion generation, better maintenance of mitochondrial function and higher cell viability in vitamin D treated cells (Uberti et al., 2014). Lower vitamin D status was associated with higher oxidative stress (assessed by plasma malondialdehyde (MDA) and myeloperoxidase) in obese children aged 7-14, however obesity itself is associated with both lower vitamin D and higher oxidative stress, and so it is not clear if there was a direct link between vitamin D and oxidative stress (Fernández-Sánchez et al., 2011; Codoñer-Franch et al., 2012). In a study with stroke patients aged 63-65y, no significant association was seen between vitamin D and antioxidants (measured using SOD and GPx activity) in either stroke patients or healthy controls (Afshari et al., 2015). Vitamin D supplementation was reported to be beneficial in diabetic patients, who have elevated oxidative stress, and people with impaired fasting glucose, assessed by advanced oxidation protein products and oxidized LDL

(OxLDL) (Gradinaru et al., 2012; Nikooyeh and Neyestani, 2016). However, to date the evidence linking vitamin D status and oxidative stress is scarce and conflicting. Furthermore, it is noted that in healthy non-obese people, the link between vitamin D status and oxidative stress and antioxidant status has not been studied to date.

In this part of the study, the association between vitamin D status and antioxidant status and oxidative stress was investigated in a group of young (18-26y), healthy adults. The fasting plasma FRAP value and ascorbic acid concentrations, as well as plasma allantoin and urine 8-oxodG concentrations were measured, and their inter-relationships with vitamin D status, as plasma 25(OH)D concentration, were explored. In addition, gender differences in biomarkers in this group were also investigated.

MATERIALS AND METHODS (PLEASE ALSO REFER TO CHAPTER 3 FOR DETAILED DESCRIPTIONS)

Fasting venous blood and urine samples were collected from 196 subjects (63 males, 133 females) with their written informed consent. Plasma, from heparinized blood samples, was separated within one hour of blood collection. Total antioxidant power (as the FRAP value) and ascorbic acid concentration (using FRAP/FRASC method) were measured using fresh plasma samples. Plasma allantoin was measured using an established LC-MS/MS method (Chung and Benzie, 2013), and samples were stored at -80 ^{o}C, and thawed for once only before use. Urinary 8-oxodG was measured using an established LC-MS/MS method (Lee et al., 2010), and the results were corrected by

urinary creatinine concentration. It is noted that in this study, the FRAP value was also presented as that corrected for urate as follows:

FRAP corrected for urate = FRAP value - 2*uric acid concentration (µmol/l)

(Benzie and Strain, 1996a and b)

For more details of the principles of measurement and sample handling, please refer to Chapter 3.

Data handling and statistical analysis: Results are presented as mean and standard deviation (SD), median and range (low to high). The association between antioxidant status and oxidative stress biomarkers and vitamin D status was analyzed by Pearson's correlation or Spearman's correlation (for skewed data). The biomarker values across plasma 25(OH)D quartiles were compared using one-way ANOVA (with Newman-Kuels post hoc test) or Kruskal-Wallis test (with Dunns post hoc test) for skewed data. A p value <0.05 was considered statistically significant.

RESULTS

Descriptive statistics for the biomarker results of the 196 subjects (63 males, 133 females) are shown in Table 7.1. In relation to vitamin D status, significant direct associations were found between plasma 25(OH)D concentration and the FRAP value as well as the FRAP value corrected for urate: $r=0.252$, $p<0.001$ and $r=0.144$, $p<0.05$, respectively (Table 7.2 and Figure 7.1). No other significant correlations were seen between plasma 25(OH)D and other biomarkers of interest. According to the quartiles

of plasma 25(OH)D, significantly lower FRAP value was found in Q1 (the lowest quartile) compared with that in Q3 and Q4 ($p<0.05$), however no significant differences across quartiles were seen in the other biomarkers (Table 7.3). No significant differences were seen in the biomarkers of oxidative stress or antioxidant status when data were examined according to the vitamin D status categories of <25 nmol/l, ≥25<50nmol/l, and ≥50 nmol/l (Table 7.4). A trend of higher FRAP value, both 'total' and corrected for urate, was seen in higher vitamin D quartiles and categories, but after Newman-Kuels' multiple comparison correction, no statistically significant difference was found.

In relation to gender differences, no significant gender difference was found except for the FRAP value: mean(SD) values were 1150(203) in men and 977(177) μmol/l in women, $p<0.05$.

Table 7.1. Descriptive Results of Oxidative Status Biomarkers in 196 Apparently Healthy Adults Aged 18-26 Years:

	Total (n=196)			Male (n=63)			Female (n=133)		
	Mean(SD)	Median	Range	Mean(SD)	Median	Range	Mean(SD)	Median	Range
Plasma 25(OH)D (nmol/l)	42.1(13.0)	40.4	15.7-86.8	45.3(14.4)	44.2	17.4-86.8	40.6(12.1)	38.5	15.7-67.4
FRAP value (µmol/l)	1033(202)	1010	614-1666	*1150(203)	1143	614-1666	977(177)	948	629-1624
FRAP corrected for urate (µmol/l)	388.6(116.7)	381.0	108.0-1089.0	395.7(100.5)	397.9	109.0-685.2	385.2(123.8)	378.9	149.6-1089.0
Ascorbic acid (µmol/l)	55.3(22.0)	54.6	8.5-115.0	52.2(22.7)	49.6	8.5-112.7	56.8(21.6)	58.0	9.0-115.0
Allantoin (µmol/l) #	0.85(0.58)	0.71	0.25-4.58	0.96(0.68)	0.82	0.33-4.58	0.80(0.51)	0.67	0.25-3.42
8-oxodG (nmol/mmol creatinine) #	1.15(0.76)	0.98	0.32-7.48	1.24(0.63)	1.04	0.35-3.46	1.10(0.81)	0.91	0.32-7.48

*$p<0.05$ compared to value in females

Table 7.2. Table of Association between Plasma 25(OH)D Concentration and Antioxidant and Oxidative Stress Biomarkers in the 196 Subjects Studied

Biomarker	Pearson's r	p value
FRAP value (µmol/l)	0.2525	0.0004
FRAP corrected for urate (µmol/l)	0.1438	0.0444
Ascorbic acid (µmol/l)	0.0307	0.6706
Allantoin (µmol/l) #	0.0406	0.5732
8-oxodG (nmol/mmol creatinine) #	-0.0338	0.6385

#Because these biomarkers had a skewed distribution, Spearman's correlation was performed

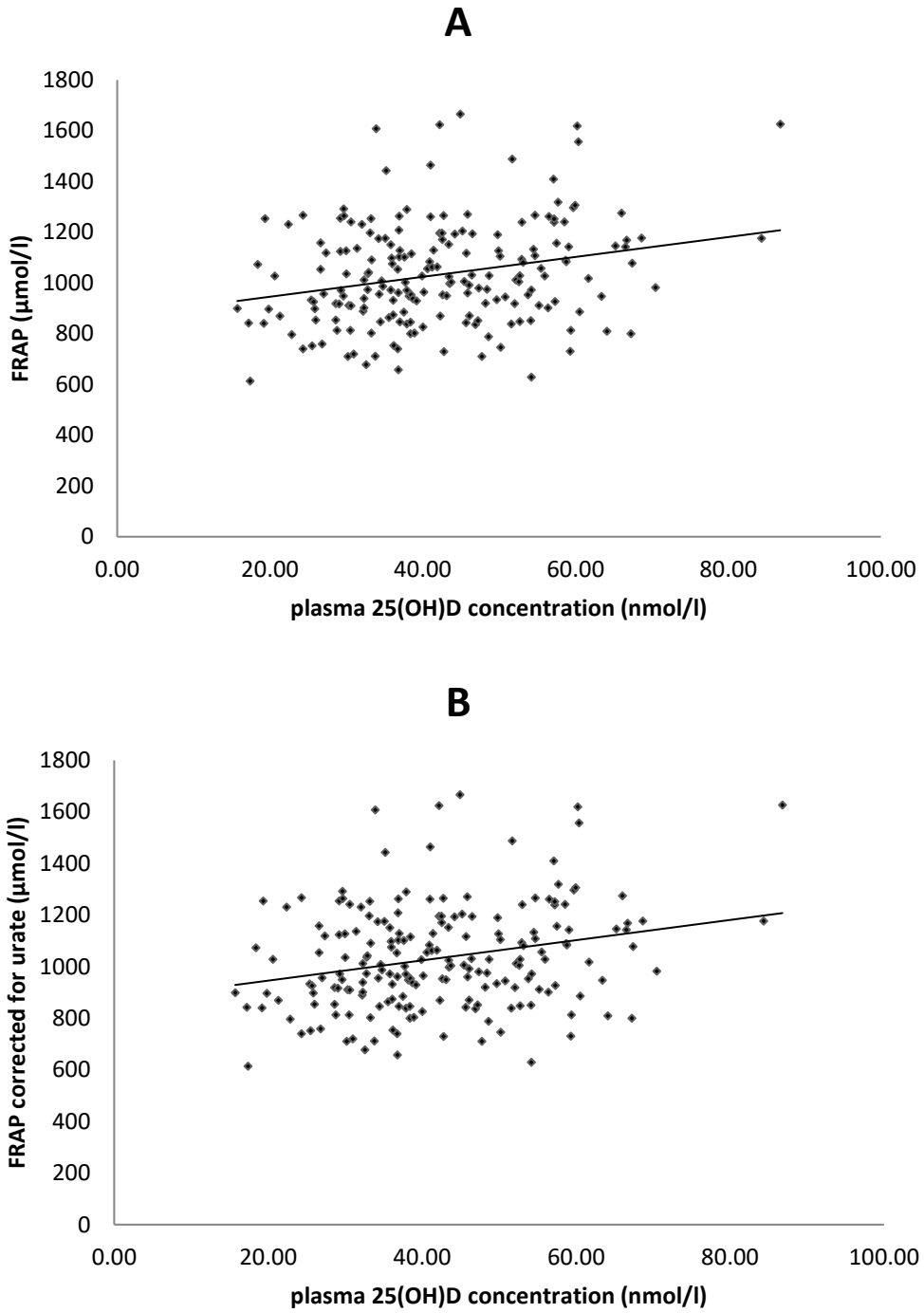


Figure 7.1. Scatter Plots of Fasting Plasma 25(OH)D Concentration and FRAP Value in 196 Healthy, Young Adults

A shows the FRAP value, r=0.2525, p=0.0004;

B shows the FRAP value corrected for urate, r=0.1438, p=0.0444;

Table 7.3. Selected Antioxidant and Oxidative Stress Biomarkers Across Quartiles (Q) of Plasma 25(OH)D Concentration

All results shown are mean(SD)

	Q1 (n=49)	Q2 (n=49)	Q3 (n=49)	Q4 (n=49)	p
25(OH)D (nmol/l)	27.0(4.7)	36.5(2.0)	45.5(3.3)	59.7(7.2)	<0.001
FRAP value (µmol/l)	966(176)	1005(187)	1061(209)*	1098(213)*	0.0058
FRAP corrected for urate (µmol/l)	357.7(93.1)	388.2(145.9)	394.5(105.2)	413.9(112.4)	0.1170
Ascorbic acid (µmol/l)	58.1(25.2)	53.4(24.0)	51.4(17.2)	59.9(23.3)	0.2205
Allantoin (µmol/l) #	0.89(0.77)	0.80(0.50)	0.92(0.59)	0.79(0.38)	0.4876
8-oxodG (nmol/mmol creatinine) #	1.24(0.65)	0.99(0.57)	1.16(1.02)	1.20(0.71)	0.0788

*p<0.05 compared to the value in Q1

Kruskal–Wallis test was performed for skewed data, and ANOVA was used for the others

Table 7.4. Oxidative Stress Biomarkers across Vitamin D Status Categories

All results shown are mean(SD)

Plasma 25(OH)D concentration (nmol/l)	<25 nmol/l n=13	≥25<50 nmol/l n=128	≥50 nmol/l n=55	p
25(OH)D (nmol/l)	20.2(2.7)	37.2(6.5)	58.8(7.3)	<0.001
FRAP value (µmol/l)	950(206)	1017(190)	1088(218)	0.0284
FRAP corrected for urate (µmol/l)	346.4(108.1)	383.5(120.4)	410.5(107.1)	0.1439
Ascorbic acid (µmol/l)	56.4(27.6)	53.6(20.6)	58.9(23.7)	0.3323
Allantoin (µmol/l) #	0.76(0.47)	0.88(0.64)	0.81(0.41)	0.7569
8-oxodG (nmol/mmol creatinine) #	1.16(0.48)	1.13(0.81)	1.17(0.69)	0.5409

Kruskal–Wallis test was performed for skewed data, and ANOVA was used for the others

DISCUSSION

The aim of this part of the study was to investigate the association between vitamin D status and selected biomarkers of antioxidant status and oxidative stress in apparently healthy young adults. Higher vitamin D status was found to be significantly associated with higher 'total' FRAP value and the FRAP value corrected for urate, implying a link between vitamin D status and total antioxidant power, however, no significant association was found between vitamin D status and fasting plasma ascorbic acid, allantoin or urinary 8-oxodG. No gender difference was found except for the FRAP value, but this difference disappeared after correction for urate, thus, it was driven by the higher levels of uric acid in men: as presented in Chapter 5, mean(SD) values were: 376.9(82.4) and 296.0(52.0) μmol/l for uric acid in men and women, respectively. It is noted that uric acid contributes more than 50% to the FRAP value, while it has clear causal effect on gout and is an independent risk factor for CHD (Benzie and Strain, 1996a), and it was found to be significantly associated with higher vitamin D status in the group of young adults studied here. It is noted that, after the correction for uric acid, the association between vitamin D status and the FRAP remained statistically significant, thus, the association found between vitamin D status and the non-enzymatic "total" antioxidant capacity in this study was not driven entirely by uric acid.

In previous studies, higher vitamin D was found to be associated with higher antioxidant status, but the results were not consistent across studies. Vitamin D status was found to be lower in obese children compared with children with normal BMI, and among the obese children, vitamin D insufficiency (defined as serum 25(OH)D <50 nmol/l) was found to associate with higher oxidative stress (Codoñer-Franch et al.,

2012). A total of 66 obese and 39 normal BMI children aged 7-14 y were recruited in that study, and their vitamin D status, as well as malondialdehyde (MDA) and myeloperoxidase and other biomarkers of inflammation and endothelial dysfunction were measured. Obese children had significantly lower mean 25(OH)D concentration, 60.7 nmol/l, compared with 75 nmol/l in normal controls, although it is noted that these values are markedly higher than those found in the healthy young (18-26 y) people studied here. In the group of obese children, vitamin D insufficiency (n=20) was significantly ($p<0.05$) associated with higher MDA and myeloperoxidase concentrations, compared with those with sufficient vitamin D (defined as >50 nmol/l in that study): mean MDA values were: 0.91 vs. 0.40 µmol/l and for myeloperoxidase were 60 vs. 41 ng/ml, respectively (Codoñer-Franch et al., 2012). It is noted that obesity has been clearly linked to lower vitamin D status, and evidence shows that obesity is also associated with higher oxidative stress (Fernández-Sánchez et al., 2011; Pereira-Santons et al., 2015). The influence of obesity was not removed in that study, thus the reported association between lower vitamin D status and higher oxidative stress was not robust.

Lower vitamin D status was also found to be associated with higher oxidative stress in those with problems with glycaemic control (Gradinaru et al., 2012). People with impaired fasting glucose (n=30) and type 2 diabetes mellitus (type 2 DM, n=35) aged 65-78 y were recruited, and their vitamin D status was found to be significantly lower, but still relatively high, compared with normal controls (n=25): mean 25(OH)D values were 79.8, 71.2 and 98.5 nmol/l, respectively. Vitamin D status in people with impaired fasting glucose and type 2 DM (n=65) was significantly inversely associated with levels of advanced oxidation protein products ($r=-0.475$, $p<0.001$) and oxidized low-density

lipoprotein (oxLDL; r=-0.413, p<0.05). Besides, those with 25(OH)D <75 nmol/l (n=35) had significantly higher oxLDL and advanced oxidation protein products, compared with those who had higher vitamin D status (n=30) (Gradinaru et al., 2012). However, it is noted that vitamin D status was significantly associated with glycaemic control; people with 25(OH)D <75 nmol/l had significantly higher HbA1c and fasting glucose levels than those with higher 25(OH)D. However, diabetes is associated with higher oxidative stress (Choi et al., 2008; Nikooyeh and Neyestani, 2016), and the link between vitamin D status and oxidative stress may also be driven by poorer glycaemic control.

Another cross-sectional study with ischaemic stroke patient (n=36) and matched controls who were free of cerebrovascular accident (n=36) found no significant association between vitamin D status and activities of enzymatic antioxidants (serum SOD and GPx) (Afshari et al., 2015). It is noted that the vitamin D status in both groups was very low, and no significant difference in vitamin D status was found between stroke patients and controls: mean values of serum 25(OH)D concentration were: 26.8 and 25.0 nmol/l, respectively. No significant association was found between vitamin D status and the antioxidant enzyme activities in either group (Afshari et al., 2015). However, it is noted that the antioxidant enzyme activity was measured in serum in that study, which is not representative of the true results in the human body, as SOD and GPx are intercellular and most commonly measured in red blood cells (Halliwell and Gutteridge, 2007). Similarly, in the study conducted by Krivošíková et al. (2015), vitamin D status was associated with the number of cardiometabolic risk factors, but was not found to be associated with plasma advanced oxidation protein products, which was used as a biomarker of oxidative stress in that study (Krivošíková et al., 2015).

The effect of vitamin D supplementation on antioxidant status and oxidative stress has been studied, but results are also conflicting. One study suggested that vitamin D supplementation significantly decreased urine isoprostanes in overweight or obese African Americans with elevated blood pressure and low serum 25(OH)D level (25-68 nmol/l) (Martins et al., 2014). The subjects received 100,000 IU vitamin D/month for 3 months (n=60) or matching placebo (n=55), and their baseline mean 25(OH)D levels were 41 and 42 nmol/l, respectively. After supplementation, the mean 25(OH)D in those who received vitamin D supplementation increased to 86 nmol/l ($p<0.0001$), while no significant difference was seen in the placebo group, and urine isoprostane decreased significantly in the vitamin D group, but not in the placebo group (Martins et al., 2014).

In major depressive disorder patients, vitamin D supplementation was found to link to improvement of higher total antioxidant capacity (TAC, measured by FRAP) and GSH. In this study, 20 patients received 50,000 IU vitamin D/week for 8 weeks, while the other 20 received matching placebo. The average serum 25(OH)D in vitamin D group was increased from 34 nmol/l to 85 nmol/l, while no significant change was seen in the placebo group. In the vitamin D group, FRAP value significantly increased by 7% and the GSH was also increased by 24%, $p<0.05$ (Sepehrmanesh et al., 2016).

With the supplementation of elderly women with 200,000 IU vitamin D /day (n=20) or placebo (n=20) for four weeks, oxidative stress and inflammation decreased in the vitamin D group (de Medeiros Cavalcante et al., 2015). It is noted that the baseline vitamin D status of both groups was quite high: mean values of 25(OH)D in both groups were each 65 nmol/l, but after supplementation with this very high dose the vitamin D

status increased only modestly, to a mean of 79 nmol/l, while the placebo group's mean was 61 nmol/l. Still, significantly lower ultra-sensitive C reactive protein and higher TAC, measured by the 2,2-diphenyl-1-picryhydrazyl (DPPH) method (Brand-Williams et al., 1995) in plasma were seen in the vitamin D supplemented group, though no significant change was seen in the oxidative stress biomarker used (MDA) (de Medeiros Cavalcante et al., 2015). The high baseline level of 25(OH)D and the small improvement of vitamin D status may limit the potential effect of vitamin D supplementation, Furthermore, even with the very high dose of vitamin D used (5 times higher than the upper limit recommended by the Institute of Medicine for this age group), the improvement of the mean 25(OH)D concentration was only 14 nmol/l.

A double-blinded, randomized, controlled trial with pregnant women aged 18-40 years found that vitamin D supplementation was beneficial for glycaemic control and the antioxidant system (Asemi et al., 2013a). In that study, 48 pregnant women at 25 weeks of gestation received either 400 IU vitamin D/day (n=24) or matching placebo (n=24) for nine weeks. After the supplementation, significant improvements in vitamin D status, TAC (measured by the FRAP value) and glutathione (GSH) levels were seen in the plasma of the vitamin D supplemented group compared with baseline values, while the changes in these biomarkers were not significant in the placebo group. Besides, significant decreases of hsCRP and fasting plasma glucose, and increase in calcium were also found in the pregnant women supplemented with vitamin D. The results indicated a beneficial role of vitamin D on calcium balance, glucose control, anti-inflammation and antioxidant status (Asemi et al., 2013a). It is noted that the difference in baseline level of vitamin D status of the two groups was fairly large, at ~22% (baseline mean of 36.5 nmol/l in placebo group vs. 44.5 nmol/l in the supplementation

group), the dose of vitamin D used (400 IU/d) was quite small, and the associated increase in 25(OH)D was modest (increase in mean (25(OH)D from 44.5 to 53.7 nmol/l). Therefore, the groups were not well matched for 25(OH)D and the improvement of vitamin D status was limited. However, as with the study of de Medeiros Cavalcante et al. (2015), a small improvement in vitamin D status of was reported to cause a significant impact on antioxidant status and inflammation. What is interesting is that in one study (Asemi et al., 2013a), most of the subjects were deficient in vitamin D (mean 44, which increased to 53 nmol/l with supplementation), while in the other, most of the subjects were likely to have had plasma 25(OH)D >50nmol/l (mean of 65 nmol/l at baseline, which increased to 77 nmol/ with supplementation), and that both studies showed an increase in TAC after supplementation. Inspection of the data shows that the increase in TAC was greater (~22%) in the study of largely deficient subjects compared to the study of those with higher baseline levels of 25(OH)D (~10% increase in TAC). Therefore, we can speculate that improvement in vitamin D status, even if limited, may have a beneficial effect for all those who have 25(OH)D <75 nmol/l, but that the effect is greater in the more deficient subjects.

In contrast, another study from the same research team on pregnant women with gestational diabetes mellitus (GDM) also found that vitamin D supplementation had no significant effect on two biomarkers of antioxidant status (plasma FRAP and GSH) was seen (Asemi et al., 2013b). The patients were allocated to two groups of 27/group. One group received 50,000 IU vitamin D at baseline and day 21 of the intervention, the other group received the placebo. Fasting blood and urine samples were collected from each subject at baseline and the end of the study period (6 weeks). Baseline mean 25(OH)D in both groups was 50 nmol/l. After intervention, the vitamin D status increased to 97

nmol/l in the supplemented group, while no significant change was seen in the placebo group. No significant differences were found in FRAP value or GSH, but a significant decrease in fasting plasma glucose in the supplemented group was found (Asemi et al., 2013b). Overall, in these two studies from Asemi et al., there is an interesting finding that antioxidant status increased with improvement in vitamin D status in pregnant women without GDM, but not in those with GDM although the baseline vitamin D status of both groups was similar (44 and 50 nmol/l) and despite the large increase after supplementation in the plasma 25(OH)D in the GDM patients (Asemi et al., 2013a & 2013b). This finding suggests that the improvement of vitamin D status before disease, such as GDM, becomes overt may be more effective and rewarding.

From the previous studies, it can be seen that the link between vitamin D status and the effect of improvement of this by supplementation on antioxidant status and oxidative stress is not yet clear. As summarized in table 7.5, cross-sectional studies have shown that vitamin D status was associated with oxidative stress in obese children and people with poor glycaemic control, but not in ischaemic stroke patients and non-diabetic patients; while supplementation studies have shown significant improvement in plasma total antioxidant capacity in major depressive disorder patients, elderly women and pregnant women without GDM after vitamin D supplementation, and oxidative stress was lowered in obese and overweight African–Americans after vitamin D supplementation, however no effect was seen in pregnant women with GDM.

Table 7.5. Summary of Association between Vitamin D and Antioxidant and Oxidative Stress in Previously Published Reports

(A. Observation Studies; B. Supplementation Trials)

A. Observational Studies		
Study	Subjects and Sample size	Results
Codoñer-Franch et al., 2012	Obese children (n=66); normal controls (n=39)	Obese children with 25(OH)D <50 nmol/l had significantly higher MDA and myeloperoxidase than those with higher vitamin D status
Gradinaru et al., 2012	Type 2 DM patients (n=35); people with impaired glucose control (n=30); normal controls (n=25)	In type 2 DM patients and people with impaired fasting glucose: 1. Significant inverse association was found between vitamin D status and advanced oxidation protein products and oxLDL in plasma; 2. significantly higher oxLDL and advanced oxidation protein products were found in those with 25(OH)D <75 nmol/l
Afshari et al., 2015	Ischaemic stroke patients (n=36); Matched controls (n=36)	No significant association was found between vitamin D status and serum SOD and GPx activities
Krivošíková et al., 2015	Non-diabetic subjects (n=411)	Vitamin D status was not associated with advanced oxidation protein products

B. Supplementation Trials			
Study	Subjects	Intervention	Results
Martins et al., 2014	African Americans	100,000 IU vitamin D/month (n=60); placebo (n=55) for 3 months	Urine isoprostane decreased by 24% significantly in the vitamin D group only
Sepehrmanesh et al., 2016	Major depressive disorder patients	50,000 IU/week (n=20); Placebo (n=20) for 8 weeks	Significant increase in TAC (measured by FRAP value) by 7% and GSH by 24% were found in vitamin D supplemented group
de Medeiros Cavalcante et al., 2015	Elderly women	200,000 IU/day (n=20); placebo (n=20) for 4 weeks	In vitamin D supplemented group: 1. Significantly higher TAC (measured by the DPPH method) by 10% in plasma were seen 2. no significant plasma MDA change was seen
Asemi et al., 2013a	Pregnant women at 25 weeks of gestation	400 IU vitamin D/day (n=24); placebo (n=24) for 9 weeks	Significantly increase on plasma TAC (measured by FRAP value) by 22% and GSH by 34% were in the vitamin D group only
Asemi et al., 2013b	Pregnant women with GDM	50,000 IU vitamin D at baseline and day 21 (n=27); placebo (n=27)	No significant changes were found in plasma TAC (measured by FRAP value) and GSH levels in either group after 6 weeks' intervention

It is noted that there are several factors in previous studies that may have influenced the final outcome when we are comparing findings. The methods to measure total antioxidant capacity vary between research teams (i.e. FRAP used in our team and those

of Asemi et al., and DPPH was used in de Medeiros Cavalcante et al.), the method difference makes the direct comparison of different studies difficult. Secondly, the vitamin D status in a cross-sectional study and the baseline levels of the subjects in supplementation studies varies a lot. In our study, the mean plasma 25(OH)D concentration was 42 nmol/l, while this figure was ~40 nmol/l in the study of pregnant women and African-Americans, 50 nmol/l in GDM patients, 61 nmol/l in the obese children studied, 65 nmol/l in the elderly women studied (Martins et al., 2014).

None of the studies investigated association between vitamin D status and antioxidant status and oxidative stress in young healthy, non-pregnant or non-obese subjects. In addition, clearly, some studies supplemented subjects who were not deficient (if 50 nmol/l is taken as the cut-off point for deficiency). Furthermore, there was very large difference in the doses of vitamin D that were used. Another limitation is the biomarkers of oxidative stress used in published studies. GSH, SOD and GPx are mainly intracellular, and serum levels are very low; and MDA, though commonly used in the past, is a non-specific and insensitive biomarker of oxidative stress (Benzie, 1996; Halliwell and Gutteridge, 2007).

There are also some limitations in this current study with young adults. While the range of vitamin D status in this group was wide (15.7-86.8 nmol/l), only 2/196 had vitamin D sufficiency (plasma 25(OH)D ≥75 nmol/l). Thus, the link between vitamin D status and oxidative stress was not clear at a higher range. Besides, the biomarkers involved in this study, though sensitive and specific and well validated, were limited in number, and enzymatic antioxidant biomarkers like erythrocyte SOD and GPx, and other sensitive biomarkers such as F2 isoprostanes were not measured because of resource limitation.

In conclusion, this study is the first to investigate the association between vitamin D status and antioxidant status and oxidative stress in young, healthy, non-obese adults. Significant association was seen between vitamin D status and total antioxidant power (FRAP value – both 'total' and corrected for uric acid), but the link was not found with other individual antioxidants, or with the two sensitive and specific biomarkers of oxidative stress used. Further study of possible links is needed in subjects who are at the upper end of vitamin D status (75-100 nmol/l), but this will require a supplementation study to be performed, as the number of young subjects with 25(OH)D in this range is very low (only 2/196 in this current study).

Some of the information presented in this chapter was presented as:

9. *Presentation in the 4th International Vitamin Conference, Copenhagen, Denmark in May 2016*

CHAPTER 8

A PILOT SUPPLEMENTATION TRIAL: PRELIMINARY RESULTS

INTRODUCTION

Low vitamin D status is very common in many parts of the world (Hilger et al., 2014), and as found in Part 1 of this current study (please refer to Chapter 4), there is very high prevalence of low vitamin D status in the young healthy adults studied here. As reported in Chapter 4, 99% of the 196 subjects had plasma 25(OH)D <75 nmol/l, and 72% <50 nmol/l. Therefore, the poor vitamin D status in this population is alarming. Previous studies have shown association between poor vitamin D status and NCD risk (Holick, 2007). Furthermore, this current study revealed that low vitamin D status in the young healthy non-smoking and non-obese subject studied associated with higher fasting plasma glucose, HbA1c, TC/HDL-C ratio and lower HDL-C and FRAP value corrected for urate (please refer to Chapters 5 and 7), although no significant association was seen with DNA damage. It is noted that, even though the plasma 25(OH)D concentration in this group of young adults covered a wide range (15.7-86.8 nmol/l), only 2/196 subjects had plasma 25(OH)D ≥75 nmol/l, the suggested threshold for sufficiency (Holick et al., 2011; Spedding et al., 2013), thus, the link between vitamin D status and the biomarkers of interest in those with sufficient vitamin D status was not able to be adequately probed in our observational arm. As the prevalence of vitamin D insufficiency (<75 nmol/l) in this group is very high, a supplementation study is a reasonable approach to explore the

association between changes in vitamin D status in those who are deficient at baseline and supplementation-related changes in biomarkers that link to the underlying biological changes that underpin NCD development.

In this Chapter, preliminary results of a pilot double-blinded, placebo-controlled supplementation trial are presented. A sub-group of subjects who were found to have low vitamin D status (plasma 25(OH)D <40 nmol/l) in the observational arm of the study were re-recruited, with separately obtained written informed consent, and the biomarkers of interest were measured (listed in Chapter 2) at immediately before and after a 12 weeks period of supplementation with 2,400 IU/ vitamin D or matching placebo. The vitamin D status and biomarker response to vitamin D supplementation was investigated, and the association between vitamin D status and the biomarkers of interest was also investigated post-supplementation.

MATERIALS AND METHODS (PLEASE ALSO REFER TO CHAPTER 3 FOR DETAILED DESCRIPTIONS)

Study design

This part of the study was an exploratory, double-blinded, placebo-controlled clinical trial. A total of 22 subjects from the observational study who were identified as having vitamin D deficiency (plasma 25(OH)D <40 nmol/l) were recruited into the supplementation trial. The 22 participants were assigned to the vitamin D group (n=11, 2 males and 9 females) or the placebo group (n=11, 4 males and 7 females). In the vitamin D group, each participant received 2,400 IU vitamin D/day for 12 weeks, while the participants in the placebo group received matched placebo.

Notes:

- Group allocation was randomized (by the Chief Supervisor, and this student investigator was blinded to the treatment allocation). Groups were stratified as much as possible for initial plasma 25(OH)D concentration, and as far as possible for gender.
- One female subject (who had been allocated to the vitamin D group) dropped out of the trial. Despite many efforts to contact this volunteer by phone and email, we were unable to contact her and so do not know the reason.
- It is noted that, to increase the power of the sample size of this exploratory study, six of the volunteers (3 male and 3 female) who were allocated to the placebo group were agreed, and gave their informed consent, to continue for a further 12 weeks, during which time they took the vitamin D supplement.

Therefore, the total number of subjects who took 12 weeks of vitamin D supplement (2,400IU/day) was 16 and the total number of subjects in the placebo group remained at 11.

- Compliance: The subjects were contacted (by the Chief Supervisor) at least once per week to inquire about their compliance and general well-being. Compliance was assessed by counting the pills returned by each subject. If the number returned was <20% of the expected number (i.e. 80% or more of the pills had been taken), this was regarded as satisfactory compliance.
- The vitamin D supplements and placebo were kindly provided by VITA GREEN PHARMACEUTICAL, (HK) LTD (Appendix II A and B).
- This trial was a registered clinical trial with US National Institutes of Health (ClinicalTrials.gov, Identifier NCT02451787).

Sample handling

Fasting venous blood and urine samples were collected from each subject at baseline and week 12. For the six subjects from the placebo group took another 12 weeks of vitamin D supplements, their samples collected at week 12 after taking placebo were regarded as the baseline sample of their supplementation, and their samples collected after vitamin D supplementation for 12 weeks were used as post-supplementation samples. Blood pressure and BMI were measured on the sampling day or within one week. Twenty millilitres of venous blood were collected from each subject at each timepoint, and allocated as: 2 ml into EDTA blood collection tube, 2 ml into sodium fluoride (Flox) tube and 16 ml into heparin tube. Flox and heparinized plasma was separated within one hour of the blood collection, and EDTA whole blood was stored at 4 ^{o}C for HbA1c (within one week). Fasting plasma glucose (using Flox plasma) and

urine creatinine were measured on the sampling day. The FRAP value and ascorbic acid were measured using fresh heparinized plasma, also on the day of collection, and the FRAP value was also corrected for urate. The rest of the heparinized plasma and aliquots of centrifuged urine were stored at -80 ^{o}C for later use. White blood cells from each subject, to be tested using the Fpg-assisted comet assay, were harvested from heparinized blood samples, and stored in freezing medium (90% foetal bovine serum and 10% dimethylsulfoxide) at -80 ^{o}C. Plasma lipids, uric acid and hsCRP were measured by commercial kits, and LDL-C was calculated by Friedewald equation (LDL-C = TC - HDL-C - Tg/2.2, with unit of mmol/l of all biomarkers involved) (Friedewald et al., 1972). Plasma allantoin, urine 8-oxodG were measured by LC-MS/MS. Please refer to Chapter 3 for more detail on the measurement of these biomarkers.

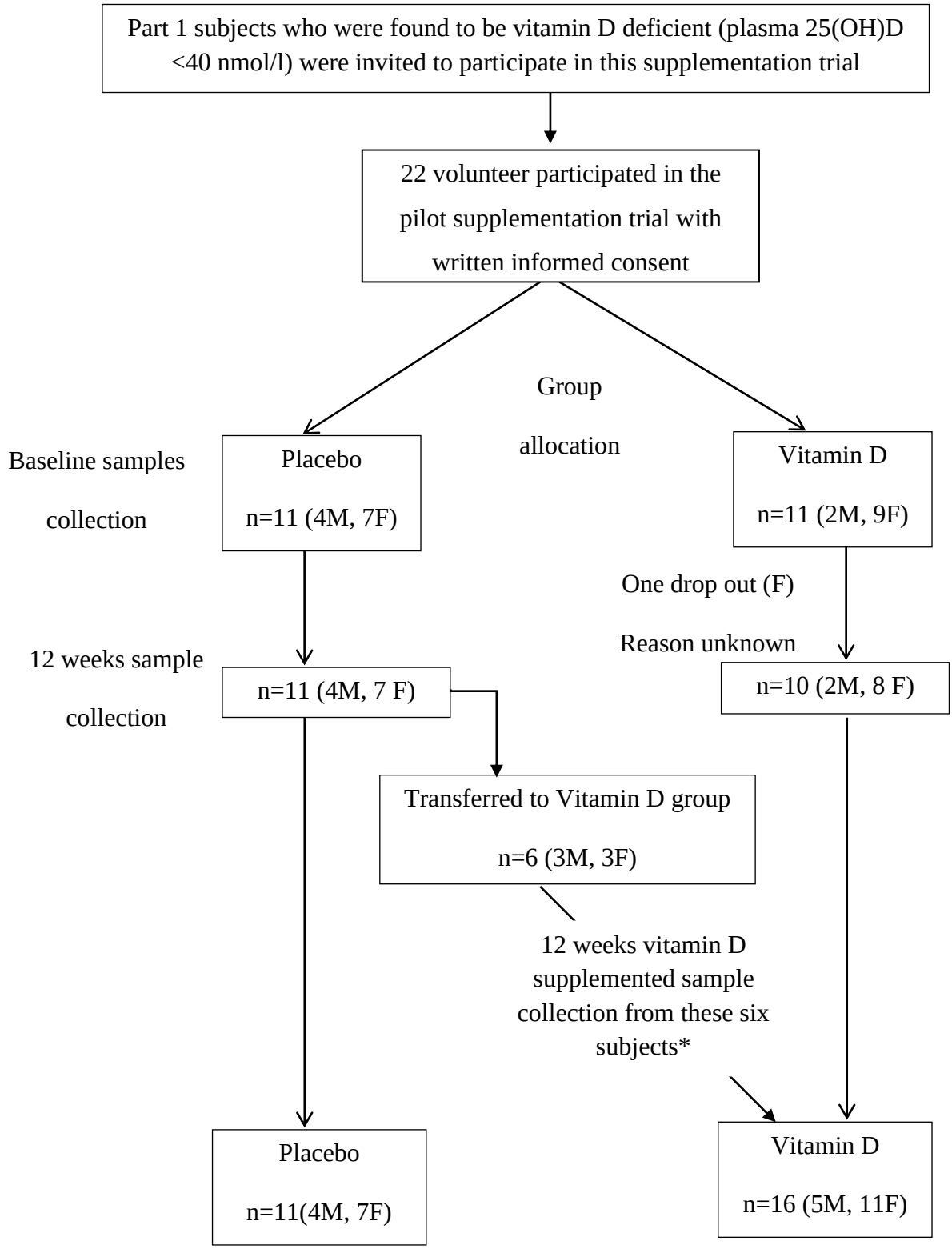


Figure 8.1. Overview of the Pilot Supplementation Study

*the baseline (pre-vitamin D supplementation) samples used for these six subjects were the samples collected after 12 weeks of placebo.

Data handling and statistical analysis

Results are presented as mean and standard deviation (SD), median and range (low to high). All of the data were tested by Kolmogorov-Smirvov test with Dallal-Wilkinson-Lilliefor p value for normal distribution. The within-treatment response to vitamin D supplementation or placebo (i.e. the pre-post difference) was analyzed by the paired t test or Wilcoxon matched pairs test. The across-treatment response (i.e. the pre-post difference of response to each treatment) was investigated by the unpaired t test or the Mann-Whitney test. In order to further explore the association between plasma 25(OH)D and the biomarker of interest with 25(OH)D values in the higher range, the post-supplementation values in both groups combined were analyzed by Spearman's correlation (due to the skewed distribution of the 25(OH)D data). A p value <0.05 was considered statistically significant.

RESULTS

Baseline comparison and compliance

The descriptive results of the baseline levels of both groups are shown in Table 8.1. No significant difference was found in 25(OH)D concentration or any biomarker between the groups at baseline (Table 8.1).

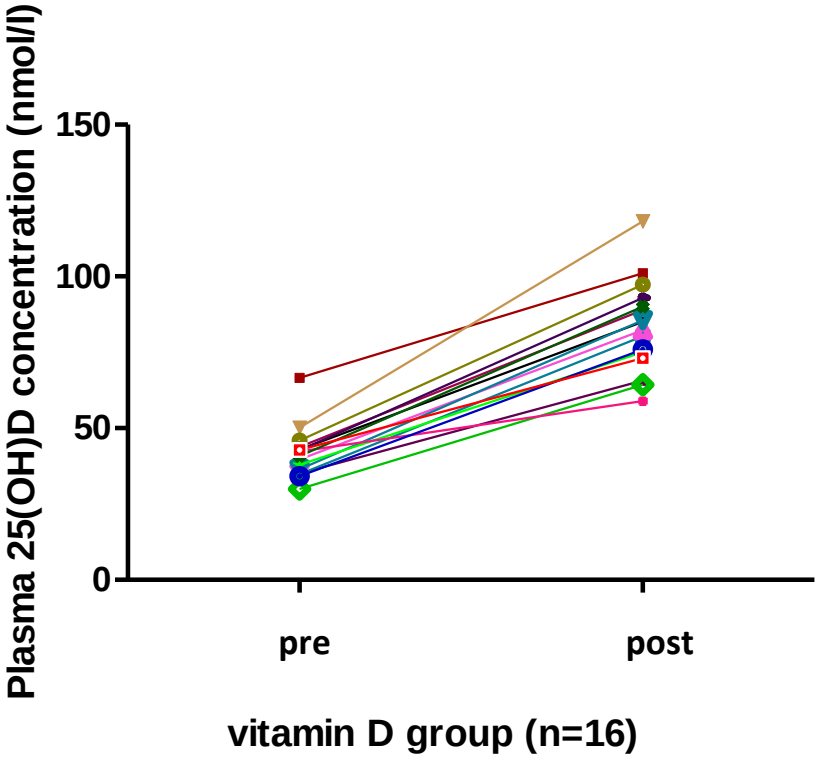
All of the subjects who completed the trial had satisfactory compliance (>80%). The response of plasma 25(OH)D concentration in both groups is shown in Figure 8.2. All of the subjects in the supplementation group showed an increase in their plasma 25(OH)D concentration after vitamin D supplementation, which ranged from 17-68 nmol/l, while the plasma 25(OH)D concentration in the placebo group was quite stable compared with the baseline. It can be seen from Figure 8.2 that the 25(OH)D concentration doubled in the vitamin D group. All of the subjects who received vitamin D had achieved plasma 25(OH)D ≥50 nmol/l after 12 weeks, and 12/16 (75%) subjects had achieved plasma 25(OH)D ≥75 nmol/l after the supplementation period. In the placebo group, none of the subjects had achieved plasma 25(OH)D ≥50 nmol/l after 12 weeks. Mean(SD) plasma 25(OH)D concentrations in the placebo group and vitamin D group were, respectively, 43.0(8.32) and 41.62(8.38) nmol/l ($p>0.05$) at baseline and 38.51(5.83) and 83.41(15.08) nmol/l after supplementation ($p<0.05$) (Figure 8.3).

Table 8.1. Descriptive Results for Both Groups at Baseline (Immediately Prior to Supplementation)

	Placebo group (n=11)			Vitamin D supplemented group (n=16)			p
	Mean(SD)	Median	Range	Mean(SD)	Median	Range	
25(OH)D (nmol/l)	43.0(8.32)	42.8	27.1-61.13	41.62(8.38)	41.53	29.95-66.61	0.6778
BMI (kg/m2) #	21.3(3.5)	20.4	17.9-30.9	21.1(3.4)	20.4	16.8-30.9	0.9409
SYS BP (mmHg)	112.9(5.8)	113	104.0-122.0	114.1(8.1)	112.5	101.0-131.0	0.6725
DIA BP (mmHg)	65.1(6.3)	65	55.0-74.0	65.6(6.5)	68.0	53.0-75.0	0.8532
Fasting glucose (mmol/l)	5.17(0.27)	5.2	4.80-5.60	5.04(0.27)	5.03	4.57-5.50	0.2585
HbA1c (%)	5.29(0.29)	5.3	4.90-5.80	5.08(0.39)	5.10	4.40-5.90	0.1382
TC (mmol/l)	4.56(0.73)	4.71	3.57-5.76	4.72(0.88)	4.63	3.69-6.53	0.6184
HDL-C (mmol/l)	1.47(0.39)	1.43	0.91-2.28	1.57(0.41)	1.45	1.02-2.43	0.5175
LDL-C (mmol/l)	2.65(0.49)	2.81	1.77-3.22	2.80(0.59)	2.89	1.79-3.88	0.5033
TC/HDL-C ratio #	3.23(0.67)	3.22	2.17-4.06	3.08(0.52)	2.93	2.13-3.98	0.5053
Tg (mmol/l)	0.96(0.38)	0.96	0.42-1.50	0.77(0.37)	0.70	0.42-1.66	0.2028
Log(Tg/HDL-C) #	-0.21(0.26)	-0.20	-0.60- 0.11	-0.34(0.25)	-0.43	-0.69- 0.11	0.1910
Uric acid (µmol/l)	350.4(50.8)	343	281.5-421.0	320.4(78.6)	345.8	211.4-508.0	0.2753
hsCRP (mg/l) #	0.23(0.10)	0.24	0.06-0.45	0.36(0.47)	0.17	0.06-1.87	0.8047

Buffer (DNA% in tail)	1.61(0.70)	1.57	0.42-2.99	1.62(0.56)	1.56	0.83-2.85	0.9420
Fpg (DNA% in tail)	21.99(3.68)	20.95	16.93-29.81	24.52(3.62)	24.62	17.83-32.71	0.0885
Fpg-buffer (DNA% in tail)	20.39(3.54)	19.99	15.45-26.81	22.89(3.74)	22.69	15.42-31.05	0.0926
FRAP (µmol/l)	1275(174)	1245	1032-1546	1128(200)	1129	789-1412	0.0595
FRAP corrected for urate (µmol/l)	574.4(89.3)	576.9	436.5-716.5	534.7(96.1)	552.7	382.3-691.1	0.2941
Ascorbic acid (µmol/l)	77.4(35.4)	71.5	18.5-133.5	83.93(38.7)	74.0	31.5-158.5	0.6581
Allantoin (µmol/l)	1.53(1.40)	1.06	0.55-2.07	1.04(0.65)	0.89	0.34-2.36	0.2269
8-oxodG (nmol/mmol creatinine)	1.06(0.42)	1.06	0.55-2.07	1.19(0.46)	1.20	0.58-2.26	0.4736

#Mann-Whitney test was used for skewed data; unpaired t test was used for the others



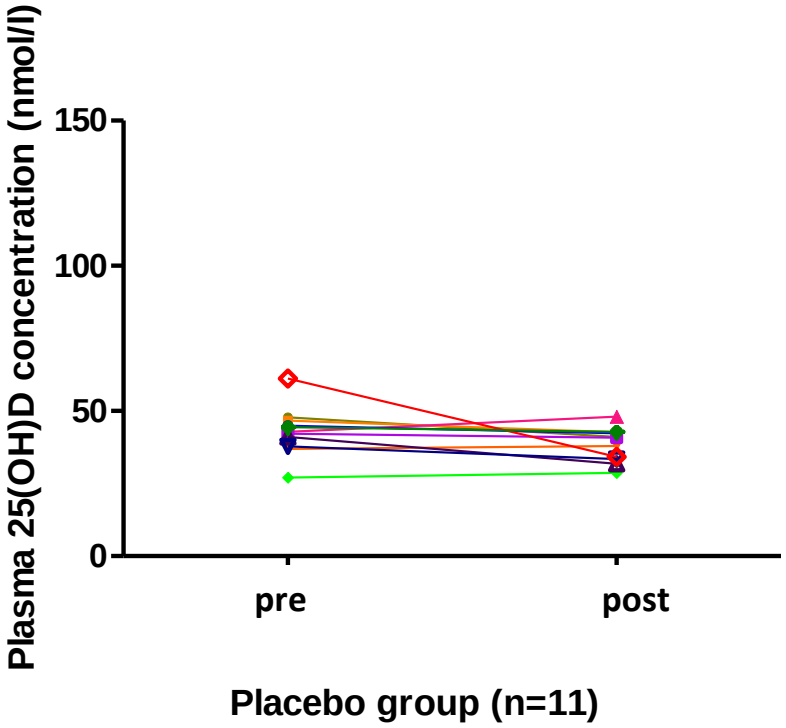


Figure 8.2. The Individual Responses in Plasma 25(OH)D to Vitamin D Supplementation or Placebo for 12 Weeks

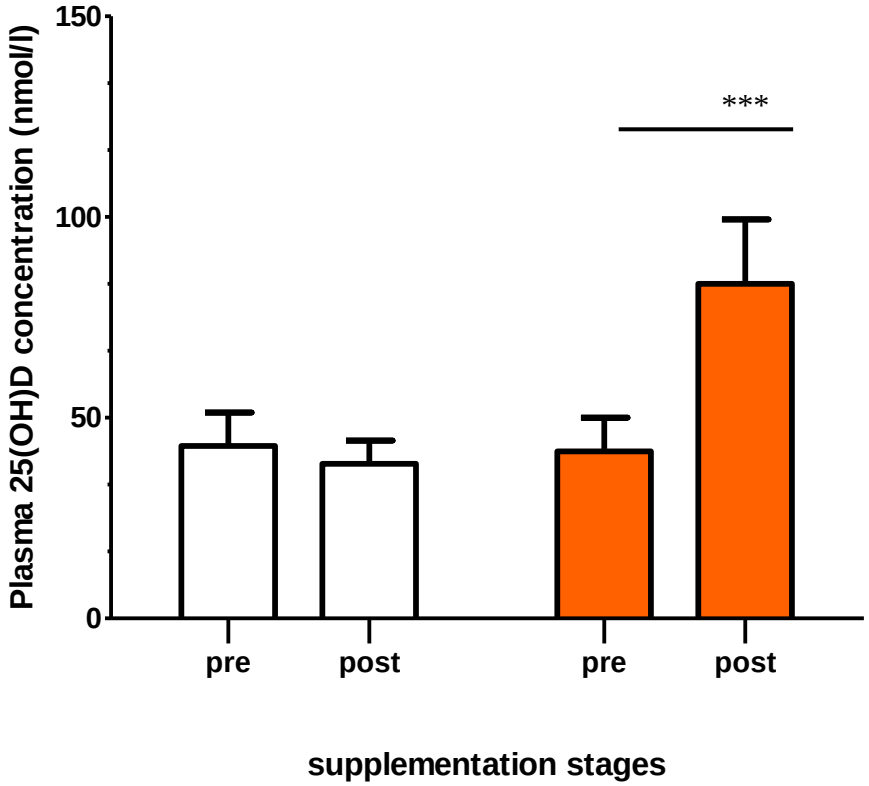


Figure 8.3. The Plasma 25(OH)D Concentration Results in the Pilot Supplementation Trial

All data are Mean+SD. The open bars represent the placebo group (n=11), the orange bars represent the vitamin D supplemented group (n=16); ***p<0.001 compared with the pre-supplementation value for this group.

Within-treatment comparison

After supplementation, for the placebo group, a decrease was seen in the vitamin D status, mean plasma 25(OH)D values were: 43.0 and 38.5 nmol/l at baseline and after 12 weeks, respectively, but the decrease was not statistically significant ($p=0.1087$). However, significantly ($p<0.05$) lower triglyceride and higher buffer-treated DNA damage score (i.e. pre-existing strand breaks) were seen after 12 weeks on placebo (Table 8.2). In the placebo group, the triglyceride was decreased by 15% after 12 weeks, from 0.96 mmol/l to 0.82 mmol/l, and for the buffer treated DNA damage score, it was increased from a mean of 1.61% to a mean of 2.31 %, by 43%.

For the vitamin D supplemented group, the average plasma 25(OH)D was significantly increased by 100%, from a mean value of 41.6 to a mean value of 83.4 nmol/l, $p<0.0001$ (Figure 8.3). In regard to the other biomarkers, no significant change was seen after supplementation (Table 8.3). But it is noted that there was a trend of decrease in oxidation induced DNA damage after supplementation, the mean value Fpg-assisted DNA damage score (pre-existing strand breaks combined with oxidation-induced DNA damage) was decreased from 24.5% to 22.4%, and the oxidation-induced DNA damage (by ΔFpg-buffer DNA damage score) also had this trend, mean value was decreased from 22.9% to 20.73%, they neither of them reached statistical significance (p value = 0.0946 and 0.1075, respectively). In addition, a potential increase of allantoin was also seen in this group, the mean allantoin was increased from 1.04 to 1.83 μmol/l, but the variation was large (seen from the large SD values), and this change also did not reach statistical significance ($p=0.0525$).

Table 8.2. Biomarkers of Interest in the Placebo Group (Placebo for 12 Weeks)

All results shown are mean(SD)

	pre	post	p
25(OH)D (nmol/l)	43.00(8.32)	38.51(5.83)	0.1087
SYS BP (mmHg)	112.9(5.8)	109.6(6.0)	0.1006
DIA BP (mmHg)	65.1(6.3)	61.8(4.6)	0.1080
FPG (mmol/l)	5.17(0.27)	5.10(0.31)	0.2530
HbA1c (%)	5.29(0.29)	5.26(0.47)	0.8230
TC (mmol/l)	4.56(0.73)	4.75(0.66)	0.1411
HDL-C (mmol/l)	1.47(0.39)	1.57(0.33)	0.0942
LDL-C (mmol/l)	2.65(0.49)	2.81(0.50)	0.4601
TC/HDL-C ratio#	3.23(0.67)	3.12(0.63)	0.1168
Tg (mmol/l)	0.96(0.38)	0.82(0.42)	0.0476
Log(Tg/HDL-C)#	-0.21(0.26)	-0.33(0.27)	0.3246
Uric acid (μmol/l)	350.4(50.8)	352.4(65.3)	0.9013
hsCRP (mg/l)	0.23(0.10)	0.40(0.27)	0.1380
Buffer (DNA% in tail)	1.61(0.70)	2.31(0.87)	0.0261
Fpg (DNA% in tail)	21.99(3.68)	24.17(3.24)	0.1626
ΔFpg-buffer (DNA% in tail)#	20.39(3.54)	21.86(3.01)	0.2907
FRAP (μmol/l)	1275(174)	1157(179)	0.1178
FRAP corrected for urate (μmol/l)	574.4(89.3)	519.0(111.8)	0.2507
Ascorbic acid (μmol/l)#	77.4(35.4)	90.5(34.8)	0.2402
Allantoin (μmol/l)#	1.53(1.40)	0.94(0.48)	0.3503
8-oxodG (nmol/mmol creatinine)	1.06(0.42)	1.11(0.39)	0.3138

Wilcoxon matched pairs test was used for skewed data; paired t test was used for the others

Table 8.3. Biomarkers of Interest in the Supplementation Group

(2,400 IU Vitamin D/day for 12 Weeks)

All results shown are mean(SD)

	pre	post	p
25(OH)D (nmol/l)	41.62(8.38)	83.41(15.08)	<0.0001
SYS BP (mmHg)	114.1(8.1)	113.1(8.7)	0.3358
DIA BP (mmHg)	65.6(6.5)	66.6(6.0)	0.7667
Fasting glucose (mmol/l)#	5.04(0.27)	5.07(0.31)	0.5133
HbA1c (%)	5.08(0.39)	5.17(0.41)	0.2749
TC (mmol/l)	4.72(0.88)	4.64(0.69)	0.6111
HDL-C (mmol/l)	1.57(0.41)	1.52(0.39)	0.4235
LDL-C (mmol/l)	2.80(0.59)	2.74(0.49)	0.6496
TC/HDL-C ratio#	3.08(0.52)	3.19(0.84)	0.6603
Tg (mmol/l)#	0.77(0.37)	0.82(0.33)	0.2663
Log(Tg/HDL-C)#	-0.34(0.25)	-0.29(0.22)	0.1406
Uric acid (µmol/l)	320.4(78.6)	298.3(60.2)	0.2878
hsCRP (mg/l)#	0.36(0.47)	0.52(0.86)	0.9382
Buffer (DNA% in tail)	1.62(0.56)	1.64(0.54)	0.9298
Fpg (DNA% in tail)	24.52(3.62)	22.38(4.14)	0.0946
ΔFpg-buffer (DNA% in tail)	22.89(3.74)	20.73(4.33)	0.1075
FRAP (µmol/l)	1128(200)	1155(189)	0.6705
FRAP corrected for urate (µmol/l)#	534.7(96.1)	558.1(174.3)	0.5614
Ascorbic acid (µmol/l)	83.93(38.7)	65.8(32.2)	0.1845
Allantoin (µmol/l)#	1.04(0.65)	1.83(1.33)	0.0525
8-oxodG (nmol/mmol creatinine)	1.19(0.46)	1.17(0.50)	0.8500

Wilcoxon matched pairs test was used for skewed data; paired t test was used for the others

Across-treatment comparison

For the biomarker response to supplementation (i.e. comparison of the post-pre differences) across the two groups, significant differences were seen in plasma 25(OH)D, allantoin and comet assay results (Table 8.4). For vitamin D status, the 25(OH)D responses in the placebo and vitamin D group were, respectively, -4.5 and +39.5 nmol/l, $p<0.0001$. For allantoin, there was a decrease of 0.59 µmol/l in the placebo group, while an increase of 0.67 µmol/l was seen in the vitamin D group, $p<0.05$. For the comet assay results, significant differences were seen in the buffer-treated DNA damage score (i.e. pre-existing strand breaks) and in the Fpg-assisted DNA damage score (i.e. pre-existing strand breaks and oxidation induced DNA damage combined); mean values were +0.70 vs. +0.02 % for the buffer-treated DNA damage scores ($p<0.05$), and +2.18 vs. -2.14 % for the Fpg-assisted DNA damage score ($p<0.05$) in the placebo group and vitamin D group respectively, $p<0.05$. For the oxidation-induced DNA damage (ΔFpg-buffer DNA damage score), a decrease was also seen in the vitamin D supplemented group, but the difference between groups did not reach statistical significance: mean changes were +1.5% in placebo vs. -2.16% in the vitamin D supplemented group, $p=0.0645$ (Figure 8.4). It is noted that even though lower triglycerides result was seen after supplementation in the placebo group (Table 8.2), no significant difference was detected across treatments (Table 8.4), although a small difference was seen in the FRAP value corrected for urate: this biomarker had an average change of -102.6 µmol/l (an 18% decrease) in the placebo group, and +56.8 µmol/l (an increase of 11%) in the vitamin D group, but this difference did not quite reach statistical significance ($p=0.068$).

Table 8.4. Comparison of Biomarker Response (Post-Pre Change) in the Placebo and Vitamin D Supplementation Groups; All results shown are mean(SD)

	Placebo group (n=11)	Vitamin D supplemented group (n=16)	p
25(OH)D (nmol/l)	-4.49(8.46)	+41.78(11.58)	<0.001
SYS BP (mmHg)#	-3.27(6.00)	-15.13(37.98)	0.9212
DIA BP (mmHg)#	-3.27(6.15)	-6.69(23.04)	0.2261
Fasting glucose (mmol/l)	-0.066(0.182)	+0.02(0.24)	0.3136
HbA1c (%)	-0.036(0.525)	+0.088(0.311)	0.4458
TC (mmol/l)	+0.188(0.391)	-0.086(0.659)	0.2294
HDL-C (mmol/l)	+0.097(0.175)	-0.051(0.246)	0.0988
LDL-C (mmol/l)	+0.158(0.245)	-0.056(0.483)	0.1892
TC/HDL-C ratio#	-0.111(0.214)	+0.108(0.471)	0.4155
Tg (mmol/l)	-0.148(0.218)	+0.046(0.307)	0.0826
Log(Tg/HDL-C)#	-0.12(0.10)	+0.05(0.14)	0.0045
Uric acid (µmol/l)	+1.95(50.93)	-22.03(79.95)	0.3888
hsCRP (mg/l)#	+0.166(0.342)	+0.163(0.821)	0.5050
Buffer (DNA% in tail)	+0.704(0.892)	+0.015(0.697)	0.0334
Fpg (DNA% in tail)	+2.177(4.785)	-2.144(4.809)	0.0301
ΔFpg-buffer (DNA% in tail)	+1.474(4.394)	-2.161(5.050)	0.0645
FRAP (µmol/l)#	-118.6(229.8)	+26.4(243.2)	0.2462
FRAP corrected for urate (µmol/l)	-102.6(219.8)	+56.8(209.4)	0.0682
Ascorbic acid (µmol/l)	+13.14(40.94)	-18.14(52.18)	0.1086
Allantoin (µmol/l)	-0.59(1.42)	+0.80(1.59)	0.0283
8-oxodG (nmol/mmol creatinine)	+0.049(0.150)	-0.015(0.365)	0.5859

#Mann-Whitney test was used for skewed data; unpaired t test was used for the others

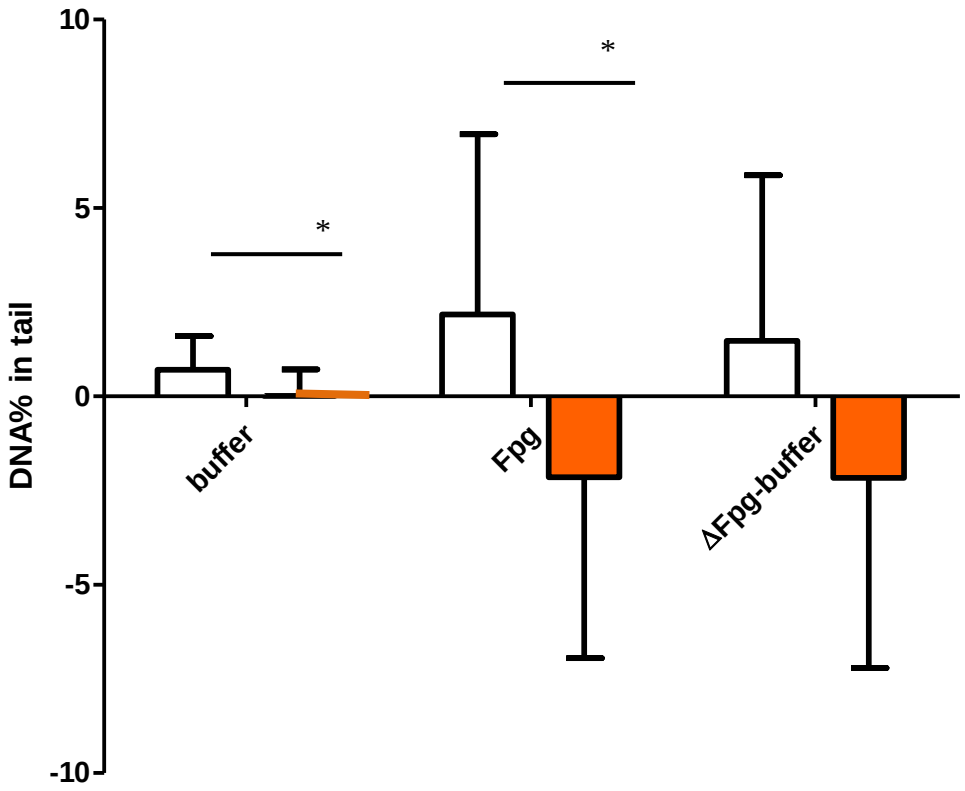


Figure 8.4. The Response (post-pre) in DNA Damage Score (in the Fpg-Assisted Comet Assay) after 12 weeks' Vitamin D Supplementation or Placebo

All results shown are mean+SD; the open bars represent the placebo group (n=11), the orange bars represent the vitamin D supplemented group (n=16); *p<0.05 compared with the other group

Correlation between vitamin D status and biomarkers of interest

In relation to vitamin D status and biomarkers of interest after supplementation, no significant association was found with any of the biomarkers (Table 8.5), although it can be seen that for three of the biomarkers (diastolic blood pressure (DIA BP), Buffer-treated DNA damage, and uric acid), the p values approached 0.05.

Table 8.5. Correlation Table of Association between Plasma 25(OH)D Concentration and Individual Biomarkers after 12 Weeks' Supplementation with Vitamin D (2,400 IU/day) or Placebo

All post-supplementation data were used, n=27

Biomarker	Spearman's r	p value
SYS BP (mmHg)	0.0694	0.7307
DIA BP (mmHg)	0.3500	0.0735
FPG (mmol/l)	-0.2584	0.1931
HbA1c (%)	-0.0351	0.8619
TC (mmol/l)	0.0275	0.8918
HDL-C (mmol/l)	0.2803	0.1567
LDL-C (mmol/l)	-0.0421	0.8347
TC/HDL-C ratio	-0.2613	0.1880
Tg (mmol/l)	-0.2110	0.2908
Log(Tg/HDL-C)#	-0.2314	0.2455
Uric acid (µmol/l)	-0.3333	0.0893
hsCRP (mg/l)	-0.2975	0.1319
Buffer (DNA% in tail)	-0.3435	0.0794
Fpg (DNA% in tail)	-0.1132	0.5740
ΔFpg-buffer (DNA% in tail)	-0.0459	0.8201
FRAP (µmol/l)	-0.1350	0.5020
FRAP corrected for urate (µmol/l)	0.0544	0.7920
Ascorbic acid (µmol/l)	-0.2198	0.2706
Allantoin (µmol/l)	0.2424	0.2231
8-oxodG (nmol/mmol creatinine)	-0.0101	0.9602

DISCUSSION

Results of this small pilot study showed that supplementation of 2,400 IU vitamin D/day for 12 weeks improved vitamin D status significantly, and comparing the response across treatments, vitamin D supplementation was linked to small but statistically significant effects on allantoin (which increased) and DNA damage (which decreased), compared with the placebo group, though no effects were seen in any of the other biomarkers. Furthermore, despite the large overall range of vitamin D status (28.6-118.2 nmol/l in the placebo and vitamin D groups combined), no significant association was seen between 25(OH)D and any of the biomarkers of interest at the end of the trial.

This pilot study, though small, was placebo controlled and is the first to examine the effect of vitamin D supplementation on a wide range of biomarkers of cardiometabolic disease risk, DNA damage, oxidative stress and antioxidant status in a group of young, still-healthy, non-obese, non-smoking adults with vitamin D deficiency (plasma 25(OH)D <40 nmol/l). There are many supplementation trials that have investigated the effects of vitamin D supplementation, but most have focused on elderly subjects of those with pre-existing disease or at high risk of disease. Furthermore, some trials have supplemented subjects who were not clearly deficient in vitamin D.

Significant improvements in glycaemic control were found in several supplementation trials. Two studies from Asemi et al., found that vitamin D supplementation was associated with better glycaemic control in both healthy pregnant women and women with gestational diabetes mellitus (GDM) (Asemi et al., 2013a and 2013b). For healthy

pregnant women, with 400 IU vitamin D/day for 9 weeks (n=24), a significant increase in serum 25(OH)D from a mean value of 45 nmol/l to 53 nmol/l was seen, significant decreases in fasting plasma glucose (by 14%) and insulin (by 15%) were seen, and though no difference was detected in HOMA-IR, an increase in the quantitative insulin sensitivity check index score (QUIKI, by 5%) was seen in the vitamin D supplemented group ($p<0.05$), while no significant changes were seen in the placebo group (n=24) (Asemi et al., 2013a). For across treatment comparison, the vitamin D group had significant ($p<0.05$) decrease in fasting plasma glucose and insulin, and an increase in QUIKI, compared with the placebo group, but it is noted that the baseline vitamin D status was not matched in the placebo group and the vitamin D group (Asemi et al., 2013a). It is noted also that the effect of vitamin D supplementation on glycaemic control after was surprisingly large given that the supplementation dose was small and the improvement in vitamin D status was only ~10% (Asemi et al., 2013a). For the GDM patients (n=27), a dose of 50,000 IU of vitamin D was given at baseline and day 21, and at the end of the study (6 weeks), the serum 25(OH)D concentration had increased significantly: mean values were 51 and 97 nmol/l at baseline and the end of the study, respectively ($p<0.001$), and a significant ($p<0.05$) within-treatment decrease was also seen in the fasting plasma glucose (by 18%), insulin (by 19%) and insulin resistance (by 34%, assessed by HOMA-IR), as well as higher QUIKI (by 9%), while no significant change was seen in any of these biomarkers in the placebo group (n=27) (Asemi et al., 2013b). For across treatment comparison, vitamin D supplementation was associated with a decrease in fasting plasma glucose, insulin, HOMA-IR and an increase in QUIKI, compared with the placebo group ($p<0.05$). Comparing these two studies by this group, it can be seen that the improvement in glycaemic control after supplementation was larger in the GDM patients, compared with healthy pregnant

women. This difference may have been due to the higher baseline fasting glucose in GDM patients and a more significant improvement on vitamin D status: ~10% in pregnant women and ~90% in GDM patients (Asemi et al., 2013a and b). In another study of 40 major depressive disorder patients, 50,000 IU vitamin D/week was given to 20 patients for 8 weeks, while the other 20 patients received matching placebo (Sepehrmanesh et al., 2016). The mean serum 25(OH)D in the vitamin D group significantly increased ($p<0.05$) from 34 nmol/l to 85 nmol/l, while no significant change was found in the placebo group, although it is noted that the baseline vitamin D status in the vitamin D group was higher than that in the placebo group: mean(SD) of 25(OH)D was 34(20) and 23(15) nmol/l, respectively. In the vitamin D supplemented group, no significant change within-treatment was seen in fasting plasma glucose, but significant decrease was seen in insulin (by 28%), and HOMA-IR (by 36%). For across treatment comparison, significantly lower insulin, HOMA-IR and better β cell function were seen with vitamin D supplementation, but no difference was seen in the fasting plasma glucose (Sepehrmanesh et al., 2016). Overall, from these three studies, it can be seen that vitamin D supplementation appears to play a better role in improving glycaemic control in those with higher baseline fasting glucose patient (GDM patients) than those with normal glucose (pregnant women and major depressive disorder patients), but significant improvement (including insulin, insulin resistance and/or β cell function) was seen in all supplemented groups, though effect on fasting plasma glucose are less clear. It is noted that no significant change was seen in fasting plasma glucose or HbA1c in this current pilot supplementation trial. This may have been due to the fasting plasma glucose concentrations at baseline, which were not high, and the short duration of supplementation. In regard to this point, a supplementation trial with elderly women found that even after achieving vitamin D sufficiency (defined in that study as

plasma 25(OH)D >80 nmol/l), the response of β cell function still needed a longer time for a detectable change in HOMA-B (von Hurst et al., 2010). In this study, 4,000 IU vitamin D per day (n=42) or placebo (n=39) was given to elderly women with plasma 25(OH)D <50 nmol/l for 6 months. The 25(OH)D increased significantly from 21 to 80 nmol/l in the supplemented group, and from 19 to 23 nmol/l in the placebo group (both $p<0.05$). The changes of insulin, insulin sensitivity, and β cell function were significantly larger in the vitamin D group, compared with the changes in the placebo group, but no significant difference was seen in fasting serum glucose. Interestingly, this study also collected samples at the half-way (3 months) point of supplementation, and in the people (n=16) in the vitamin D group who had achieved 25(OH)D >80 nmol/l in 3 months, the β cell function had not significantly changed, suggesting that the effect on glycaemic control of correcting vitamin D deficiency may need weeks or months after sufficiency is achieved (von Hurst et al., 2010).

For the response of antioxidant status and oxidative stress to vitamin D supplementation, only five published studies were found and these, as discussed in some detail in Chapter 7, showed significant improvements in antioxidant status or decreases in oxidative stress (assessed by plasma TAC or GSH or urine F2 isoprostanes) in elderly women, obese and hypertensive African-Americans, healthy pregnant women and major depressive disorder patients, but no effect was seen in GDM patients (please refer to Table 7.5B) (Asemi et al., 2013a and b; Martins et al., 2014; de Medeiros Cavalcante et al., 2015; Sepehrmanesh et al., 2016). It is noted that, in this current pilot study, no statistically significant improvement was seen in any of the biomarkers, though a potential beneficial effect on FRAP value corrected for urate was seen. Overall, previous supplementation trials indicate that vitamin D supplementation may

be beneficial in terms of improved antioxidant status and lower oxidative stress. However, no significant improvement was seen in this current exploratory pilot study. This may be due to the small sample size and the limited number of biomarkers tested.

In regard to DNA damage and vitamin D supplementation, in this current study, vitamin D supplementation was associated with a significant ($p<0.05$) decrease in oxidation-induced DNA damage and pre-existing strand breaks. There are very few published human studies of effects of vitamin D supplementation on DNA damage. A 2*2 factorial clinical trial found that in healthy people, 2 g/day calcium (n=23), 800 IU/day vitamin D (n=23), or calcium plus vitamin D (n=23) supplementation for 6 months had no statistically significant impact on 8-oxodG labeling in normal-appearing colorectal mucosa biopsy samples compared with placebo treatment (n=23) (Fedirko et al., 2010). The baseline mean values of 25(OH)D of the vitamin D group and calcium plus vitamin D group were both 53 nmol/l, and both increased to ~72 nmol/l ($p<0.05$) after supplementation. No statistically significant effect on 8-oxodG was seen (in comparison with the placebo group) in any of the supplemented groups, although some decreases were seen: 8-oxodG labeling was 22% lowered in the calcium group ($p=0.14$), 25% lowered in vitamin D group ($p=0.10$) and 6% lowered in the calcium plus vitamin D group ($p=0.70$) (Fedirko et al., 2010). In contrast, in a small study of severe asthmatic patients, vitamin D supplementation in those with vitamin D deficiency (defined as 25(OH)D <75 nmol/l in that study) showed a significant decrease in DNA damage using the alkaline comet assay without enzyme treatment. In that study, a large dose (300,000 IU) vitamin D was given to 8 subjects at day 1 and day 4, while both the vitamin D group and the placebo group (n=8) received 80 mg/day of methylprednisolone for 7 days. In the end of trial (day 7), DNA damage in lymphocytes was significantly lower

DNA in the supplemented group compared to the placebo group (Lan et al., 2014). However, in this study, no across treatment comparison was done on the post-pre changes, and no baseline or post supplementation 25(OH)D concentration data on these subjects were given. Furthermore, it is noted that, for the DNA damage score, though no data were directly provided, but from the published figure it can be seen that DNA damage was very high, with day 7 scores of ~80% in the placebo group and ~10% in the vitamin D group (Lan et al., 2014). To reach such a high assay score is very unusual, especially in the basic version of the assay. Indeed, in our hands the typical score obtained without use of Fpg is 3% or less, and even with the enzyme the score generally <25% (Wong et al., 2011; Ho et al., 2013, and as shown in chapter 6). Besides, the duration of supplementation in the study of Lan et al. (2014) was very short, and although the dose given was large, the difference in these two groups was remarkably and difficult to accept: the DNA damage score presented was ~8 times higher in the placebo group (Lan et al., 2014). Still, from the preliminary results of this current study, vitamin D supplementation was also found to lower the DNA damage as assessed by the comet assay.

Overall, from the previous studies discussed above, it can be seen that vitamin D supplementation is linked to better glycaemic control, higher antioxidant status and lower oxidative stress, but the findings on DNA damage are conflicting. It is noted that most previous supplementation trials have targeted in elderly people or people with existing diseases or at high risk of disease. However, considering the high prevalence of vitamin D deficiency and insufficiency in various regions and countries in the world, as well as in the young people living in Hong Kong (Hilger et al., 2014 and the results present here), the investigation of vitamin D and the risk of disease in still-healthy

people is also very important. This has been little studied to date, but the sensitive biomarker approach is needed in order to further our understanding of the inter-relationship between vitamin D status and the emerging risks or changes associated with NCD, if indeed this exists. This current study is the first to explore the effect of correction of vitamin D deficiency on still-healthy young people, and employing a wide range of biomarkers of interest. However, there are limitations in this pilot study. The recruitment strategy of this supplementation trial was to target those who were found to have vitamin D deficiency (plasma 25(OH)D <40 nmol/l) in the observational part of the study. However, when they were recalled to participate this study, the vitamin D status of some subjects was found (from the baseline sample) to be at a higher level: one subject from the placebo group and one from the vitamin D group had baseline plasma 25(OH)D >60 nmol/l. This improvement of vitamin D status may have been due to the seasonal change (although this effect is very limited in Hong Kong) or their awareness of the importance of vitamin D may have led them to more sunshine exposure since their first participation in the observational study and the fact that they knew their vitamin D status was low before they agreed to participate in Part 2 (the supplementation study). However, it is noted that the overall vitamin D status in both groups was still low, 24/27 were still vitamin D deficient (plasma 25(OH)D <50 nmol/l) at entry, with mean 25(OH)D values at ~42 nmol/l in both groups. Secondly, the sample size in this current study is very small due to the resource and time limitations, with 16 who received vitamin D and 11 who received placebo. The small sample size leads to insufficient power to detect small changes in the biomarkers of interest. In addition, the duration of supplementation was also a limitation. Even though the vitamin D status had increased significantly (by ~100%) at the end of the 12 weeks supplementation period in the vitamin D group, changes to other biomarkers, especially HbA1c and lipids, may

need a longer time to have detectable change after the correction of vitamin D deficiency.

In summary, this small pilot supplementation trial provides novel data on the effects of vitamin D supplementation on a wide range of biomarkers related with NCD risk in a group of young, still-healthy but vitamin D deficient adults. The preliminary results suggest that correcting deficiency of vitamin D may be beneficial for lowering DNA damage, a change that likely lowers risk of mutations and promotes cellular well-being. However, on the basis of the results presented here, there is no convincing evidence that improvement of vitamin D status in young adults has detectable beneficial effects on biomarkers of cardiometabolic health, antioxidant status or oxidative stress, at least in the short term. A larger study of longer duration may reveal effects of the correction of vitamin D deficiency.

CHAPTER 9

SUMMARY OF BACKGROUND OF THE STUDY, ITS MAIN FINDINGS & LIMITATIONS, SUGGESTIONS FOR FUTURE WORK & CONCLUDING REMARKS

SUMMARY OF BACKGROUND

Poor vitamin D status is highly prevalent in many parts of the world (Hilger et al., 2014). Apart from its impact on the skeletal system, vitamin D may play a role in determining risk of non-skeletal NCDs. Globally, as a group, NCD is the leading cause of death and disability, and brings a huge burden in both medical care and economic cost, and these burdens are estimated to increase rapidly in the next decades due to the ageing of the population (Beaglehole et al., 2011; Bloom et al., 2012; WHO, NCD fact sheet, data accessed in July, 2016). Low vitamin D status was suggested to be an emerging risk factor of NCDs, including CVD, cancer and diabetes, as association between vitamin D deficiency and mortality and incidence of NCD has been reported in many, though not all, epidemiological studies of disease outcomes (Holick, 2007; Vuolo et al., 2012; Wang et al., 2012; Afzal et al., 2013a and b; Anglin et al., 2013; Khan et al., 2013; Pludowski et al., 2013; van der Schaft et al., 2013; Feldman et al., 2014; Krivošíková et al., 2015; Pliz et al., 2016). Furthermore, there is some evidence that low

vitamin D status associates with poor glycaemic control, inflammation, and increased oxidative stress, but again, links are not clearly confirmed (Luo et al., 2009; Codoñer-Franch et al., 2012; George et al., 2012; Yadav et al., 2012; Asemi et al., 2013a and b; Lan et al., 2014). Most of the previous studies on vitamin D and biomarkers of disease risk have investigated elderly people or those with pre-existing disease or under high risk, and studies on young apparently healthy people are lacking. Still, the accumulation of the underlying biochemical and cellular changes that lead to higher risk of developing NCDs in a later stage of life begins much earlier, and importantly these changes are often reversible in their early stages. For example, poor glycaemic control, high lipids and elevated inflammation can be lowered by lifestyle changes or drugs, and DNA damage and oxidative stress can also be decreased by dietary agents (Collins et al., 2001; Ma et al., 2005; Ho et al., 2013; Choi et al., 2015). It is noted that, for most NCDs, to reverse the pathological changes after the disease becomes overt is very difficult or even impossible, but to control or reverse the early changes is much easier. If the link between low vitamin D status and the 'hidden' biological changes associated with higher risk of NCDs, which can be detected by the use of sensitive biomarkers, is confirmed, then public health strategies to address these changes by improving their vitamin D status can be initiated. Thus, the study of young, still-healthy people is important, and yet is still lacking up to now. This forms the background of this study (Figure 9.1). In regard to Hong Kong, a sub-tropical, affluent, modern city, some previous studies have indicated that low vitamin D status is very common (Woo et al., 2008; Xu et al., 2015), but no data have been published on young healthy people, especially on young men. Vitamin D status is not assessed in routine health checks in Hong Kong, and is an expensive biomarker to measure, generally costing around HK$1,200 (~US$154). Thus, many of our young people may have vitamin D deficiency

and not be aware of this. In this study, the vitamin D status of a group of young healthy people living in Hong Kong was determined, and the inter-relationships between vitamin D status and the 'hidden' changes of NCD risk were also investigated though a biomarker method. In addition, a pilot study to explore the effects of vitamin D supplementation on vitamin D deficient young still-healthy adults was performed.

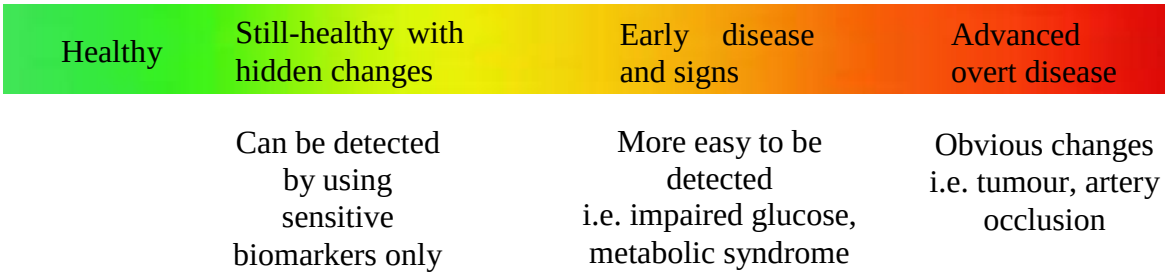


Figure 9.1. The Process of NCDs

MAIN FINDINGS

To achieve the aims (as presented in Chapter 2), this study was in two parts. In part 1, 204 subjects were recruited, with viable samples from 196 collected, and in part 2 a pilot supplementation trial was performed on 22 subjects who were found to have vitamin D deficiency (plasma 25(OH)D <40 nmol/l). Biomarkers of interest (Table 2.1) were measured in samples from both parts of the study. The findings in relation to aims are summarized below.

Main aims

Aim 1: To determine the vitamin D status (plasma 25(OH)D concentration) of a group of young, apparently healthy adults in Hong Kong.

Main findings: The mean(SD) of plasma 25(OH)D was 42.1(13.0) nmol/l. Only 2/196 subjects (1%) had plasma 25(OH)D ≥75 nmol/l; 53/196 (27%) had insufficient vitamin D (plasma 25(OH)D ≥50<75 nmol/l); 128/196 (65%) had plasma 25(OH)D ≥25<50 nmol/l, and 13/196 (7%) had severe vitamin D deficiency (plasma 25(OH)D <25 nmol/l). Overall, 141/196 (72%) were clearly deficient in vitamin D, and if 75 nmol/l is taken as the threshold for sufficiency, 194/196 (99%) had insufficient vitamin D. Males had significantly ($p<0.05$) higher 25(OH)D concentration than females, the mean(SD) values were 45.3(14.4) and 40.6(12.1) nmol/l, respectively (please refer to Chapter 4). No significant difference across seasons was found.

Aim 2: To investigate the inter-relationships between vitamin D status and selected biomarkers of interest (cardiometabolic risk factors, DNA damage, and antioxidant status and oxidative stress).

Main findings:

Cardiometabolic risk factors

Significant inverse correlation was found between vitamin D status and fasting plasma glucose ($r=-0.18$, $p<0.05$). Subjects with severe vitamin D deficiency (plasma 25(OH)D <25 nmol/l) had higher ($p<0.05$) HbA1c and TC/HDL-C ratio and lower HDL-C, compared with those who had plasma 25(OH)D ≥25<50 nmol/l and those with plasma

25(OH)D ≥50 nmol/l. Significant direct correlation was also found between plasma 25(OH)D concentration and uric acid (r=0.23, p<0.01) (please refer to Chapter 5).

DNA damage

The mean(SD) plasma 25(OH)D concentration in the subgroup (n=121) whose lymphocytes were tested for DNA damage was 44.7(13.0) nmol/l. No significant association was found between vitamin D status and pre-existing strand breaks, pre-existing strand breaks plus oxidation-induced damage combined, or oxidation-induced DNA damage alone. No significant difference was found in DNA damage scores between subjects with plasma 25(OH)D <50 nmol/l and those with plasma 25(OH)D ≥50 nmol/l (please refer to Chapter 6).

Antioxidant status and oxidative stress

Significant direct correlations were found between plasma 25(OH)D and total FRAP value, as well as the FRAP value corrected for urate, but no significant associations were found between 25(OH)D and plasma ascorbic acid, plasma allantoin or urine 8-oxodG. A trend of higher FRAP value (total and corrected for urate) in the higher quartiles and higher categories of 25(OH)D was seen, but this trend did not reach statistical significance (p>0.05) (please refer to Chapter 7).

To investigate the overall relationship between vitamin D status and the biomarkers of disease, multivariate analysis of variance (MANOVA) based on vitamin D status categories (<25 nmol/l, ≥25<50 nmol/l, ≥50 nmol/l) was performed. However, it is noted that not all subjects had the comet assay performed, and because of this missing data, MANOVA was only performed with the biomarkers of cardiometabolic risk,

antioxidant status and oxidative stress. From the results, HbA1c, HDL-C, TC/HDL-C ratio, total FRAP value, which were found to be significantly associated with vitamin D status according to the vitamin D status categories (please refer to chapter 5 and 7), remained statistically significant in the MANOVA test. In addition, the Log(Tg/HDL-C), which was found to be approaching statistical significance in analysis of variance, reached statistical significance with MANOVA ($p<0.05$) (Table 9.1). This multivariate analysis of variance provided further evidence that very low vitamin D status (25(OH)D<25 nmol/l) is significantly associated with poorer cardiometabolic profile and lower antioxidant status, even in apparently healthy young adults.

Table 9.1. Overall Inter-relationships between Vitamin D Status and Biomarkers of Interest According to Categories of Vitamin D Status

Vitamin D status category	<25 nmol/l	≥25<50	≥50 nmol/l	P
SYS BP (mmHg)	113.8(13.2)	111.9(10.1)	114.5(9.1)	0.345
DIA BP (mmHg)	61.9(7.5)	63.7(8.0)	65.2(7.1)	0.333
Fasting glucose (mmol/l)	5.26(0.42)	5.25(0.46)	5.12(0.41)	0.127
HbA1c (%)	*5.69(0.52)	5.25(0.50)	5.30(0.49)	0.012
TC (mmol/l)	4.45(0.82)	4.54(0.70)	4.76(0.91)	0.258
HDL-C (mmol/l)	*1.30(0.23)	1.53(0.31)	1.50(0.29)	0.029
LDL-C (mmol/l)	2.72(0.68)	2.65(0.56)	2.87(0.75)	0.173
TC/HDL-C ratio	*3.50(0.79)	3.03(0.52)	3.23(0.59)	0.009
Tg (mmol/l)	0.95(0.46)	0.78(0.29)	0.86(0.36)	0.152
Log(Tg/HDL-C)	-0.17(0.22)	-0.31(0.19	-0.26(0.19)	0.042
Uric acid (µmol/l)	301.9(66.1)	316.8(70.2)	338.8(80.9)	0.105
hsCRP (mg/l)	0.37(0.50)	0.52(0.86)	0.47(0.88)	0.774
FRAP (µmol/l)	950(206)	1017(190)	1088(218)	0.021
FRAP corrected for urate (µmol/l)	346.4(108.1)	383.5(120.4)	410.5(107.1)	0.111
Ascorbic acid (µmol/l)	56.4(27.6)	53.6(20.6)	58.9(23.7)	0.266
Allantoin (µmol/l)	0.76(0.47)	0.88(0.64)	0.81(0.41)	0.696
8-oxodG (nmol/mmol creatinine)	1.16(0.48)	1.13(0.81)	1.17(0.69)	0.858

*$p<0.05$ in post-hoc test compared to the other two groups of vitamin D status

Aim 3: To explore the response of the biomarkers with 12 weeks of vitamin D supplementation in a sub-group of subjects who were found to have low vitamin D status.

Main findings: In the 16 subjects who received supplementation of 2,400 IU vitamin D/day for 12 weeks, significant ($p<0.05$) improvement in vitamin D status was found, with 25(OH)D on average doubling in this group, while no significant change in 25(OH)D was seen in the placebo group. Comparing the biomarker changes (post-pre change) across treatments, vitamin D supplementation was linked to an increase of plasma allantoin and a decrease of lymphocytic DNA damage ($p<0.05$). Potential benefit on the total antioxidant power (FRAP corrected for urate) was seen, but this did not reach statistical significance ($p=0.068$). After supplementation, the 25(OH)D had reached or exceeded the 75 nmol/l threshold in 12 people, but no significant association was found between 25(OH)D concentration and any of biomarkers of interest in the post-12 week samples (please refer to Chapter 8).

Secondary aim

Aim 4: To investigate the cardiometabolic risk factors profile (blood pressure, HbA1c, fasting plasma glucose, TC, HDL-C, LDL-C, TC/HDL-C ratio, Tg, Log(Tg/HDL-C), hsCRP and uric acid) in a group of young, apparently healthy adults in Hong Kong.

Main findings: Evidence of elevated cardiometabolic risk was found in this group of young healthy adults, with 74% having at least one biomarker in the elevated risk group; of these 83/196 (42%) had one elevated risk factor, and 11% (n=21) of the subjects had three or more increased risk factors. High LDL-C was the most commonly prevalent risk factor found, 62/196 (32%) of the subjects had LDL-C >3.0 mmol/l, followed by elevated TC and uric acid, which were each found in 56/196 subjects studied (28.6%).

In terms of gender difference, males were found to have larger number of risk factors in the elevated risk categories than females: the mean numbers were 1.73 and 0.94 respectively, $p<0.001$. It is noted that high uric acid was found in over half of the young men, with 37/63 (59%) of them having uric acid >420 μmol/l (please refer to Chapter 5).

In summary, the aims of the study were achieved.

NOVELTY AND IMPACT OF THIS STUDY

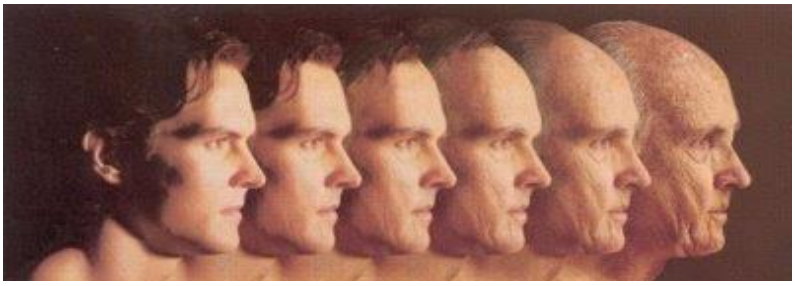
This study is the first to investigate vitamin D status and its inter-relationships with the risk of NCDs (assessed using a wide range of biomarkers) in young, apparently healthy, non-obese and non-smoking people. Results show that vitamin D deficiency or insufficiency was present in all but two of the 196 subjects, and that low vitamin D status was found to be associated with biomarker signs of increased risk of cardiometabolic disease. However, no significant effect of vitamin D supplementation was seen in any of these biomarkers. For antioxidant status, a link between higher 25(OH)D and the FRAP value (total and 'urate corrected') was found, and a potential improvement of the FRAP corrected for urate was found in those who received vitamin D supplementation, but this improvement was not statistically significant. No significant association between vitamin D status and oxidative stress biomarkers was seen in either part of this study. In regard to DNA damage, no significant association between vitamin D status and DNA damage found in the observational part, but interestingly, the preliminary results from the pilot supplementation trial found that

correction of vitamin D deficiency may be beneficial to lower DNA damage. For the correlation between 25(OH)D and biomarkers of interest, significant correlation between 25(OH)D and fasting plasma glucose and FRAP value (total and 'urate corrected') was found in the 196 young adults, however, no significant association between vitamin D status and any of these biomarkers was found after supplementation, even though the range of 25(OH)D was greatly widened. Overall, data generated in the observational arm and the pilot supplementation arm are somewhat conflicting (Table 9.2).

Table 9.2. Comparison of Results in the Observational and the Pilot Supplementation Arms of the Study

Aspect	Observational study	Pilot supplementation trial
Glycaemic control	Significant direct association was seen between 25(OH)D and fasting plasma glucose, and those with 25(OH)D<25 nmol/l had significantly higher HbA1c compared with those with 25(OH)D ≥25<50 nmol/l and with plasma 25(OH)D ≥50 nmol/l.	No significant association or improvement was seen after supplementation
Lipids	Significantly higher TC/HDL-C ratio and lower HDL-C were seen in those with 25(OH)D<25 nmol/l, compared with those with 25(OH)D ≥25<50 nmol/l and with plasma 25(OH)D ≥50 nmol/l.	No significant association or improvement was seen after supplementation
Antioxidant status	Significant direct association was seen between 25(OH)D and the FRAP value ('total' and corrected for urate)	Potential improvement was seen in the FRAP value corrected for urate after supplementation, but this was not statistically significant (p=0.068)
DNA damage	No significant association with vitamin D status was seen	Significant improvement was seen in DNA damage after supplementation

It is noted that all of the subjects recruited into this study were young, apparently healthy people, and most had baseline glucose and lipids that were well within the normal range (as shown in Chapter 5). Thus, any improvements in these biomarkers that may have been induced by vitamin D supplementation, if indeed vitamin D plays a role, would undoubtedly have been small. This is in contrast to the magnitude of potential changes that could be achieved in those with CVD or diabetes, the elderly, or in others under high risk of NCD in whom there is a larger potential for improvement. For example, as reported by George et al. (2012), vitamin D supplementation was associated with lower fasting plasma glucose in diabetes patients and those with impaired fasting glucose, but not in normal subjects (George et al., 2012). For young people, the potential improvement is much smaller, because they are closer to optimal biomarker levels than those with disease or under higher risk of NCD. Thus, the potential short-term biomarker improvement induced by correction of vitamin D deficiency is difficult to detect in young healthy adults, though such improvement from their early adult life would bring greater beneficial impact on health and NCD risk in comparison with effects on elderly people or those with pre-established disease, in whom short-term effects may be easier to detect but in whom the impact of this would be more limited due to the advanced nature of their health problems (Figure 9.2). It is noted that this pilot study was performed with a small group of subjects and so lacks power to adequately detect small changes. Furthermore, the duration of supplementation was short (12 weeks), and while effective in correcting deficiency of vitamin D, it must be noted that some biomarkers, such as HbA1c, need a longer time for improvement to reach a detectable level. Therefore, a supplementation trial with more people and of a longer duration is needed to more completely investigate the potential for biomarker improvement induced by the correction of vitamin D deficiency in young adults.



	Targeting Young Healthy Adults	Targeting the Elderly or those with Disease
Advantages	Long-term impact to health potentially profound Risk factors and changes are at early, reversible stage	The improvement induced by short-term supplementation is easier to detect as the biomarker levels are higher
Disadvantages	The improvement with short-term supplementation is very difficult to be detect, as the room for improvement is limited	The impact on health for this age group is limited, as most of the pathological changes are advanced and irreversible

Figure 9.2. The Advantages and Disadvantages in Studies with Different Target Populations (Figure from Naturessunshine website)

Another reason why potential effects may be difficult to see and manifest as well as some apparent conflict in results from the two arms of the study (Table 9.2) is the possibility that the biomarker improvement induced by the correction of vitamin D deficiency has a threshold effect, and that the response to vitamin D status improvement and threshold for different biomarkers is different.

Results from this current study found that the fasting glucose was inversely associated with plasma 25(OH)D when the overall vitamin D status was low (in the observational study), but no significant improvement was found when the vitamin D status was enhanced to a higher level (in the pilot supplementation trial), this may be due to the threshold effect for glycaemic control. In contrast, for DNA damage, no significant association was found in the observational study but significant improvement was seen after the 25(OH)D concentration doubled in the supplementation trial (Table 9.2). This may indicate that the response of DNA damage to the improvement of vitamin D status is less 'sensitive' than that of fasting glucose (thus, difficult to be detected when the vitamin D status was generally low in the observational study), but the threshold for DNA damage is higher (thus, the enhancement of vitamin D status to a much higher level had significant impact on lowering DNA damage).

In a study of DNA methylation (Zhu et al., 2016), significant association between 25(OH)D and DNA methylation was found in African Americans, who had lower vitamin D status, but not in the other group (Caucasians) who had higher vitamin D status: mean(SD) values were 25(OH)D were 46(20) and 95(38) nmol/l, respectively (Zhu et al., 2016). Besides, a subgroup of African Americans (n=70) with low vitamin D status (25(OH)D <50 nmol/l) were recruited for a supplementation trial, and received

placebo (n=17), monthly oral vitamin D of 18,000 IU (n=17), 60,000 IU (n=18) or 120,000 IU (n=18) for 16 weeks. After supplementation, a significant increase of DNA methylation was found in all groups given vitamin D, and the increase was directly associated with the dose they received, p for trend <0.05. In addition, this change of DNA methylation was also found to be directly associated with the change of 25(OH)D concentrations in all subjects (n=70), r=0.60, p<0.05. Results from Zhu et al. (2016) suggests that the DNA methylation was increased with the improvement of vitamin D status, but when the vitamin D status reached a certain level (i.e. for Caucasians), this association disappeared. In other studies, Spedding et al. (2013) suggested that higher vitamin D status was associated with lower risk of various disease, and the thresholds were different: for example, 75 nmol/l for mortality, 80 nmol/l for type 2 diabetes and CVD, 95 nmol/l for depression and 100 nmol/l for cancer. Findings from Spedding et al. (2013), Zhu et al., (2016) and from this current study are supportive of the hypothesis that the impact of vitamin D on different aspects of health has a threshold based rather than linear effect, and that the effect threshold for different biomarkers is different. This hypothesis is presented in Figure 9.3.

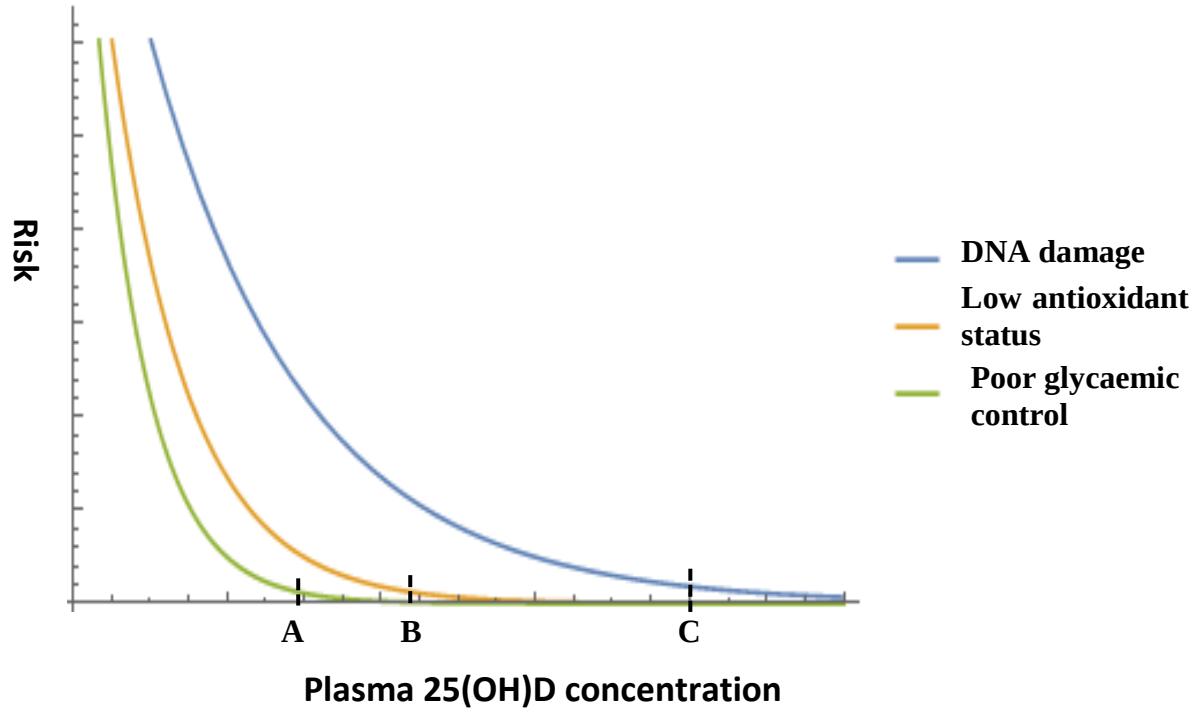


Figure 9.3. The Hypothesis of Risk Response in Different Biomarkers to Vitamin D Status

A, B, C represent the thresholds for poor glycaemic control, low antioxidant status and DNA damage, respectively.

Compared with previous studies, this current study supports existing evidence that poor vitamin D status is associated with higher risk of diabetes and dyslipidaemia. In addition, this link is further expanded to those young, still-healthy people in this study, not just limited to elderly people, sick patients or those already with impaired fasting glucose. Besides, this current study also reveals that low vitamin D is associated with lower antioxidant status. Furthermore, this current study generated novel data on the effect of vitamin D improvement by supplementation on DNA damage, even though the results are preliminary, it still provides evidence to support potential beneficial effect on DNA damage of the correction of vitamin D deficiency. Overall, the findings from this current study suggest that low vitamin D status is associated with various risk factors in young, healthy people.

Another potentially valuable finding from this study is the high prevalence of poor vitamin D status and also cardiometabolic risk profile in the 196 young, apparently healthy, non-obese, non-smoking subjects studied. In this group, 99% had insufficient or deficient vitamin D and 74% had at least one cardiometabolic risk factor in the high risk category. These new data show a worrying combination of low vitamin D status and poor cardiometabolic risk profile in these young adults that indicates a public health problem. Public health measures, including educational programmes, vitamin D food fortification programme and early health screening for vitamin D status and cardiometabolic disease risk should be implemented in Hong Kong to address these problems.

LIMITATIONS

For the subjects studied, because of the resource and time limitations, only 196 subjects were tested, while there are 447,900 people aged 20-25 living in Hong Kong (Population Estimates Report in 2015 from Census and Statistics Department of Hong Kong SAR Government, http://www.censtatd.gov.hk/hkstat/sub/sp150.jsp?tableID=002&ID=0&productType=8, data accessed in July, 2016). The majority of the subjects were university or college students, and the group was quite homogeneous, with various confounding factors such as age, obesity, smoking habit and education level well controlled. Still, the group cannot be regarded as completely representative of all healthy adults in this age group living in Hong Kong, although we have no reason to believe that their lifestyle differs a lot from others in the same age group living in this sub-tropical, modern, affluent city.

In relation to the biomarkers measured in this study, they covered a wide range and were measured by sensitive specific methods, however some important biomarkers, for example, insulin, HOMA-IR, F2 isoprostanes, enzymatic antioxidants and cytokines, were not measured because of the resource limitation. In addition, $1,25(OH)_2D$ was not measured. This may have provided more information of vitamin D status, but it is not a commonly used indicator for vitamin D status and its concentration in plasma is very low and it is difficult to measure it accurately. Another limitation is that polymorphisms in VDR and DBP were not measured. These were outside the scope of this study but may be of interest in future studies.

In regard to the supplementation trial, the sample size in this pilot study was very small, and as noted the supplementation period was 12 weeks only. The duration of the supplement was guided by previous studies, and achieved the correction of vitamin D deficiency in all subjects who receive vitamin D. However, as noted above, the biomarker response to the vitamin D status improvement may need a longer follow-up time. Also, the initial target number was larger and this study aimed to target only those who were vitamin D deficient. However, because of serious technical problems with equipment, there was an unavoidable delay in recruitment for the supplementation trial. This large gap between the recall to join the supplementation trial and their first participation led to low response rate and some of the subjects were subsequently found to have higher vitamin D status at part 2 baseline compared to at part 1 entry; two (one in each group) subjects in the supplementation trial had plasma 25(OH)D >60 nmol/l at baseline. This unexpected finding may have been due to their awareness that they had low vitamin D status as all potential part 2 subjects were informed of that.

SUGGESTIONS FOR FURTHER WORK

In this study, the link between low vitamin D status and the 'hidden' changes associated with NCD risks was found as the results of a cross-sectional observation study. To further confirm the impact of low vitamin D status and other biomarkers tested here to their health and disease outcomes in a later stage of life, a prospective study to follow up this group of young people in the decades to come would be ideal to reveal the real NCD risk in relation to vitamin D status and cardiometabolic profile in their early adulthood.

For the supplementation trial, this current study generated preliminary but useful information. The 12 weeks' supplementation with 2,400 IU vitamin D/day was effective to correct vitamin D deficiency. In addition, some potentially valuable effect was seen in terms of lowering DNA damage, which is associated with lower risk of mutations and cellular dysfunction. For DNA damage, it is noted that the apparent DNA damage measured is the result of the balance of both DNA damage and DNA repair, thus, to also measure the DNA repair will provide more information on the relationship between vitamin D and DNA. To further confirm the finding found in this current study, to expand the sample size with a longer duration of supplementation will be very helpful.

In regard to the biomarkers measured in this study, to expand the biomarker panel by involving other sensitive biomarkers associated with the risk of NCDs would be useful. For example, insulin concentration, insulin sensitivity and β cell function may be more sensitive for detecting the changes of glycaemic control (George et al., 2012; Asemi et al., 2013a; Sepehrmanesh et al., 2016). In addition, inflammatory cytokines may provide more information on this aspect of the 'common soil'. In this regard, it is noted that data on several biomarkers, including PTH, oxLDL, IL-6, TNF-α, sVCAM-1, sICAM-1, E-selectin and P-selectin, and two questionnaires of lifestyle and mood are under analysis by other members of the research team, and are outside the scope of this thesis.

GUIDE IN PLANNING AND DESIGN OF FUTURE STUDIES

Sample size calculation

The pilot supplementation trial generated useful preliminary data for the design and planning of further study in sample size estimation. In this section, power calculations were performed using GPower 3.1 to estimate the sample size per group needed for a parallel, placebo-controlled supplementation trial in regard to statistical significance, at 80% power and a significance level of 0.05, of the across treatment differences in selected biomarkers that were actually seen in the pilot study (Table 9.3). It is noted that, while HbA1c was regarded as a key biomarker, no decrease with vitamin D supplementation was seen in the pilot study. This may have been due to no true effect or association, or to the short duration of the trial. HbA1c represents the average plasma glucose level over the previous three months, and so needs a longer time to respond to the correction of vitamin D deficiency. Thus, a longer duration, as well as larger sample size, is important to investigate the effect of vitamin D supplementation on HbA1c. Based on a targeted across treatment response of 0.5% change in HbA1c (our study group SD, and a change that is likely to be clinically significant (WHO, Use of Glycated Haemoglobin (HbA1c) in the Diagnosis of Diabetes Mellitus, 2011), it is estimated that 17 subjects in each group would be needed.

Table 9.3. Estimation of Sample Size to Guide Further Supplementation Trials to Achieve Statistical Significance in Responses Seen in the Pilot Study (i.e. Across Treatment Difference in Post-Pre Responses)

	Mean difference in post-pre response across treatments	SD in post-pre response	Sample size in each group
LDL-C (mmol/l)	-0.214	0.412	47
ΔFpg-buffer (DNA% in tail)	-3.63	5.05	25
FRAP corrected for urate (μmol/l)	+159.1	224.1	26

Vitamin D status, other metabolites, vitamin D activity and the influence of genotype

According to the survey of Xu et al. (2016), the determinants of vitamin D status in children (6-17 years) living in Hong Kong are gender, dietary vitamin D intake (fish and milk) and sunshine exposure, while no data were shown in other age groups (Xu et al., 2016). In this current study, the subjects were not taking any supplements (please refer to the inclusion criteria in Chapter 2), and there is no mandatory vitamin D food fortification programme in Hong Kong, thus, the impact of dietary intake on vitamin D status is limited and very difficult to estimate reliably (Pearce and Cheetham, 2010). Exposure to sunshine has the largest effect on vitamin D status in non-supplemented subjects, and this may vary a lot among young people. Exposure to sunlight can be estimated by questionnaire, by 'tanning index' of skin and by use of wearable devices that can measure solar radiation (Palmer et al., 2016). Still, the key index of vitamin D

status at this time is plasma 25(OH)D concentration, and all future studies must include this measurement, regardless of the estimation of intake and sun exposure. It would also be desirable to measure $1,25(OH)_2D$, the active form of vitamin D, as well as $1,24,25(OH)_3D$, which is inactive, and $24,25(OH)_2D$ which is also inactive, as including these three vitamin D metabolites would give a more complete picture of vitamin D status. However, these are currently not often measured due to technical difficulties, levels are in the picomolar range, half-lives are very short, and some or most of the transformation of 25(OH)D is intracellular (Birke, 2014; van der Meijden et al., 2016).

In addition, some studies have suggested that the activity (effect) of vitamin D may be different with the same plasma/serum 25(OH)D concentrations, due to variations in vitamin D binding protein (DBP), vitamin D receptors (VDR) and cytochrome P450 enzymes (CYPs). It is noted that the genomic factors have very limited impact on vitamin D status, and explain only 1-4% variance of 25(OH)D concentration (Wang et al., 2010b). A cohort study in Europe suggested that significant impact was found in the genotypic variants in three loci including *GC* (for the vitamin D binding protein), *DHCR7* (for 7-dehydrocholesterol reductase, that converts 7-dehydrocholesterol to cholesterol) and *CYP2R1* (for 25-hydroxylase). No confirmatory evidence was found in the association between 25(OH)D concentration and polymorphism near/in the vitamin D receptor gene, *CYP24A1* (for 24-hydroxylase) or *CYP27B1* (for 1α-hydroxylase) (Wang et al., 2010b). In a study with 506 northeastern Han Chinese children the 25(OH)D concentration was significantly ($p<0.05$) associated with single nucleotide polymorphisms in *GC*, *CYP2R1* and *DHCR7*, but the genotypes in the vitamin D binding protein, *CYP24A1* and *CYP27B1* were not involved (Zhang et al., 2012). However, genomic differences may impact on the activity of vitamin D (rather than the

amount). A study in US suggested that the activity of vitamin D with the same 25(OH)D concentration may be different across ethnic groups. Although no difference in the biological function of free 25(OH)D and DBP-bound 25(OH)D was seen, free 25(OH)D is more easily transformed to $1,25(OH)_2D$. It was shown that people with darker skin had relatively lower 25(OH)D concentrations than light-skinned Caucasians, but the activity of vitamin D remained similar, as the proportion of free 25(OH)D was higher in darker skinned subjects (Powe et al., 2014). It is noted that the association between the vitamin D activity and genotypes of VDR and CYPs is still unclear. As mentioned above, to measure circulating $1,25(OH)_2D$ directly is not an ideal way to identify the activity of vitamin D, while 25(OH)D concentration does not explain the vitamin D activity entirely, and further study to investigate the impact of genotype on the activity of vitamin D according to 25(OH)D concentration would be of interest.

DNA repair

In this current study, it was found that after correction of vitamin D deficiency there was less DNA damage ($p<0.05$). Measured damage is the balance between damage incurred and its repair. DNA repair was not included in this current study due to resource limitations. Previous studies suggested that vitamin D plays a role in the repair of UVB-induced damage, as vitamin D receptor knock-out mice have higher risk of developing UVB-induced epidermal tumours, and $1,25(OH)_2D$ was found to be associated with nucleotide excision repair (NER), which is the major pathway for repair of UVB-induced DNA damage (Pawlowska et al, 2016). One animal study showed that vitamin D was involved in the diminishment of UV-induced cyclobutene pyrimidine dimers (CPDs) in epidermal keratinocytes (Demetriou et al., 2012). Both the wild type and vitamin D receptor knock-out mice received UV radiation, and CPDs in epidermal

keratinocyte were measured at 1 hour, 24 hours and 48 hours later. Both groups presented higher CPD signals under immunofluorescence, but this was significantly lower ($p<0.05$) in the wild type mice. The CPD signal was decreased significantly at 24 hours and undetectable at 48 hours post UV radiation in the wild type mice, but the signal still remained significant in the knock-out mice at 48 hours. For oxidation-induced damage, 10 nmol/l $1,25(OH)_2D$ was found effectively lower (by ~20%) the phosphorylation of histone H2AX induced by H_2O_2 treatment compared with the control condition, indicating that vitamin D is protective against oxidation-induced double strand breaks (Halicka et al., 2012). However, it is noted that although vitamin D was found to be beneficial for DNA repair in some cell culture and animal studies, evidence from human study is still lacking. Further human study investigating the link between vitamin D status and DNA repair is warranted, and is supported by the results of the pilot study presented here.

CONCLUDING REMARKS

Non-communicable disease is the leading cause of death globally, and causes heavy socio-economic burden to all countries, especially for the low and middle income countries. To control the burden of NCD is an important public health concern worldwide. NCD development is associated with several life-style factors, including obesity, smoking, physical inactivity and alcohol overconsumption, which cause various biochemical and cellular changes such as high glucose, hyperlipidaemia, inflammation, DNA damage and oxidative stress. Furthermore, vitamin D deficiency was found to be a possible emerging risk factor of NCD in previous studies. This study is the first human study to target young, apparently healthy, non-obese, non-smoking people using

a wide range of sensitive e biomarkers measured in relation vitamin D status, and to explore the effect of correction of vitamin D deficiency on these biomarkers. This study has shown that the vitamin D status was poor in a group of 196 young adults living in Hong Kong, and low vitamin D status was shown to be associated with poor glycaemic control, dyslipidaemia and low antioxidant status, Further, the correction of low vitamin D status was associated with a lowering of DNA damage. Besides, from the data generated in this study, it can be seen that a poor cardiometabolic risk profile was highly prevalent in this group of young people. If indeed low vitamin D status is confirmed to be a determinant of NCD risk, results have important implications for the future health of this group. It is noted that low vitamin D status is easily corrected. For most healthy people, only 10-15 min sunshine exposure to arms and legs per day is sufficient to maintain normal vitamin D status, and for those who are not able to receive sunshine exposure, taking fortified food or supplements is effective to correct deficiency. Public health measures are recommended to improve the generally low vitamin D status of people in Hong Kong. Further study is needed to confirm the role of vitamin D in NCD risk and development.

APPENDICES

Appendix 1. Ethical Approval

Appendix II.A. Certificate for Vitamin D Supplementation

Appendix II.B. Certificate for Placebo

APPENDIX I. ETHICAL APPROVAL

THE HONG KONG POLYTECHNIC UNIVERSITY
香港理工大學

To	Benzie Iris Frances Forster (Department of Health Technology and Informatics)		
From	WONG Kam Yuet, Chair, Human Subjects Ethics Sub-committee		
Email	hsfwong@	Date	19-Oct-2013

Application for Ethical Review for Teaching/Research Involving Human Subjects

I write to inform you that approval has been given to your application for human subjects ethics review of the following project for a period from 01-Nov-2013 to 31-Aug-2016:

Project Title:	Vitamin D Status in Young Adults Hong Kong: a biomarker approach to inter-connections for insight into a public health: Part 1 and Part 2
Department:	Department of Health Technology and Informatics
Principal Investigator:	Benzie Iris Frances Forster

Please note that you will be held responsible for the ethical approval granted for the project and the ethical conduct of the personnel involved in the project. In the case of the Co-PI, if any, has also obtained ethical approval for the project, the Co-PI will also assume the responsibility in respect of the ethical approval (in relation to the areas of expertise of respective Co-PI in accordance with the stipulations given by the approving authority).

You are responsible for informing the Human Subjects Ethics Sub-committee in advance of any changes in the proposal or procedures which may affect the validity of this ethical approval.

You will receive separate email notification should you be required to obtain fresh approval.

WONG Kam Yuet

Chair

Human Subjects Ethics Sub-committee

APPENDIX II.A CERTIFICATE OF VITAMIN D SUPPLEMENTATION

維特健靈®
VITA GREEN

VITA GREEN PHARMACEUTICAL, (HK) LTD.
維特健靈大藥廠

CERTIFICATE OF ANALYSIS

DATE: 9 August 2013
PRODUCT NAME: Doctor's Choice Vitamin D3 1200IU 60's
BATCH NO.: 418042A

MICROBIOLOGICAL TEST	SPECIFICATIONS	FOUND
1. Aerobic Bacteria Count	≤ 1,000 cfu/g	< 10 cfu/g
2. Yeast and Mould	≤ 100 cfu/g	< 10 cfu/g
3. *E. coli*	Negative/g	Negative/g
4. *Salmonella*	Negative/10g	Negative/10g
5. *Staphylococcus aureus*	Negative/g	Negative/g

Timothy Tam
QC and Laboratory Manager

APPENDIX II.B CERTIFICATE OF PLACEBO

維特健靈®
VITA GREEN

VITA GREEN PHARMACEUTICAL, (HK) LTD.
維特健靈大藥廠

CERTIFICATE OF ANALYSIS

DATE: 9 August 2013
PRODUCT NAME: Vitamin D Supplementation 60's placebo tablet
BATCH NO.: HKCTX429

MICROBIOLOGICAL TEST	SPECIFICATIONS	FOUND
1. Aerobic Bacteria Count	≤ 1,000 cfu/g	< 10 cfu/g
2. Yeast and Mould	≤ 100 cfu/g	< 10 cfu/g
3. *E. coli*	Negative/g	Negative/g
4. *Salmonella*	Negative/10g	Negative/10g
5. *Staphylococcus aureus*	Negative/g	Negative/g

Timothy Tam
QC and Laboratory Manager

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