Effect Of Vitamin D Supplementation On Bone Status, Glucose Homeostasis And Immune Function In Children With Vitamin D Deficiency

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A Thesis Submitted in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

to

The School of Medicine, University of Glasgow (January 2016)

Based on work conducted in the
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Author's declaration

I hereby declare that all the work in this thesis unless otherwise indicated is entirely my own contribution and is a record of work performed by me in the department child health between November 2010- July 2014.

I declare that no portion of the work in this thesis has been submitted in support of any application for any other degree or qualification from this or any other university or institute of learning and was performed under the supervision of Dr Helen McDevitt and Professor Syed Faisal Ahmed.

Dr Nagla M El fakhri

I certify that the work reported in this thesis has been performed by Dr El fakhri and that during the period of study she has fulfilled the conditions of the ordinances and regulations governing the Degree of Doctor of Philosophy, University of Glasgow.

Dr Helen McDevitt

Abstract

Background

Between 1961-1971 vitamin D deficiency was recognized as a public health issue in the UK, because of the lack of effective sunlight and the population mix [1, 2]. In recent years, health care professionals have cited evidence suggesting a re-emergence of the vitamin D deficiency linked to a number of health consequences as a concern [3-6]. Evidence from observational studies has linked low vitamin D status with impairment in glucose homeostasis and immune dysfunction [7-9]. However, interventional studies, particularly those focused on paediatric populations, have been limited and inconsistent. There is a need for detailed studies, to clarify the therapeutic benefits of vitamin D in these important clinical areas.

Objective

The aims of this PhD thesis were two-fold. Firstly, to perform preliminary work assessing the association between vitamin D deficiency and bone status, glucose homeostasis and immune function, and to explore any changes in these parameters following short term vitamin D3 replacement therapy. Secondly, to assess the effectiveness of an electronic surveillance system (ScotPSU) as a tool to determine the current incidence of hospital-based presentation of childhood vitamin D deficiency in Scotland.

Methods

Active surveillance was performed for a period of two years as a part of an electronic web-based surveillance programme performed by the Scottish Paediatric Surveillance Unit (ScotPSU). The validity of the system was assessed by identifying cases with profound vitamin D deficiency (in Glasgow and Edinburgh) from the regional laboratory. All clinical details were checked against those identified using the surveillance system. Thirty-seven children aged 3 months to 10 years, who had been diagnosed with vitamin D deficiency, were recruited for the bone, glucose and immunity studies over a period of 24 months. Twenty-five samples were analysed for the glucose and bone studies; of these, 18 samples were further analysed for immune study. Treatment consisted of six weeks taking 5000 IU units cholecalciferol orally once a day. At baseline and after completion of treatment, 25 hydroxyvitamin D (25(OH)D), parathyroid hormone (PTH), alkaline phosphatase (ALP), collagen type 1 cross-linked C-telopeptide (CTX), osteocalcin (OCN),

calcium, phosphate, insulin, glucose, homeostasis model assessment index, estimated insulin resistance (HOMA IR), glycated hemoglobin (HbA1c), sex hormone binding globulin (SHBG), lipids profiles, T helper 1 (Th1) cytokines (interleukin-2 (IL-2), tumor necrosis factors-alpha (TNF-α), interferon-gamma (INF-γ)), T helper 2 (Th2) cytokines (interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6)), T helper 17 (Th17) cytokine (interleukin-17 (IL-17)), Regulatory T (Treg) cytokine (interleukin-10 (IL-10)) and chemokines/cytokines, linked with Th1/Th2 subset balance and/or differentiation (interleukin-8 (IL-8), interleukin-12 (IL-12), eosinophil chemotactic protein (EOTAXIN), macrophage inflammatory proteins-1beta (MIP-1β), interferon-gamma-induced protein-10 (IP-10), regulated on activation, normal T cell expressed and secreted (RANTES), monocyte chemoattractant protein-1(MCP-1)) were measured. Leukoocyte subset analysis was performed for T cells, B cells and T regulatory cells and a luminex assay was used to measure the cytokiens.

Results

Between September 2009 and August 2011, 163 cases of vitamin D deficiency were brought to the attention of the ScotPSU, and the majority of cases (n = 82) were reported in Glasgow. The cross-validation checking in Glasgow and Edinburgh over a one-year period revealed only 3 (11%) cases of clearly symptomatic vitamin D deficiency, which had been missed by the ScotPSU survey in Glasgow. While 16 (67%) symptomatic cases had failed to be reported through the ScotPSU survey in Edinburgh.

For the 23 children who are included in bone and glucose studies, 22 (96%) children had basal serum 25(OH)D in the deficiency range (< 50 nmol/l) and one (4%) child had serum 25(OH)D in the insufficiency range (51-75 nmol/l). Following vitamin D3 treatment, 2 (9%) children had final serum 25(OH)D lower than 50 nmol/l, 6 (26%) children had final serum 25(OH)D between >50-75 nmol/l, 12 (52%) children reached a final serum 25(OH)D >75-150 nmol/l and finally 3 (13%) exceeded the normal reference range with a final 25(OH)D >150 nmol/l. Markers for remodelling ALP and PTH had significantly decreased ($p = 0.001$ and <0.0001 for ALP and PTH respectively).

In 17 patients for whom insulin and HOMA IR data were available and enrolled in glucose study, significant improvements in insulin resistance ($p = 0.04$) with a trend toward a reduction in serum insulin ($p = 0.05$) was observed. Of those 14 children who had their cytokines profile data analysed and enrolled in the immunity study, insulin and HOMA IR

data were missed in one child. A significant increase in the main Th2 secreted cytokine IL-4 ($p = 0.001$) and a tendency for significant increases in other Th2 secreted cytokines IL-5 ($p = 0.05$) and IL-6 ($p = 0.05$) was observed following vitamin D3 supplementation.

Conclusion

An electronic surveillance system can provide data for studying the epidemiology of vitamin D deficiency. However, it may underestimate the number of positive cases. Improving vitamin D status in vitamin D deficient otherwise healthy children significantly improved their vitamin D deficient status, and was associated with an improvement in bone profile, improvements in insulin resistance and an alteration in main Th2 secreting cytokines.

Acknowledgement

(In the Name of Allah, the Most Beneficent, the Most Merciful)

First and foremost, I would like to express my gratitude to Dr Helen McDevitt and Professor Syed Faisal Ahmed for giving me the opportunity to undertake this PhD, and for their continual guidance and support throughout the duration of my studies.

I would like to thank Dr Chris Halsey for her supervision in the immunology section of the work. I am grateful and thankful for her guidance and friendship, which made this part of my PhD studies most enjoyable and memorable.

Many of the results described in this thesis would not have been obtained without close collaboration with several people. I owe a great deal to Mr Martin McMillan and Dr Jane McNeilly for analysing the blood samples, to Dr Michelle LeBrocq and Dr Yasar Yousafzai for introducing me to, and assisting me with immunity lab work, to Dr Massoud Boroujerdi for advice concerning statistics. To all the families and children who are participated in this study I extend my thanks. Without them, this study would not have been possible. I also gratefully acknowledge the help of the secretary for the Department of Child Health, Mrs Karen Cooper, for her continued support at various stages of the PhD. Her co-operation and assistance in several administrative matters. Finally to all ward 1C staff.

I would like to express my gratitude to my colleagues at the Department of Child Health, Dr Mabrouka Altowati, Dr Mahjouba Ahmid and Dr Ifeyinwa Ezeofor for their discussion, assistance and friendships.

The financial support I received from the Libyan Government is most gratefully acknowledged.

Dedication

I lovingly dedicate this thesis;

To my parents Mahmoud and Huda El fakhri, for all their years of love and encouragement;

To my Husband Sanad Isswiasi, for the immense amounts of patience, support and encouragement;

To my children: Asir, Owias and Maria who shine my life with brightness;

To my brothers: Ahmed and Mohammed, and my sisters: Ghada, Toto, Nour and Rawa.

Publications

Full paper

1. El-Fakhri N, Williams C, Cox K, McDevitt H, Galloway P, McIntosh N, Ahmed SF. An electronic surveillance system for monitoring the hospital presentation of nutritional vitamin D deficiency in children in Scotland. Journal of Pediatric Endocrinology and Metabolism 2013;26(11-12):1053-8.
2. El-Fakhri N, McDevitt H, Shaikh MG, Halsey C, Ahmed SF. Vitamin D and its effects on glucose homeostasis, cardiovascular function and immune function. Hormone Research in Paediatrics 2014;81(6):363-78.
3. El-Fakhri1 N, Le Brocq M, Boroujerdi M, McMillan M, McNeilly J, Halsey C, Ahmed SF, McDevitt H. Effect of vitamin D treatment on glucose homeostasis, immune function and bone status in children with symptomatic vitamin D deficiency (in progress).

Oral presentations

1. An electronic surveillance system for monitoring the hospital presentation of nutritional vitamin D deficiency in children in Scotland. Yorkhill Research Day, Glasgow, UK Nov 2011.
2. An electronic surveillance system for monitoring the hospital presentation of nutritional vitamin D deficiency in children in Scotland. Complex Bone Disorders In Children (educational Day), Stirling, UK Nov 2011.
3. The effect of high dose vitamin D supplementation on markers of bone metabolism & immune status in children with vitamin D deficiency. Yorkhill Research Day, Glasgow, UK Oct 2015.

Poster presentations

1. An electronic surveillance system for monitoring the hospital presentation of nutritional vitamin D deficiency in children in Scotland. The 50th Annual Meeting for The European Society for Paediatric Endocrinology (ESPE) Sep 2011 Glasgow, UK.
2. Effect of vitamin D treatment on glucose metabolism and bone turnover in children with symptomatic vitamin D deficiency. The 53rd Annual Meeting for The European Society for Paediatric Endocrinology (ESPE) Sep 2014 Dublin, Ireland.
3. Effect of vitamin D supplementation on immune function and bone status in children with vitamin D deficiency. Annual conference for the British Society for Paediatric Endocrinology and Diabetes (BSPED) Nov 2014 Winchester, UK.

4. Effect of vitamin D supplementation on glucose metabolism, immune function and bone status in children with vitamin D deficiency. The 7th International Conference on Children's Bone Health (ICCBH) June 2015 Salzburg, Austria.

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Definitions/Abbreviations

AB	Antibiotics
APC	Antigen presenting cell
AD	Atopic dermatitis
ADP	Adenosine diphosphate
1α25(OH)ase	1alpha 25 hydroxylase
1,25(OH)2D	1,25 dihydroxyvitamin D
24(OH)D	24 hydroxyvitamin D
25(OH)D	25 hydroxyvitamin D
AIDS	Acquired immune deficiency syndrome
ALP	Alkaline phosphatase
AMPs	Antimicrobial peptides
aOR	Adjusted odds ratio
ARTs	Respiratory infections
ATP	Adenosine triphosphate
Akt	Protein kinase B
ALTM	All-laboratory Trimmed Mean
BMI	Body mass index
BPSU	British Paediatric Surveillance Unit
Ca^{+2}	Calcium ion
CI	Confidence interval
CasR	Calcium sensing receptor
CCL	Chemotactic chemokine ligand
CCR	Chemotactic chemokine receptor
CCRL	Chemotactic chemokine receptor-like
CD4+	Cluster of differentiation 4 positive
CD8+	Cluster of differentiation 8 positive
CL^{-}	Chloride ion
CLIA	Chemiluminescent microparticle immunoassay
CRF	Case report form
CSF-1	Colony-stimulating factor-1
CT	Calcitonin
CTX	Collagen type 1 cross-linked C-telopeptide
CXCR	CXC chemokine receptor

CXCL	CXC chemokine ligand
CYP27B1	Cytochrome p450 27B1 or 1 alpha hydroxylase
DBP	Vitamin D binding protein
DC	Dendritic cell
DEQAS	International Vitamin D External Quality Assessment Scheme
DEXA	Dual-energy x-ray absorptiometry
DMP1	Dentin Matrix Protein
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EOTAXIN	Eosinophil chemotactic protein
FACS	Flow assisted cell sorting
FFQ	Food Frequency Questionnaire
FGFs	Fibroblast growth factors
FOXP3	Forkhead box P3
FOXO1	Forkhead box protein O1
FSG	Fasting serum glucose
FSI	Fasting serum insulin
FSC	Forward Scatter
GATA3	Transcription factor GATA-binding protein 3
GH	Growth hormone
GP	General practitioner
Glu	Glutamic acid
GLUT	Glucose transporter
Grb2	Growth factor receptor-bound protein 2
GSK-3	Glycogen synthase kinase 3
HbA1c	Glycated haemoglobin
Hbd	Human β-defensin
Hcap18	Human cationic antimicrobial protein 18
HDL	High density lipoprotein
HNP	Neutrophil Peptide
HOMA IR	Homeostatic model assessment for insulin resistance
HPLC	High-pressure liquid chromatography

Ht	Height
ICTP	Cross-linked carboxyterminal telopeptide of type I collagen
HIV	Human immunodeficiency virus
IFN γ	Interferon gamma
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor
IL	Interleukin
IOM	Institute Of Medicine
IP3	Inositol triphosphate
IP-10	Interferon gamma inducible protein 10
IRS	Insulin receptor substrate
IU	International Units
IVGTT	Intravenous glucose tolerance test
Kg	Kilogram
L	Litre
LDL	Low density lipoprotein
LL	Lower limit
MAP kinase	Mitogen-activated protein kinases
MCP-1	Monocyte chemoattractant protein-1
MHC class II	Major histocompatibility complex class II
MIP-1β	Macrophage inflammatory protein-1β
MED	Minimal Erythemal Dose
Mg	Milligram
MME	Mixed model effect
mmol/l	Millimole/liter
MMP	Metalloproteinases
mRNA	Messenger Ribonucleic acid
MS	Multiple sclerosis
NADPH	Nicotinamide adenine dinucleotide phosphate
NADH	Nicotinamide adenine dinucleotide+ hydrogen
NAFLD	Non-alcoholic fatty liver disease
Ng	Nanogram
NHANES	National health and Nutrition Examination Survey
NHS	National Health service
NICE	The National Institute for Health and Care Excellence

NK cells	Natural killer
OCN	Osteocalcin
OGTT	Oral glucose tolerance test
OPG	Osteoprotegerin
OSX	Osterix
Oz	Ounces
PAMPs	Pathogen-associated molecular patterns
Patient ID	Patient identification number
PHEX	Phosphate-regulating gene homologous on the X chromosome
PI3-kinase	Phosphatidylinositol 3-kinase
PICP	Procollagen type 1 C-terminal
PINP	Procollagen type-1 propetides
P70s6k	A serine/threonine protein kinase
PPAR-δ	peroxisome proliferator-activated receptor
PPP	Pentose phosphate pathway
PRRs	Pattern-recognition receptors
PTH	Parathyroid hormone
PTH1R	Parathyroid Hormone 1 Receptor
QUICKI	Quantitative insulin sensitivity check index
RANK	Receptor activator nuclear factor-κB
RANKL	Receptor activator nuclear factor-Kb ligand
RANTES	Regulated on activation, normal T cell expressed and secreted
RCT	Randomized Controlled Trial
RDA	Recommended Dietary Allowance
RHSC	Royal hospital for sick children
RORγt	Retinoic acid-related orphan receptor gamma
RXR	Retinoid X receptor
Runx2	Runt-related transcription factor 2
ScotPSU	Scottish Paediatric Surveillance Unit
SD	Standard deviation
SDS	Standard deviation score
SHBG	Six hormone binding globulin
SHC	Src Homology 2 domain containing
SLE	Systemic lupus erythematosus

SOS	Son of Sevenless
SRM	Standard reference material
SSC	Side Scatter
STAT	Signal transducer and activator of transcription
TB	Tuberculosis
T1DM	Type 1 diabetes mellitus
T-bet	T-box expressed in T cells
T2DM	Type 2 diabetes mellitus
TCA cycle	Tricarboxylic acid cycle
TGF B	Transforming growth factors B
Th	T helper
TLR	Toll like receptor
TNF	Tumour necrosis factor
TRP	transient receptor potential
Treg	T regulatory
Mg	Microgram
UL	Upper limit
UVB	Ultraviolet beta radiation
UK	Untied Kindgom
USA	United States of America
USPSTF	United States Preventive Services Task Force
WHO	World Health organization
Wt	Weight
VDR	Vitamin D receptor
VDRE	Vitamin D response element

Chapter 1

Introduction

1.1 Vitamin D: Background

1.1.1 History of vitamin D

Vitamin D, which is also known as the 'sunshine vitamin' is a family of fat-soluble molecules. Vitamin D is not actually a true vitamin as the molecules are not obtained exclusively from the diet. Most of the plants and animals that are exposed to sunlight have an ability to produce vitamin D.

Studies of rickets and related bone disorders throughout history have led to the discovery of vitamin D. Soranus, a Roman physician in the 1st and 2nd century AD is often credited as one of the early describers of some rickets deformities (the backbone bending and legs twisting at the thigh). Soranus attributed these changes to inappropriate maternal hygiene and lifestyle [10]. Around the middle of 17th century, the first clear scientific descriptions of rickets were provided by Dr Whistler and Professor Glisson, in 1645 and 1650, respectively. Whistler described the rickets skeleton as poorly mineralized and deformed while Professor Glisson provided a detailed published description [11]. During that period, rickets was endemic in England. Known as 'the English disease', it was explained by the population's urbanization [12]. However, the mystery of nutritional vitamin D deficiency and its relationship with rickets was solved by Sir Edward Mellanby in 1920 through an experimental demonstration and dietary manipulation of captive dogs. Sir Mellanby was able to cure nutritional rickets with regular doses of cod liver oil or egg yolk, although he incorrectly concluded that the causative factor was a newly-diagnosed fat-soluble vitamin A [13].

In 1921, Dr Elmer McCollum, by the process of aerating and heating of cod liver oil was able to distinguish between vitamin A, can be inactivated, and vitamin D, retains its antirachitic activity [14]. A year later, Hess et al.(1922) were able to cure rickets in rats by exposing them to sunlight without dietary modification. This group was also able to demonstrate the ability of artificial radiation and sunlight to induce the skin synthesis of

vitamin D and prevent the disease [15]. Clinical use of fish liver oil, and later exposure to sunlight or mercury arc lamp to treat children with rickets started shortly after that [16]. Vitamin D refers to two biological precursors: vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol), collectively known as Calciferol. The chemical structure of vitamin D2 or ergocalciferol was first discovered in the 1930s by a British group led by Askew. They showed that vitamin D2 is produced by ultraviolet irradiation of mixtures of plant sterols [17]. This discovery was followed by identification of 7-dehydrocholesterol by Windaus et al.(1935) [18]. Esvelt et al.(1978) were able to identify and isolate vitamin D3 in rat skins via irradiation of 7-dehydrocholesterol [19] (see Figure 1-1).

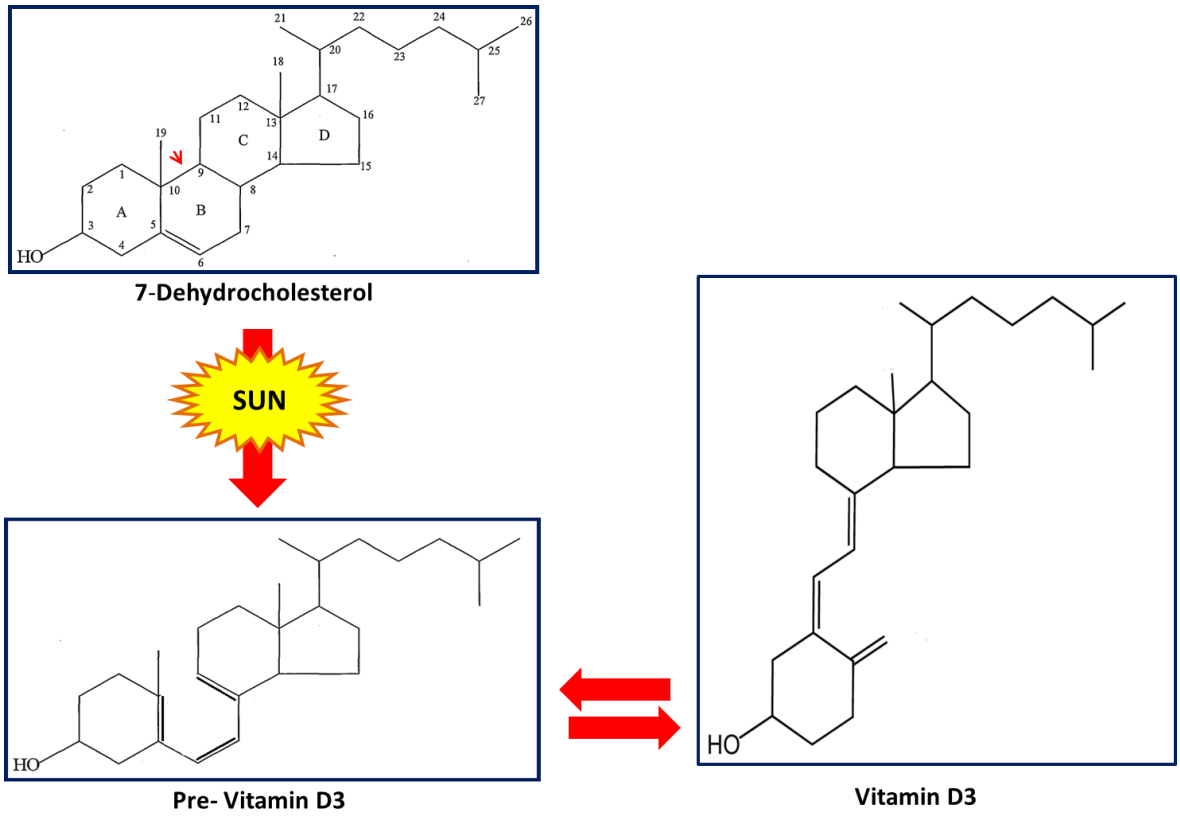


Figure 1-1: Photolysis of the skin's 7- dehydrocholesterol (pro-vitamin D3) under the influence of UV-B light to vitamin D3, via its intermediate previtamin D3.

1.1.2 Sources

Vitamin D3 constitutes around 80-90% of the circulating metabolites that can be obtained exogenously from sunlight exposure, from diet, or from supplements. Vitamin D2 can be obtained through supplements or diet [20]. Both vitamin D2 and vitamin D3 are suggested to have the same functional consequence, though vitamin D3 may be more potent than vitamin D2 in maintaining serum vitamin D status [21].

1.1.2.1 Sunlight

The main natural source of vitamin D is derived from the photolysis of the skin's 7-dehydrocholesterol (pro-vitamin D3) under the influence of UV-B light (see figure 1-1). Photosynthesized vitamin D3 is believed to stay longer in the circulation compared with the externally digested [22]. Ultraviolet radiation can provide approximately 20,000 IU of vitamin D which is equivalent to exposure to 1 minimal erythemal dose (MED), the dose of radiation that causes a slight pinkness to the skin 24 hours after exposure in a bathing suit. Therefore, exposure of arms and legs to 0.5 MED is equivalent to ingesting ~ 3000 IU vitamin D3 [23]. Dark skinned adults require 10-15 times longer sun exposure than a white adult in order to synthesize a similar amount of vitamin D [24].

1.1.2.2 Natural dietary sources

Few foods in nature contain vitamin D3 and vitamin D2. Oily fish including salmon, mackerel and herring or fish liver oil are considered as main dietary sources of vitamin D3. Cheese, beef liver and egg yolks contain small amount of vitamin D3. Sun dried mushrooms, because of the UV-B irradiation of the yeast sterol ergosterol, are considered as main sources of vitamin D2 [23]. Table 1-1 provides some of the nutritional vitamin D content of selected dietary products.

Table 1-1: Nutritional vitamin D content of selected dietary products.

Natural sources	Vitamin D content
Cod liver oil, 1 tablespoon	~ 400-1000 IU vitamin D3
Salmon, fresh wild, 3.5 oz	~ 600-1000 IU vitamin D3
Salmon, fresh farmed, 3.5 oz	~100- 250 IU vitamin D3, D2
Salmon, canned, 3.5 oz	~ 300- 600 IU vitamin D3
Sardines, canned, 3.5 oz	~ 300 IU vitamin D3
Mackerel, canned, 3.5 oz	~ 250 IU vitamin D3
Tuna, canned, 3.5 oz	~ 230 IU/ 3.6 oz of vitamin D3
Shiitake mushrooms, fresh, 3.5 oz	~ 100 IU/ 3.5 oz of vitamin D2
Shiitake mushrooms, sun-dried, 3.5 oz	~ 1600 IU/ 3.5 oz of vitamin D2
Egg yolk, 1	~ 20 IU/ yolk vitamin D3 or D2

To convert from International Unit (IU) to micrograms, divide by 40.Taken from [23].

1.1.2.3 Supplements

Most people are able to obtain the required vitamin D through a healthy balanced diet accompanied with summer sun exposure. However, the high-risk population such as pregnant women, under-five children and elderly people may need an extra supplement. Vitamin D supplementation remains the most effective way to boost vitamin D levels in vitamin D-deficient or high-risk individuals [25]. Both forms of vitamin D, ergocalciferol (vitamin D2) and cholecalciferol (vitamin D3) are available as supplements in the form of an isolated capsule (prepared with glycerol due to their solubility in fat) or as vitamin/multivitamin preparations, containing from 400 IU to 5000 IU vitamin D2/D3 [23].

Both vitamins D2 and D3 are considered as metabolically equal based on their ability to cure rickets. However, vitamin D2 has been found to be less potent, with shorter duration of action compared with vitamin D3 [26]. Vitamin D is available commercially as a supplement in a range of licensed medicines and multivitamin preparations (including fish oil products and calcium supplements) for infants and children. The bioavailability of these supplements depends on the type of vehicle providing the supplement and the individual's body mass index, whether they have malabsorption or are on medications such as anticonvulsants, glucocorticoids, antifungals such as ketoconazole, and medications for AIDS [27, 28]. Table 1-2 lists the vitamin D content of selected multivitamin preparation for children available in the UK market.

Table 1-2: Vitamin D content of selected children's supplements available in the UK.

Supplement	Vitamin D content (IU)*
Dalivit and Abidec: 0.3ml-0.6 ml	200- 400
Healthy start**	300
Well Baby, Well Kid, Well Teen	200- 340
Haliborange Kids, Haliborange Teen	100-200

When the product is labelled as vitamin D or calciferol, it usually contains vitamin D2; cholecalciferol or vitamin D3 indicates that the product contains vitamin D3.
* To convert from International Units (IU) to micrograms, divide by 40. ** Product is only available through NHS channels.

1.1.2.4 Vitamin D fortified food

The high calcium and phosphorus content of dairy products facilitates vitamin D absorption. Therefore, milk and other dairy products can be considered the ideal target for vitamin D fortification. However, fortified cows milk may not be suitable for people with milk allergies or lactose intolerance, or to prevent vitamin D deficiency among people with special dietary requirements [29]. Therefore, bread, orange juice and breakfast cereal may be suitable alternative options for fortification [30-32].

In the 1920s, ergosterol (pro-vitamin D2) was found in yeast and fungi, added to milk and then irradiated by UVB to stimulate antirachitic activity. By 1930s, an ergosterol analogue had become commercially available in the United States and was added directly to the milk without irradiation [33]. Currently, both the United States and Canada use the same dietary guidelines for optimal and safe levels of vitamin D intake, with fortification being optional in the US and mandatory for some nutrients in Canada. For instance, cheese, enriched rice, ready-to-eat cereals, orange juice and macaroni products are fortified in the US but not in Canada. Margarine is mandatorily fortified in Canada and optional in the US. Infant milk and evaporated milk are mandatorily fortified in both the United States and Canada [34].

In the UK, the Welfare Food Scheme was originally introduced in 1940 as a universal measure to ensure an adequate diet during World War II. Under that scheme, children were given cod liver oil and dried milk fortified with vitamin D [35]. However, since 1957 vitamin D fortification has been strictly regulated and even forbidden for some dairy products, due to outbreaks in the 1950s of hypercalcaemia, which is occasionally fatal and is linked to vitamin D intoxication [36]. The scheme was progressively changed to focus on people with special circumstances such as low income families and job seekers.
In 2002, the Department of Health proposed a Healthy Start Scheme for reforming the Welfare Food Scheme which came into practise in 2006. The Healthy Start Scheme targeted low income families, job seekers, pregnant and lactating mothers and under-18s and aimed to provide free milk, fruit, vegetables and vitamins for women, and children under 5 [37]. Under UK legislation, vitamin D fortification of infant formula and margarine is mandatory. Breakfast cereals, yoghurts, some processed milk and some powdered milk are optionally fortified by commercial manufacturers. Addition of vitamins and minerals to fortify any kind of unprocessed nutrients, vegetables, meat, poultry and fish is unlawful [38]. It has been found that the dietary vitamin D intake of preschool

children in the UK is very low and there is a need to reconsider milk fortification as a strategy to boost their intake [39].

1.1.3 Current intake recommendation for vitamin D

The recognized level for a safe intake of vitamin D is known as the tolerable upper intake level (UL), i.e. the amount of vitamin D that can be taken chronically without adverse health consequences. There is a number of guidelines which have been published over the last decade to evaluate, manage and prevent vitamin D deficiency [28, 40]. However, there is little consensus on the threshold which reflects biological vitamin D sufficiency at the end-organ level. In addition, there is substantial uncertainty about the extra-skeletal benefits of vitamin D supplementation, especially in those who have a relatively mild vitamin D deficiency [28, 41]. In 2011, the Endocrine Society established a guideline for clinicians for prevention and treatment of vitamin D deficiency with special emphasis on high-risk individuals. This guideline assumed minimal sun exposure and aimed to provide the essential requirement of vitamin D to maintain normal calcium homeostasis for all age groups [28]. Table 1-3 shows the Endocrine Society's Recommended Dietary Allowance (RDA) of vitamin D.

Table 1-3: Endocrine Society dietary reference intake for vitamin D for patients at risk.

	Recommended Dietary Allowance (Maintenance)		
Age	***LL (IU/day)**	****UL (IU/day)**	**Upper tolerable maintenance (IU/day)**
0-6 months	400	At least 1000	1000
6-12 months	400	At least 1000	1500
1-3 years	600	At least 1000	2500
4-8 years	600	At least 1000	3000
8-19 years	600	At least 1000	4000
19- <70 years	600-800	At least 1500-2000	4000
Pregnant & lactating	600	At least 1500-2000	4000

***LL: Lower limit. **UL: Upper limit of safe intake. Minimum needs to be maintained at a 25(OH)D level of above 75 nmol/l (30 ng/ml). Upper tolerable maintenance should not be exceeded without medical supervision [42].

1.1.4 Toxicity

Toxicity is extremely rare and may happen due to excessive vitamin D intake via food or supplements [43, 44]. The precise vitamin D metabolites contributing to intoxication is still a matter of debate [45]. Several theories have tried to explain the mechanisms by which hypervitaminosis D can lead to vitamin D intoxication. Most of the suggestions involve an increase in one of the vitamin D metabolites, 25(OH)D3, 24,25(OH)2D3, 25,26(OH)2D3, or 25(OH)D3-26,23-lactone. Another suggestion is either an increase or a reduction of free 1,25(OH)2D [45, 46]. During intoxication, increased levels of 25(OH)D will exceed the vitamin D binding protein (DBP) binding capacity and bind directly with VDR in target cells. Alternatively, the elevated 25(OH)D concentrations can compete and displace 1,25(OH)2D from its transport protein, DBP causing a release of 'free' 1,25(OH)2D which enters the target cells and stimulates gene expression. Vitamin D signal transduction procedure becomes overwhelmed, and fails to effectively clear the excessive metabolites [45, 47].

The outcome of vitamin D intoxication is hypercalcaemia as a result of increasing intestinal calcium absorption and the stimulation of bone resorption [48]. Initially, it can be presented as loss of appetite, fatigue, weight loss, excessive thirst, excessive urination, dehydration, constipation, muscle weakness, irritability and confusion. Prolonged vitamin D intoxication may lead to chronic toxicity that affects bones, tissues and other organs, which can result in calcification of soft tissue and bone resorption causing kidney and heart damage [45, 49]. Toxicity thresholds are a little less debated than sufficient or deficient levels [50]. The Institute of Medicine (IOM) suggested that 25(OH)D of lower than 125-150 nmol/l and up to 250 nmol/l is safe for both children and adults. Serum concentration of more than 375 nmol/l is dangerous and may lead to hypercalcaemia. It has been shown that adult 25(OH)D levels remain around 125-150 nmol/l after maximal sun exposure [45, 51, 52].

Due to the relatively short biological half-life of 1,25(OH)2D (1 to 2 days) there is no specific treatment for vitamin D toxicity. Stopping taking vitamin D supplements, and if necessary rehydrating using intravenous saline solution to enhance the glomerular filtration rate and calcium excretion should be sufficient. In some cases, corticosteroids or bisphosphonates may be used to enhance renal calcium reabsorption and inhibit the production and activity of 1,25(OH)2D [49, 53].

1.1.5 Assessment of vitamin D status

1.1.5.1 Dietary methods

Dietary intake assessment is a part of the process of defining nutritional status and may reflect the likelihood of developing vitamin D deficiency or to predict outcomes in clinical studies. Precise assessment of vitamin D intake is challenging to achieve and is very difficult to obtain due to the effect of multiple confounders. For example, vitamin D intake estimated from food and dietary supplement can only be compared on group mean rather than individuals data. Furthermore, the effect of sun exposure and lifestyle factors is very difficult to measure [54].

The most frequently used method for dietary assessment is the Food Frequency Questionnaires (FFQ), pre-printed lists of foods which could be either long or short and can be self-administered or performed by a professional. Other methods include 24-hour food recalls, three-day dietary records, observed food intake and weighted food inventories, in which subjects are taught to describe and measure all food prior to consumption and then to record any leftovers [55, 56]. For more detailed dietary information such as for assessment of vitamin D and calcium intake, using a FFQ designed to ask pointed questions specific to the nutrient and age group in a study could be an effective and cost-effective method to collect data [57]. Other methods such as weighted food inventories may be more accurate but require more time, trained staff and specific equipment, and are more expensive, time consuming and difficult to apply. For larger studies other inexpensive and quicker methods such as 24-hour recall and the three-day food record may be preferable. However, the last two methods may provide a better idea about the habitual intake rather than nutritional assessment as there may be an overestimation of the intake [58].

1.1.5.2 Biochemical measurement

Measurement of the major circulating form of vitamin D, 25(OH)D is currently considered as the best parameter that reflects the circulatory vitamin D status from either cutaneous synthesis or nutritional consumption [59]. However, 25(OH)D is an indicator of the supply rather than the biological function. Biologically the most active vitamin D metabolite, 1α,25(OH)D, is unsuitable for measuring the vitamin D status for many reasons. Firstly, in secondary hyperparathyroidism due to mild or moderate vitamin D deficiency, plasma 1α,25(OH)D is maintained at normal or slight elevated levels [60]. Secondly, plasma levels of 25(OH)D are nearly 100 times higher than for 1α,25(OH)2D, and thirdly, circulating 25(OH)D can be converted locally in almost all peripheral tissues to 1α,25(OH)2D in order to cover local needs [61-63].

There are many commercially available 25(OH)D assays used to assess vitamin D status such as high-pressure liquid chromatography (HPLC) mass spectrometry [64, 65], radio immunoassays, enzyme immunoassays, competitive protein binding assays, automated chemiluminescence protein binding assays, and chemiluminescence immunoassays (CLIA) [66]. Significant variability in results between assay methods and laboratories has been reported in the literature and the concern regarding performance and accuracy of 25(OH)D has risen resulting in the recommendation to adopt the same standards between laboratories to reduce the variability [67].

The International Vitamin D External Quality Assessment Scheme (DEQAS) was established in 1989 with the aim of ensuring the analytical reliability and performance of individual laboratories [68]. In this scheme, five samples of unprocessed human serum are distributed quarterly and the results analysed to give an All-Laboratory Trimmed Mean (ALTM) and standard deviation (SD). The participating laboratories require that 80% or more of their results are within 30% of the ALTM in order to be awarded a certificate for meeting the performance targets [68]. However, using the ALTM as the standard has its own issues. The ALTM has deviated from the gold standard, gas chromatography-mass spectrometry, due to the increasing number of methods used to measure 25(OH)D [69]. Additionally, the available results by DEQAS do not judge the absolute accuracy of the method but only the relative accuracy of laboratories using the same method [69]. Recently, the National Institute of Standards and Technology (NIST) has introduced a standard reference material in human serum (SRM 972) in order to improve accuracy and assay comparability [70].

1.1.6 Vitamin D metabolism

At the initial stages, both vitamin D2 and D3 undergo different pathways of metabolism before joining pathways to become biologically available, or activated. Dietary vitamin D gets combined into chylomicrons, subsequent to ingestion. Exposure to UV radiation (280-315 nm) converts 7-dehydrocholesterol in the skin into pre-vitamin D3, which is rapidly transformed to vitamin D3. Vitamin D3 in turn binds to the DBP and is transported to the liver. Both vitamin D molecules, externally ingested and photosynthesized, then travel via the lymphatic system and subsequently enter circulation [42]. Figure 1-2 shows that the vitamin D metabolic pathway including several steps of degradation and activation.

Vitamin D from the skin and diet has no biological activity without a two-step hydroxylation process in the liver and kidneys. The first step, in the liver, requires P450 enzymes such as CYP2R1 and CYP27A1 (25-hydroxylases) [71, 72]. In this step a hydroxyl group is attached at the carbon-25 atom (C-25) to form the major circulating form of 25-hydroxyvitamin D, 25(OH)D. 25-hydroxyvitamin D is also considered to be the most abundant circulating form of vitamin D, even though it is biologically inactive. 25-hydroxyvitamin D is also used to determine human vitamin D status. The role of the liver in this stage of hydroxylation was first reported in 1966 by DeLuca et al. [73]. The second step, in the kidneys, requires the P450 enzyme, CYP27B1 (1α-25 hydroxylase), to form the main active metabolite 1,25-hydroxyvitamin D (1,25(OH)2D or calcitriol). 1α-25 hydroxylase is considered the major rate-limiting step in 1,25(OH)2D synthesis. The renal hydroxylation reaction is tightly regulated by plasma parathyroid hormone (PTH) and serum calcium and phosphate levels. Fibroblast growth factor 23 (FGF-23), which is secreted mainly by osteocytes, can suppress the expression of calcium and phosphate co-transporters and inhibit 1,25(OH)2D synthesis [74, 75]. Another significant enzyme in vitamin D metabolism is 24-hydroxylase (CYP24A1). This enzyme has been identified in almost all cells and is highly unregulated by the kidneys. The main defined role of CYP24A1 is to convert 1,25(OH)D to its inactive metabolites, including 1,24,25-(OH)3D and calcitroic acid and converting 25(OH)D to 24,25-(OH)3D [76].

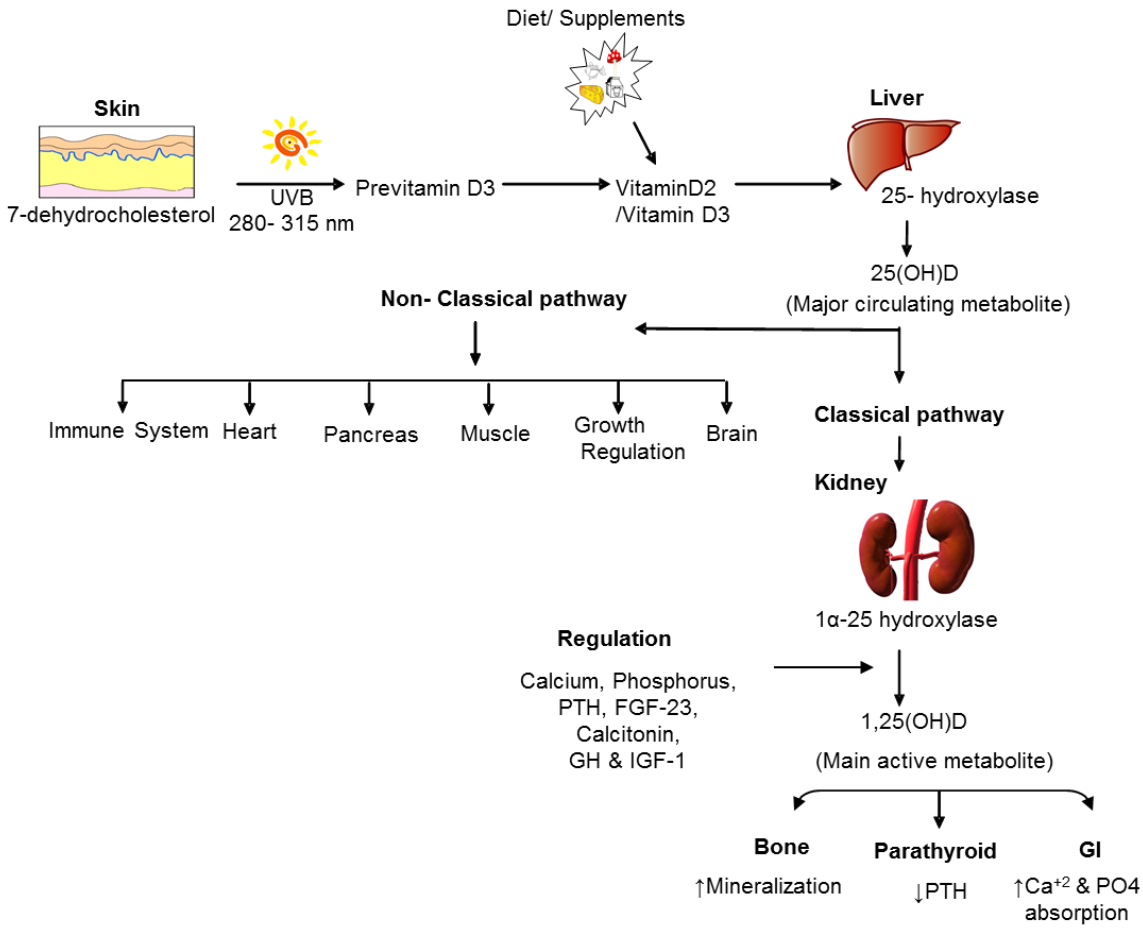


Figure 1-2: Photochemical synthesis of vitamin D and main target tissues.

PTH: parathyroid hormone. FGF-23: fibroblast growth factor-23. GH: growth hormone. IGF-1: insulin-like growth factor-1. GI: gastrointestinal.

1.1.7 Mechanism of action of vitamin D

Experimental evidence supports the hypothesis that 1,25(OH)2D can produce its effect at the target tissues by transmitting signals and regulating gene transcription that results in both genomic and non-genomic responses. Genomic responses are involved in the formation of a ligand-receptor complex with vitamin D nuclear receptors (VDR). Through non-genomic action, 1,25(OH)2D can generate a rapid cellular response, a mechanism which can be mediated at the plasma membrane involving a membranous localized vitamin D nuclear receptor (VDRmem) (see Figure 1-3).

1.1.7.1 Genomic action

The examination of the primary sequence of vitamin D receptor cDNAs showed that VDRs are members of the nuclear receptor superfamily (NHR) which consists of more than 350 different receptors. A common feature for all NHRs is that they act by regulating gene expression [77].

The genomic actions of the active form of 1,25(OH)2D is mediated through nuclear vitamin D receptors (VDRs). The VDRs act as transcriptional factors which modulate gene expression in a ligand-dependent way. Human VDR protein consists of three main regions: an N-terminal dual zinc finger domain that binds to DNA, a C-terminal domain that binds to 1,25(OH)2D, and an extensive unstructured region that links these two functional domains. The process of transcriptional activation of the target genes is initiated by ligand binding of 1,25(OH)2D to the VDR. This leads to conformational changes within the receptors that stimulate heterodimerization with retinoic X receptors (RXR) and high specificity and affinity binding to the vitamin D-responsive element (VDRE) in the DNA of the target gene [78]. In addition to VDR/RXR heterodimers, VDR can form homodimers that bind DNA and regulate target gene expression [79]. The classical role of 1,25(OH)2D is the control of serum calcium and phosphorus levels through its transcriptional regulation of calbindin D38d, a specific calcium–binding protein in the intestine, and osteocalcin, osteopontin, and receptor activator of the nuclear factor κβ ligand (RANKL) in bone [80].

The 1,25D-VDR complex plays an important role in the regulation of VDR, CYP27B1, and CYP24A1. As will be discussed later in this chapter, both CYP27B mRNA and VDRs have been identified in many other tissues such as β cells of the pancreas, skeletal muscles

and immune cells. They are suggested to play a critical role in many cellular functions such insulin secretion, insulin sensitivity and modulation of immune responses.

1.1.7.2 Non-genomic action

The active form of vitamin D, 1,25(OH)2D, can bind to putative cell membrane receptors 1,25(OH)2D (mVDR) and generates a non-genomic response [81]. This action is mediated through several signal transduction systems such as protein kinases and phospholipase C pathways and involving the rapid opening of voltage-gated Ca^{2+} and Cl^- channels [81]. Evidence has suggested that through the non-genomic response, 1,25(OH)2D may be able to modulate the genomic pathway via effects on the function of the VDR in the nucleus.

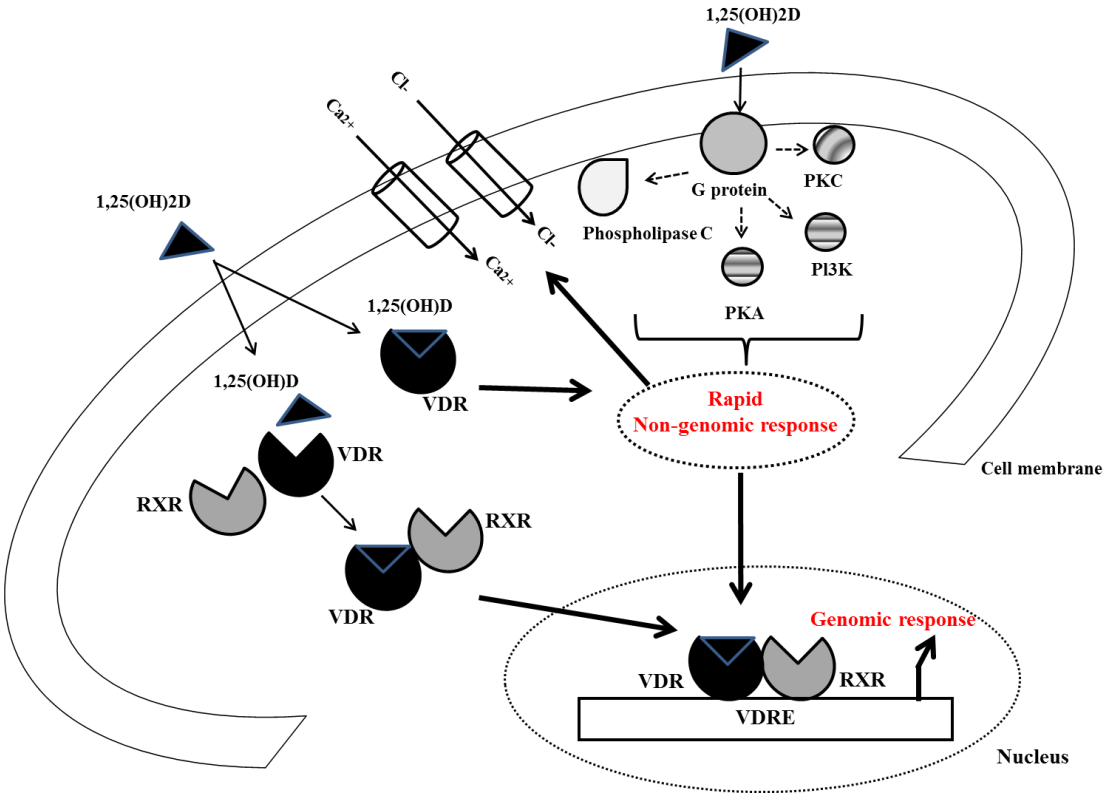


Figure 1-3: Mechanism of action of vitamin D: Up-regulation of genomic and non-genomic signalling via 1,25(OH)2D.

1,25(OH)2D interacts with the VDR in the nucleus to initiate the genomic response through regulation of gene transcription. It can also bind to the membranous VDR to generate the non-genomic responses. In the genomic pathway, association of the nuclear VDR with 1,25(OH)2D with the ligand will result in heterodimerization with RXR, leading to either suppression or stimulation of the transcription of genes subject to hormone regulation. Binding of 1,25(OH)2D to the plasma membrane pits-associated VDR results in the activation of second messenger systems to elicit short-term non-genomic responses. Probable consequences include opening of voltage-gated chloride or calcium channels or initiation of second messengers. Some of these mechanisms may lead to changes in gene expression through effects on the function of the VDR in the nucleus.

1.2 Epidemiology of vitamin D deficiency

1.2.1 Definition of vitamin D deficiency

Although there is no consensus on optimal levels of 25-hydroxyvitamin D, vitamin D deficiency has been defined by the Institute of Medicine (IOM) as a measured serum 25(OH)D of <50 nmol/l (20 ng/ml) and vitamin D insufficiency when the serum 25(OH)D is 52-73 nmol/l (21-29 ng/ml) [42]. There is a number of guidelines which have been published over the last decade to evaluate, manage and prevent vitamin D deficiency [40, 42]. Nevertheless, there is a little consensus on the threshold which reflects biological vitamin D sufficiency at the end-organ level. In addition, there is a substantial uncertainty about the extra-skeletal benefits of vitamin D supplementation especially in those who have relatively mild vitamin D deficiency [28, 40].

1.2.2 Risk factors

The risk factors of vitamin D deficiency can be categorized as environmental, personal and genetic risk factors.

1.2.2.1 Environmental risk factors

Although the relationship between vitamin D and sunlight is clearly identified, vitamin D deficiency still continues to exist even in those areas where there is plenty of sunshine. For example, poor vitamin D status has been reported among children, young adults and elderly across Europe, Canada, USA and Africa [23]. The higher prevalence of hypovitaminosis D during the winter and autumn has been reported and attributed to a decrease in outdoor activities and an increase in wearing heavy winter clothing, thus decreasing the surface area exposed to sunlight [20, 82]. Additionally, the significant impact of seasonal and latitudinal changes on vitamin D status is mainly related to both the quantity and quality of solar radiation reaching the globe's surface. The solar zenith angle, defined as the angle between the sun and the vertical (zenith sky), increases during winter time and the amount of solar ultraviolet radiation that reaches the global surface is markedly reduced which significantly affects vitamin D3 skin production [23].

In addition to season, the duration of vitamin D production depends on many other environmental parameters such as time of day, total ozone, clouds, aerosols, surface reflectivity and altitude. For instance, in winter, heavy cloudy conditions and the ozone layer can absorb or reduce the necessary UVB rays to induce the photosynthesis of vitamin D [83].

1.2.2.2 Personal risk factors

There are several factors which may contribute to an individual's risk for vitamin D deficiency through their diet and sun exposure and including personal, social and cultural factors such as age, gender, obesity, ethnicity, use of sun screen, cultural background and working patterns. Analysis of NHANES 2001–2004, showed that older, female, obese, non-white individuals who drank milk less frequent and had a sedentary lifestyle were more likely to be vitamin deficient with 25(OH)D levels of lower than 37.5 nmol/l [84].

The risk of vitamin D deficiency increases with age due to a reduced capacity to synthesize vitamin D. For instance, a 70-year-old exposed to a similar quantity of sunlight as a person in their twenties will make only around 25% of the vitamin D3 that the 20-year-old can make [24]. There are many reasons for this, including the decrease in cutaneous production of vitamin D due to a decline in 7-dehydrocholesterol, impaired intestinal absorption, and impaired hydroxylation in the liver and kidney, in addition to a lack of sunlight exposure.

There is an inverse relationship between obesity and vitamin D which has been suggested as one of the predisposing factors for the development of metabolic syndrome in obese people [85, 86]. Many mechanisms have been suggested which are related to the lipophilic nature of vitamin D which results in it being sequestered deeply in the body fat tissue, in addition to its interfering with several metabolic processes that affect glucose and insulin metabolism in obese individuals [86].

People with dark skin synthesize markedly less vitamin D than those with a light or fair complexion. This is due to the high skin content of melanin pigment which serves as a barrier that prevents UVB radiation from penetrating the skin, thereby decreasing the efficiency of photosynthesis of precholecalciferol [87]. People with dark skin require more time for sun exposure than people with light skin to make sufficient vitamin D [88]. However, the increase in serum 25(OH)D after vitamin D supplementation is similar for

both groups [89]. Sunscreen also can absorb UVB depending on the sunscreen's sun protection factor [90].

Vitamin D deficiency is believed to be significantly affected by working patterns as certain professions require many more hours to be spent indoors and allow less time for sun exposure than those who work outdoors [91]. For example, osteomalacia has been reported in night nurses who are regularly employed on the night shift for many years [92] and higher vitamin D levels have been recorded in lifeguards compared with submarine sailors [93].

Vitamin D deficiency has been correlated with specific cultural behaviours such as full-coverage clothes or prolonged breast feeding. Therefore, vitamin D deficiency is very common among black African, South Asian and Middle Eastern immigrants, and the trend is more significant in women than men [5, 94]. In many areas across North Africa and Asia, vitamin D deficiency has been reported as a major public health issue [95]. Breast-fed babies whose mothers are vitamin D deficient and those who are breast-fed for a prolonged period are at a high risk for developing vitamin D deficiency. The reason for this is the 25(OH)D passes very poorly and 1,25(OH)2D does not appear to pass at all into breast milk. It has been estimated that breast milk can provide an average of 22 IU/l (from 15 to 50 IU/l) in a vitamin D sufficient mother [96]. Therefore, exclusive breastfeeding without sun exposure would provide only 11 to 38 IU/day in babies who consume around 750 ml milk per day and therefore breast milk cannot provide the amount needed to meet the recommended dietary vitamin D intake [97].

1.2.2.3 Genetic risk factors

Researchers suggest a strong genetic influence on vitamin D status. Despite abundant sun exposure in some countries, vitamin D deficiency is still common even among healthy adults, regardless of gender or lifestyle [98, 99]. Genetic predisposition has been considered as an additional risk factor. A study conducted in the USA provided a possible explanation and showed that South Asian immigrants have a significant increase in 25(OH)D-24-hydroxylase activity compared with Caucasians [94]. Their genetic predisposition may facilitate the occurrence of symptomatic vitamin D deficiency among Asian, Middle Eastern and African immigrants, particularly when there are other risk factors involved, including early childhood, adolescence, pregnancy and lactation.

Analysis of the Framingham Offspring Study showed that the variation in circulatory vitamin D levels may be attributed to genetic susceptibility (heritability estimate = 28.8%, $p < 0.01$) [100]. A UK twin study suggested that around 60-65% of hormones regulating calcium homeostasis, PTH and vitamin D are under strict genatic control [101]. Several polymorphisms in the VDR gene have been investigated, and reviewed, with several candidate genes being suggested to contribute to this genetic effect [102]. For example, high winter vitamin D status in BB homozygotes (BsmI-polymorphism of the VDR gene) compared with other genotypes, has been reported in a sample of 93 Finnish females [103]

1.2.3 Vitamin D deficiency in the United Kingdom

In the UK, several epidemiological and regional monitoring studies have provided strong evidence that lower vitamin D status is highly prevalent and is becoming an alarming public health issue, particularly among ethnic minorities [104]. A large-scaled dataset which involved 7437 British Caucasians who were born in England, Scotland, or Wales during the same week in 1958 showed that circulatory 25(OH)D levels follow a yearly cycle pattern that is highly affected by season and geographical location. The lowest recorded levels for circulatory 25(OH)D were <25, <40, and <75 nmol/l during late winter and spring in 15.5%, 46.6%, and 87.1% of study subjects respectively [82]. In Scotland, a recent survey conducted by the Food Standards Agency and the Scottish Government between 2010-2013 included around 1400 blood samples showed that the average vitamin D level of the Scottish population was 37.5nmol/l. This provided further evidence for the high prevalence of hypovitaminosis D among the Scottish population of various background [105].

During 2000, a West Midlands prospective one year survey of 119 consultant paediatricians targeting under-5-year-old children identified 24 new cases of profound vitamin D deficiency [106]. Between May 2001 and July 2002, another group studied children from ethnic minority backgrounds who were attended a child health clinic in Central Manchester for immunisation, identified two of 124 ethnic minority children with clinical rickets with a prevalence of 1.6% [6]. However, there was a question about the validity of these surveys and it is more likely that the actual number of cases was underestimated. A further survey conducted in three hospitals in Birmingham between 2001 and 2003 reported 65 new cases of profound vitamin D deficiency with 25(OH)D levels of< 25 nmol/l including 17 cases of hypocalcaemic seizures [107].

The increase in the occurrence of vitamin D deficiency among the population in Scotland has been attributed mainly to its northerly location, insufficient dietary intake and population mix. Scotland's latitude position at 55° to 59° north explains the lack of sunlight for vitamin D synthesis. In addition, an indoor lifestyle, obesity, high alcohol intake, and poor diet, which are common among the Scottish population, further adversely affect vitamin D status [3, 4]. Since the identification of rickets and osteomalacia among the Asian community in the Glasgow in 1962, evidence has shown that despite a gradual decline in the prevalence of vitamin D deficiency, the problem was not eliminated completely [1, 108]. As a result, the Glasgow Stop Rickets campaign was held from 1979-1981 with the aim of introducing a free prophylactic supplementation (400 IU) for Asian school-age children in need. Post-campaign assessment showed that the un-supplemented group had four times higher incidence of rickets compared with the regularly supplemented children. However, there was also a lack of organization and effectiveness in this campaign [109, 110].

The impact of geographic location, decreased sun exposure and low dietary calcium on vitamin D status become evident after the development of a multivariate logistic regression model analysing the risk factors [111, 112]. Dietary pattern and the degree of lacto-vegetarianism (no meat, fish and egg intake) were significant independent risk factors for nutritional rickets and osteomalacia among Asian women [113]. Therefore, further attempts to provide more effective preventive measures were represented in the Healthy Start scheme which was launched in November 2006 to replace the Food Welfare scheme. Two years after the introduction of this new scheme, the number of cases of rickets presenting at the Royal Hospital for Sick Children (RHSC) remained unchanged at under 30 per year [5]. It remains unclear whether this new scheme is effective at reducing the incidence of rickets.

In Glasgow, a retrospective survey of suspected vitamin D deficiency cases which were identified through clinicians' own records, clinical biochemistry and diagnostic imaging records and carried out between 2002 and 2008 at RHSC showed the incidence increasing over time to reach a peak in 2008 with 42 cases. This report documented 160 cases with various clinical features providing an average annual incidence of 23 cases per year [5]. It is important to note that there are other cases of symptomatic vitamin D deficiency which were not treated in the metabolic bone clinic at RHSC as well as other suspected cases where the diagnosis was not completely clear which were not included in this survey. Therefore, the actual incidence has still not yet been determined and this survey provided

further evidence that the prevalence of vitamin D deficiency in the West of Scotland seems to be increasing.

The National Diet and Nutrition Survey (NDNS) (2008/09 – 2011/12), jointly funded by Public Health England (PHE), an executive agency of the Department of Health, and the UK Food Standards Agency (FSA), was originally created in 1992 as a series of cross-sectional age groups representing, pre-school children (aged 1.5- 4.5 years); young people (aged 4-18 years); adults (aged 19-64 years); and older adults (aged 65 years and over). The main aim of the NDNS was to collect information about dietary habits and nutritional status of adults and children in the UK. Post April 2008, the NDNS has been a rolling programme (RP), designed to collect data from a representative sample of 1,000 people per year (500 children, 500 adults), aged 1.5 years and older, living in private households. The dietary assessment and nutritional data in the survey was collected using face-to-face interviews, unweighted four-day diaries, and blood and urine samples [114].

Data from years one to four of the NDNS rolling programme (2008/09 – 2011/12) showed that the proportion of children with a serum 25(OH)D concentration lower than 25 nmol/l at the time of venepuncture, ranged from 7.5% for children aged 1.5-3 years, to 24.4% for girls aged 11-18 years. When subdivided by season, the survey revealed that more than 40 % of children aged 11-18 years had a serum 25 (OH)D concentration below 25 nmol/l in the winter months (January to March). This was in contrast to the summer months (July to September), when the proportion ranged from 1.7% for children aged 4-10 years, to 13.4% for children aged 11-18 years.

The result from the NDNS has also shown that decreased dietary intake of vitamin D among UK population was associated with an increased risk of vitamin D deficiency. The data revealed that the average vitamin D intake were 2.2 µg/day (88 IU) for boys aged 4-18 years, 1.9µg/day (76 IU) for girls aged 4-18 years and 1.9 µg/day (76 IU) for children aged 1.5-3 years. A slightly higher total dietary intake (food plus supplements) was seen among supplement users: 2.7 µg/day (108 IU) for boys aged 4-18 years, and 2.3 µg/day (92 IU) for girls aged 4-18 years and children aged 1.5-3 years.

The Reference Nutrient Intake (RNI) can be defined as the amount of a nutrient that is sufficient to meet the needs of almost the entire population (97.5%). In the UK, there are no RNIs set for those aged 4–64 years, and it is only expressed for populations aged between 1.5-3 and over 65 years (vitamin D ' RNIs for children aged 1.5-3 years is 7

µg/day (280 IU), and 10 µg/day (400 IU) for adults aged 65 years and over). Using these values, data from the NDNS showed that the mean intake of vitamin D from food sources was 27% of the RNI for children aged 1.5-3 years, compared with 29% for adults aged 65 years and over. With and the contribution of vitamin D-containing supplements, the mean vitamin D intake increased to 32% and 51% of RNI for children aged 1.5-3 years, and adults older than 65 years, respectively.

With the exception of children aged 1.5-3 years, meat and meat products have been indicated as a major contributor to vitamin D intake for all age groups (23-35% of vitamin D intake). For children aged 1.5-3 years, milk and milk products were the major contributors to vitamin D intake (providing 24% of the intake). Vitamin D-fortified fat spreads provided 19-21% of vitamin D intake for all age groups. Cereals and cereal based products, including pasta, rice, pizza, buns, cakes, pastries, and fruit pies provided 17-20% of the vitamin D intake for children aged 4-18 years and 14% for children aged 1.3-3 years. Fish and fish dishes (mainly oily fish) contributed 22% of the vitamin D intake, compared to 8-9% in children [114].

In summary, rickets and osteomalacia are associated with morbidity in children and can be prevented. Profound vitamin D deficiency, presenting mainly as rickets, is a real problem in Scotland and many other parts of the UK. The occurrence of vitamin D deficiency is becoming increasingly common among the UK population. The results from emerging data highlighted that the recommendations of the Department of Health for vitamin D supplementation may be ineffective or are just being ignored, due to changes in levels of awareness of referring clinicians. There is a need for more effective public health measures which particularly target the at-risk population or a surveillance system that monitors incidence uniformly across Scotland.

1.2.4 Screening for vitamin D deficiency

The Endocrine Society and the U.S. Preventive Services Task Force (USPSTF) have suggested that there is not sufficient evidence to prove that vitamin D screening is beneficial in routine clinical practise. Therefore, there is no need for implementation of a universal screening for serum vitamin D deficiency among the general population [28, 41, 42, 115]. Regarding the at-risk population, such as people with dark skin, malabsorption, decreased sun exposure or pregnant women, both the Endocrine Society and the USPSTF indicated that there is a lack of evidence to support the direct advantage of screening for vitamin D deficiency in adults. Additionally, there are no available studies evaluating the benefits and drawbacks of screening [115]. There is general agreement for using supplementation for the purpose of preventing skeletal consequences of vitamin D deficiency among high-risk adults. The USPSTF recommends prescribing vitamin D supplementation to prevent other diseases such as cardiovascular or immune disorders, or for improving quality of life. However, the Endocrine Society did not advocate this due to a lack of sufficient evidence [41, 42, 115].

Screening for vitamin D deficiency is recommended for high-risk children presenting with non-specific symptoms such as poor growth and irritability, children with recurrent fractures or those with chronic diseases such as malabsorption [41, 42]. A suggested screening method for vitamin D deficiency rickets is serum alkaline phosphatase (ALP) [116]. If the ALP is found to be elevated, then follow-up measurements of serum 25(OH)D, calcium, phosphorus, and PTH should be carried out, along with radiologic examination. However, there are arguments against this screening tool as not all children with subclinical vitamin D deficiency rickets have raised ALP levels [117].

1.3 Skeletal effect of vitamin D deficiency

The relationship between vitamin D and bone health has been well documented and involves the role of vitamin D in maintaining calcium and phosphorus homeostasis and supporting bone mineralization. The conventional skeletal consequences of prolonged vitamin D deficiency are: rickets among infants, young children and adolescents [118], osteomalacia among adults [1, 119] and osteoporosis in older adults [120].

1.3.1 Structural anatomy of bone

The function of the skeleton is not just as a supporter and a protector for the vital organs but also includes other essential roles such as blood cell production, participation in acid–base balance, acting as a reservoir for minerals needed for normal calcium homeostasis and acting as a pool for growth factors and cytokines [121]. Bone is a functional unit of the skeleton, defined as a metabolically active, highly vascularised and mineralised connective tissue which undergoes regular remodelling or skeletal renewing process in order to produce mechanically stronger bone which is able to adapt to biomechanical changes [122]. Generally, bones can be classified according to their shape into: flat bones such as the skull, scapula and ribs; long bones such as the ulna, radius, humerus and femur; short bones such as carpal bones (wrist); sesamoid bones such as the patella; sutural bones including the irregularly shaped bone within the skull, and, finally, irregular bones such as vertebrae.

The anatomical structure of long bones involves three parts. The peripheral parts comprise both the epiphysis and metaphysis, which are separated by a developmental growth plate or an epiphyseal cartilage plate. The mid shaft of long bones is called the diaphysis. The wall of the diaphysis is made of three layers. The outer thinner layer is called the periosteum. Periosteum is a sheet of connective tissue covering the bone except at the site of articulation with another bone where the bone is instead covered by articular cartilage. The middle layer of the diaphysis is called cortical (compact) bone. The inner layer is known as the medullary cavity or marrow cavity, which contains bone marrow and adipose tissue. The epiphysis consists of mainly spongy bone in addition to the thin layer of cortex (see Figure 1-4) [121].

Based on its gross appearance, two basic structural units of bone are distinguished; compact and spongy (cancellous) bones.

Compact bone accounts for 80% of the total bone mass and contains few cells and blood vessels. The main functional microscopic unit of the compact bone is the osteon or Haversian system. Each osteon comprises of a central canal (Haversian canal) surrounded by osteocytes and containing the blood supply to the osteon. Another canal known as Volkmann's canal runs perpendicular to the surface of the bone and penetrates deeper to the cortical bone and connects with those of the medullary cavity. The Haversian canal runs parallel to the bone surface, surrounded by the concentric lamellae forming a target-like pattern. Between the lamellae, there are small spaces, or lacunae, filled with extracellular fluid containing osteocytes. Finally, radiating through the lamellae are small microscopic canals known as canaliculi [121].

The second structural unit of bone is the spongy (cancellous) bone which accounts for 20% of the human skeleton weight. In long bone, spongy bone constitutes the interior of the epiphyses. The lamellae in the spongy bone are not organized in the osteon, but instead arranged in an irregular meshwork called the trabeculae. The macroscopic spaces between the trabeculae contain the red bone marrow, which is responsible for blood cell manufacture. In early childhood, most of the skeleton is composed of cancellous bone, and all the bone marrow is red bone marrow. As children grow up, the cancellous bone is slowly converted into cortical bone in the long bones and the red bone marrow is slowly converted to yellow bone marrow [121].

The outer cover of the bone called periosteum. This consists of an inner layer of osteoblasts which are in direct contact with the bone and being more prominent during fetal life and early childhood. The outer layer of the periosteum is abundant with blood vessels which penetrate the bone [121].

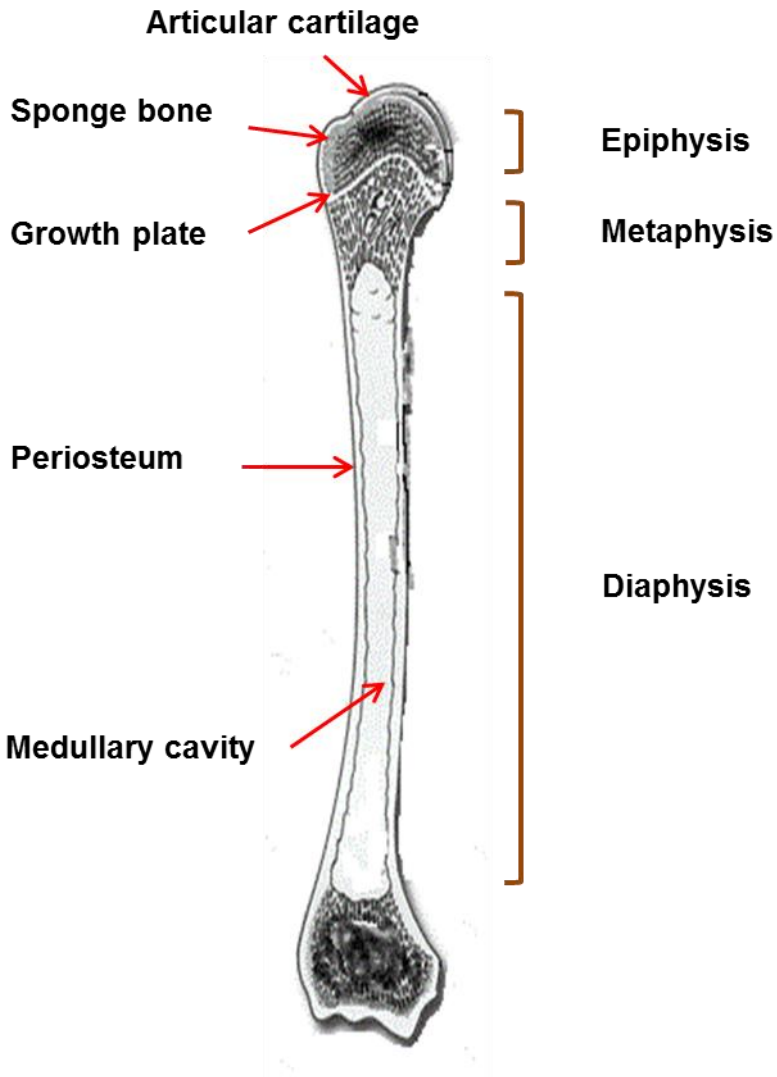


Figure 1-4: Anatomical structure of long bone.

Long bones consist of a long shaft (diaphysis) and two peripheral sections, epiphysis and metaphysis. The outer surface of the bone is covered by periosteum except at the articulation surface, at the epiphysis (covered by articular cartilage).

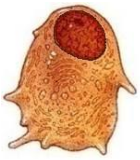
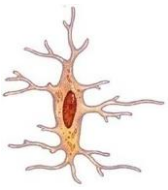
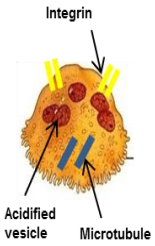
1.3.2 Bone matrix

Bone architecture is composed of two elements: around 90% is bone matrix and 10% are cellular elements. Bone matrix is further divided into organic (35%) and inorganic (65%). Around 90% of the organic component is a non-mineral collagen tissue (osteoid) and 10% is non-collagenous proteins. Type I collagen is the most abundant component of the osteoid, which is released from fibroblasts and osteoblasts. The rest of the organic matrix components are non-collagenous proteins include osteocalcin, osteopontin and osteonectin which are produced mostly by osteoblasts. Osteocalcin and osteopontin production is a unique feature of the mineralized bone and their production are further stimulated by 1,25(OH)2D [123].

1.3.3 Cellular elements of bones (Bone cells)

The actively growing bone contains cells derived from two lineages; the osteoblast lineage (osteoblast and osteocytes) and the osteoclast lineage (osteoclast). Each of the cellular elements of bone has a specific function. For example, bone tissue is formed by osteoblasts, maintained by osteocytes and removed by osteoclasts (Table 1-4) [123].

Table 1-4: Main types of bone cells, their locations and functions.

Bone cells	Origin & regulation	Location	Function
Osteoblasts 	Mononuclear derived mainly from mesenchymal stem cells. Have receptors for PTH, IGF-1 and vitamin D. Osteoblast differentiation to osteocyte requires an activation of key transcription factor Runx2 which is counteracted by another factor called Osterix (Osx) [124]. Fate; osteocytes, lining cells and apoptosis.	Bone surfaces. Particularly active at places of bone growth and repair.	**Bone formation** Through synthesis of organic bone matrix (osteoid). Control bone matrix mineralization by secretion of type 1 collagen and other non-collagenous matrix proteins such as; osteocalcin osteopontin and sialoprotein Have surface protein alkaline phosphatase which is released in the process of bone formation.
Osteocytes 	Mature bone cells derived from osteoblasts; make up 90-95% of all bone cells in adults [125]. Contain vitamin D & PTH receptors. CD44 and DMP1 molecules are the main positive regulators. Sclerostin is a main negative regulator [126, 127]. Major source of FGF23 [128].	Trapped within in bone matrix.	**Preserve mineral concentration of the matrix** Can express osteocalcin, osteonectin & osteopontin, but show little alkaline phosphatase activity. Provide a structure support for bone via cytoplasmic connections with neighboring cells. **Stimulate bone resorption**
Osteoclasts 	Giant multinucleated bone lining cells. Originated from bone marrow derived monocyte. M–CSF & RANKL (secreted and expressed by osteoblasts) molecules are critical for osteoclastogenesis [129, 130]. Contain receptors for calcitonin, PTH and vitamin D; up regulate M-CSF activity. Cytokines such as IL-6, TNF-α and IFN-γ also increase the osteoclasts activity. Fate; apoptosis.	Bone surface. Particularly active at places of bone growth and repair.	**Bone resorption** **Firstly,** osteoclasts attach to the bone through integrin, making a sealing zone, into which is secreted hydrochloric acid & acidic protease such as cathepsin K & MMP9. **Secondly,** hydrochloric acid is produced by the actions of H+ ATPase within acidified vesicles. **Thirdly,** integrin attachment to the bone results in signals that target acidified vesicles leading to secretion of the acid and proteases and finally bone resorption and degradation of exposed organic matrix.

*DMP 1, Dentin matrix protein; FGF23, fibroblast growth factor-23; IGF-1, insulin-like growth factor-1; RANKL, Receptor activator of nuclear factor kappa B ligand; M–CSF, macrophage colony stimulating factor; IFN-γ, interferon gamma; TFN-α, tumor necrosis factor alpha; IL-6, interleukin 6; MMP 9, matrix metalloproteinase. (summarised from [123], unless otherwise stated).

1.3.4 Bone remodelling

Bone remodelling is a continuous process of bone recycling and renewing which takes place throughout human life and involves two counteracting processes, bone formation and bone resorption. The balancing collaboration between bone resorption cells and bone formation cells is known as a basic multicellular unit. Osteoporosis or bone loss is a consequence of increased osteoclasts function and/or reduced osteoblasts activity. In contrast, conditions such as osteopetrosis may occur due to osteoclastic failure to resorb bone leading to an increase in the bone mass [131, 132]. Bone remodelling is divided into five consecutive phases: resting, activation, resorption, reversal and formation (see Figure 1-5). Remodelling starts with an activation phase at the resting surface by stimulation of osteoclastogenesis (generation of new osteoclasts). The resorption phase is initiated after osteoclasts are formed via acidification and destruction of bone matrix. In the formation phase, the osteoblasts are recruited via several growth factors which are produced during the process of resorption. Once an osteoblast stops secreting bone matrix, it has one of these potential fates: it may change into a quiescent cell on the bone surface, called a lining cell, it can undergo apoptosis, or it can become embedded in osteoid and differentiate into an osteocyte [133].

1.3.4.1 Role of vitamin D in bone remodelling

In bone, the active form of vitamin D, 1,25(OH) is critical for mineralization either via its direct action on bone cells, or indirectly via its role in regulation of the intestinal and renal calcium absorption [134]. Experimental studies have shown that the major bone cells, osteoblasts, osteoclasts and osteocytes are able to express the VDR and CYP27B receptor, and can locally synthesize 1,25(OH)2D from 25(OH)D [135, 136]. Moreover, it has been shown that 1,25(OH)2D can act in concert with PTH leading to inhibition of the rate of bone resorption and increasing the rate of bone formation, thereby promoting osteoblast proliferation and maturation [135-137]. However, in case of low serum calcium levels, 1,25(OH) and PTH can promote osteoclastogenesis and bone resorption. Furthermore, in the literature there are some inconsistencies regarding the effects of vitamin D on bone differentiation and mineralization. For instance, transgenic mice that overexpressed the VDR mature cells of the osteoblastic bone-forming cells showed an increase in bone formation rate as demonstrated by an increase in the trabecular thickness and bone strength [137]. Another experiment on VDR knockout mice also showed a similar observation, including an increase in the trabecular bone volume and mineralization, in addition to the

number of osteoblasts [138]. Although both of the studies support the direct role of vitamin D on bone formation, the first indicated that vitamin D is an anabolic factor whereas the second study showed of vitamin D is not required for normal skeletal formation.

The content of the extracellular matrix (ECM) is an essential determinant of mineralization. Several proteins have been found to be regulated by 1,25(OH)2D and ECM mineral contents such as osteocalcin [139], osteopontin [140] and bone sialoprotein [141]. Another important element for mineralization is matrix vesicles which are secreted into the ECM from osteoblasts and contain a large quantity of alkaline phosphatase (ALP) [142]. It has been shown that treatment of pre-osteoblastic cell lines with 1,25(OH)2D can increase the production of mature matrix vesicles prior to mineralization leading to earlier onset and higher rates of mineralization, with no change in gene expression of the extracellular matrix [143]. These studies have suggested that vitamin D plays an important role in providing the extracellular matrix with the environment necessary for mineralization rather than being a direct participant in the process of mineral deposition.

Osteocytes have been suggested as a regulator of bone mineralization via secretion of local regulatory factors that control the activity of both osteoclasts and osteoblasts such as Dentin Matrix Protein (DMP1) [127]. In addition to being critical mediators of bone metabolism by its secretion to fibroblast growth factor (FGF23), the influence of osteocytes extends to other tissues and organs such as kidneys and muscles, where they can serve as endocrine cells. Fibroblast Growth Factor is a bone-derived hormone known as a key regulator of phosphate homeostasis. Both osteocytes and osteoblasts have been suggested as the main source of circulatory FGF23, and locally produced vitamin D has been proposed as an important regulator of FGF23 production [144, 145]. Fibroblast Growth Factor can inhibit phosphate reabsorption from renal tubular and suppress CYB27B1 production, leading to a decrease in the circulating 1,25(OH)2D level in addition to stimulating CYB24 catabolism of 1,25(OH)2D. Additionally, FGF23 has also been linked to several inherited and/or acquired hypophosphatemic disorders such as autosomal dominant hypophosphatemic rickets and tumour induced osteomalacia [146]. Both 1,25(OH)2D and PTH can act on immature osteoblasts and osteoblast lining via VDR and PTH receptor type 1(PTH1R) leading to up-regulation of the expression of the RANKL and decreases the expression of its antagonist, osteoprotegerin (OPG). However, 1,25(OH)2D appears to have a regulatory effect on bone remodelling via stimulating

mature osteoblasts to secrete more OPG and less RANKL, thus reducing the rate of bone resorption [147-149].

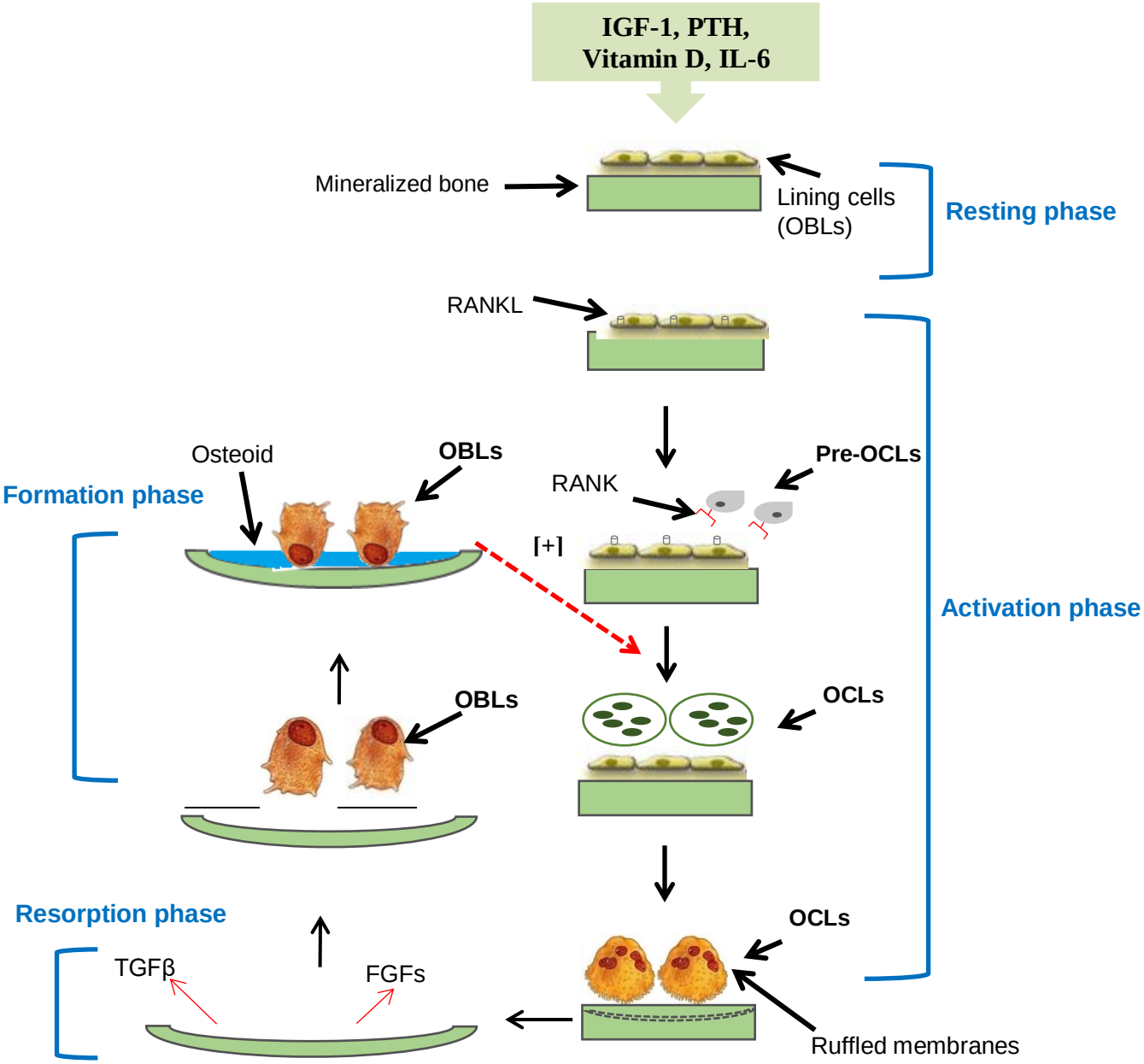


Figure 1-5: Schematic diagram summarizes the main steps of the bone remodelling process.

Bone resorption can be triggered by stimulation of the lining cells through several signals which are either related to mechanical stress or related to the bone microenvironment such as insulin-like growth factors1 (IGF1), parathyroid hormone (PTH) or interleukin-6 (IL-6). These signals stimulate osteoclasts (OCLs) formation by inducing of RANK/RANKL expression, on pre-OCLs and osteoblasts (OBLs). Additionally, OBLs signals [+] can also promote the differentiation and maturation of the osteoclast precursors (Pre-OCLs) to activated osteoclasts (OCLs). Activated osteoclasts express the resorption pits. The resorption pits dissolve the inorganic matrix and lysosomal enzymes and digest the exposed type-1 collagen. This also leads to the release of specific degradation products such as fibroblast growth factors (FGFs) and transforming growth factors-β (TGF-β) which are accountable for recruitment of osteoblasts in the resorbed area. Once recruited, osteoblasts generate new bone matrix, initially not mineralized known as osteoid, and then they stimulate its mineralization, thus completing the bone remodelling process.

1.3.4.2 Markers of bone remodelling

Biomarkers of bone metabolism are bone tissue proteins, or their derivatives or enzymes. These analytics are secreted from bone cells during various stages of bone remodelling and can be measured in blood or urine. There are several combinations of formation and resorption markers that reflect the metabolic activity of osteoblasts or osteoclasts, respectively. Measuring the rate of bone remodelling using bone remodelling markers has the advantages of being relatively cheap and non-invasive for sample collection compared with traditional methods such as bone biopsy [150].

1.3.4.2.1 Markers of bone formation

Bone formation markers are either by-products of active osteoblasts or collagen synthesis which are produced during the various stages of their development. These biomarkers include alkaline phosphatase (ALP) and osteocalcin (OCN), which are produced by osteoblasts. The N-terminal and C-terminal extension peptides of procollagen are released during collagen synthesis.

1.3.4.2.1.1 Alkaline Phosphatase (ALP)

Alkaline phosphatase is localized on the outer surface of the osteoblasts and released into circulation during the process of bone remodelling [150]. Although the exact function of this enzyme is still unclear, evidence suggested it has a role in osteoid formation and mineralisation [151]. Total serum alkaline phosphatase (TAP) consists of several isoforms which are able to express in several tissues such as intestine, liver, bone, kidney, and placenta. Three of these genes are tissue-specific, and encode the intestinal, placental and germ-line enzymes. The non-tissue-specific gene encodes bone, liver, and kidney ALP [152-154]. Therefore, measurement of serum ALP, as a marker of bone remodelling can lack sensitivity and specificity particularly in adults as a bone-specific isoenzyme constitutes only up to 50% of the total ALP.

In children and adolescents the most dominant isoform is bone-specific isoenzyme which predominates due to active skeletal development (up to 90%). Some studies have suggested the utility of serum ALP as a specific screening test to identify vitamin D deficiency in children. However, others have indicated that ALP can be used only to assess the progress of vitamin D status and to further confirm the diagnosis. The reason for this is that measurement of serum ALP may not detect the subclinical cases of vitamin

D deficiency or vitamin D deficiency in a carrier of hypophosphatasia. Immunoassays are simple and rapid methods to measure the bone-specific isoform of ALP [155].

1.3.4.2.1.2 Osteocalcin or bone gamma-carboxyglutamic acid (Gla)

Osteocalcin is a protein which is synthesized and secreted by differentiated osteoblasts constituting around 20% of the non-collagenous part of the bone matrix [156]. Osteocalcin is mainly known as a marker of bone formation [157]. However, it has been shown that it could participate in the process of osteoblast-osteoclast interaction and bone resorption by facilitating the process of recruitment and differentiation of circulating monocytes and osteoclast precursors [156, 158]. Osteocalcin consists of two forms: fully-carboxylated osteocalcin and under- carboxylated osteocalcin. The first form (carboxylated osteocalcin) is characterised by high calcium affinity and hydroxyapatite binding properties due to the presence of three γ-carboxy residues at positions 17, 21 and 24 in glutamic acid (Gla), which allow osteocalcin deposition on mineralized bone matrix. On the other hand, the lower affinity of the under- carboxylated part of osteocalcin to hydroxyapatite to be responsible for its easy release into circulation [155, 159].

Experimental studies provided evidence for the extra-skeletal role of osteocalcin. For example, activation of under-carboxylated osteocalcin in the circulation has been shown to promote insulin synthesis and to improve insulin resistance in mice. Additionally, the under-carboxylated osteocalcin stimulates β-cell proliferation and adiponectin in adipocytes [160]. In mice, daily injections of osteocalcin can improve glucose homeostasis and prevent the development of type 2 diabetes; however, human interventional studies still show inconclusive results [161, 162]. Both the carboxylated and the under-carboxylated forms are measurable in the circulation in addition to the total osteocalcin that is usually used as a marker of bone formation. Due to the presence of multiple isoforms and the fact that it easily undergoes degradation in circulation, the utility of serum osteocalcin as a marker of bone formation is very limited and there is high variability between the available assays in the detection of different fragments of osteocalcin [155].

1.3.4.2.1.3 Procollagen type-1 propetides ; C-terminal (PICP) and N-terminal (PINP) propeptides

In bone, type 1 collagen is synthesized in osteoblasts from the precursor protein, procollagen. Mature type 1 collagen is a heterotrimer consisting of two identical a1 chains and one a2 chain. During collagen synthesis, specific proteases remove amino-terminal (N-) and carboxy-terminal (C-) extensions of the precursor protein procollagen type 1. These extensions are referred to as the C- and N-terminal propeptides of procollagen type 1. Both forms can be identified in the circulation and their concentrations reflect mainly the rate of collagen type I synthesis in bone. Several extra-skeletal tissues such as skin, blood vessels, fibrocartilage and tendons can synthesize procollagen type-1 propetides; however, they have a minimal contribution to the total serum pool [155].

1.3.4.2.2 Bone resorption markers

Most bone resorption markers are the result of bone collagen degradation products, apart from tartrate-resistant acid phosphatase. Most of the available bone resorption markers are measured in urine. Bone resorption markers are classified into three groups. The first group is the degradation products of bone collagen, such as hydroxyproline, collagen cross-links and pyridinium crosslinks. The second group includes cross-linked telopeptides of type I collagens (NTX, CTX, ICTP), non-collagenous protein of bone matrix (bone sialoprotein and osteopontin) and osteocalcin fragments in urine. The third group is osteoclast enzymes [155].

1.3.4.2.2.1 Cross-linked telopeptides of type I collagens (CTX, NTX)

During the process of osteoclastic bone resorption, the collagen in the bone matrix is degraded and both collagen ends cleaved and released into circulation as amino- (N-) and carboxy- (C-) terminal telopeptides. Two degradation products have been identified at the C- terminal telopeptide: ICTP (cross-linked C-terminal telopeptide of type I collagen) and CTX (C-terminal cross-linked telopeptide of type I collagen). Two types of proteinases have been suggested to participate in collagen cleavage and degradation. The first type is cysteine proteinases such as cathepsin K, which is responsible for NTX and CTX cleavage, and the matrix metalloproteinases (MMP) which release ICTP. Several immunoassays have been developed to assess the N- and C- telopeptides in both serum and/or urine [155].

Table 1-5: Commonly used bone remodelling biomarkers.

Bone formation	Bone resorption
By-products of collagen synthesis: Procollagen type I C-terminal propeptide Procollagen type I N-terminal propeptide	**Collagen degradation products:** Hydroxyproline* Pyridinoline** Deoxypyridinoline***
Matrix protein: Osteocalcin	Cross-linked telopeptides of type I collagen N-terminal cross-linked telopeptide C-terminal cross-linked telopeptide
Osteoblast enzyme: Total alkaline phosphatase Bone alkaline phosphatase	**Osteoclast enzymes:** Tartrate-resistant acid phosphatase[4] Cathepsin K

*Measured in urine and specific for all fibrillar collagens and some collagen protein. Found in bone, cartilage, soft tissues and skin. ** Measured in urine and highly abundant in bone collage and cartilage. Found in bone tendon and cartilage. ***Abundant only in bone collagen and can be measured in urine. 4: produced by osteoclasts, erythrocytes and thrombocytes and found in bone and blood. Can be measured in serum and urine. Taken from [163].

1.3.5 Vitamin D, PTH and calcium

Vitamin D plays a central role in calcium and phosphorus metabolism, and helps to maintain sufficient levels of these minerals for both metabolic functions and bone mineralization. Vitamin D can interfere with calcium function in several ways. Vitamin D is essential for dietary calcium absorption, calcium movement across epithelium cell membranes, and calcium reabsorption from renal tubules, and calcium resorption from bone.

1.3.5.1 Vitamin D and calcium absorption

Vitamin D had multiple roles in maintaining serum calcium levels. Vitamin D increases dietary calcium absorption from 10% – 15% in case of vitamin D deficiency to around 35-40% with vitamin D sufficiency. Additionally, vitamin D plays an essential role in calcium translocation through the cytosol of the intestinal epithelium [164].

Calcium transport across cell membranes is through both an active transport process (transcellular) and a passive process (paracellular diffusion). Active or transcellular calcium transport occurs only in the beginning of the gut and involves the uptake of calcium from the apical surface (brush border of the intestine or renal epithelium), transfer through the cytoplasm and then active excretion across the basolateral cell membrane [164]. The main component of this process involves calcium binding protein, calbindin, and two ions channels called transient receptor potential channels (TRP channels), TRPV6 and TRPV5 [165].

Accumulating evidence shows that VDREs are expressed in TRPV6 promoter regions and are suggested to exert a direct effect of vitamin D on calcium influx. Additionally, both TRPV6 and TRPV5 have been identified in the epithelial cell membrane of the intestine and/or kidney [166]. Calbindin was down-regulated in vitamin D knock-out mice compared with wild type [167]. The paracellular calcium transport takes place throughout the entire length of the intestine and mainly in the more distal regions. This pathway is dependent mainly on the luminal electrochemical gradient and the integrity of the tight junction. It has been shown that vitamin D can increase permeability to tight junction ions, and VDR knockout mice have decreased levels of claudin which is a major component of transmembranous tight junction [168].

1.3.5.2 Vitamin D and endocrine control of calcium homeostasis

Calcium homeostasis, the process of regulation of calcium ions in the extracellular fluid, is a vital mechanism required for many basic body functions such as muscle contraction [169]. In this mechanism parathyroid hormone (PTH) works together with 1,25(OH)2D, and, to a lesser extent, calcitonin (CT) to maintain serum calcium levels (see Figure 1-6) [170] .

Parathyroid hormone is a true hormone which consists of a polypeptide of 84 amino acid peptide hormone synthesized and released from chief cells of parathyroid glands in response to changes in serum calcium levels. After secretion, PTH then travels through the blood stream to produce its effect at the target tissues or organs, primarily in bone, kidney and intestine [171]. The acute secretory response of the PTH is regulated tightly by extracellular calcium concentration and G protein-coupled calcium sensing receptors (CaSR) located on the surface of chief cells. Any minimal reduction in serum calcium concentration will be sensed by the CaSR and stimulate PTH secretion from parathyroid cells. For example, the reduction in serum calcium levels stimulates secretion of PTH via decreased binding to the calcium sensing receptors (CaSR).

Calcitonin (CT), is 32 amino acid peptide hormone synthesized and released from parafollicular cells of the thyroid in response to high serum calcium level. Calcitonin secretion stimulates renal calcium excretion and its actions and inhibits calcium resorption from the bone and calcium absorption from the kidney [170].

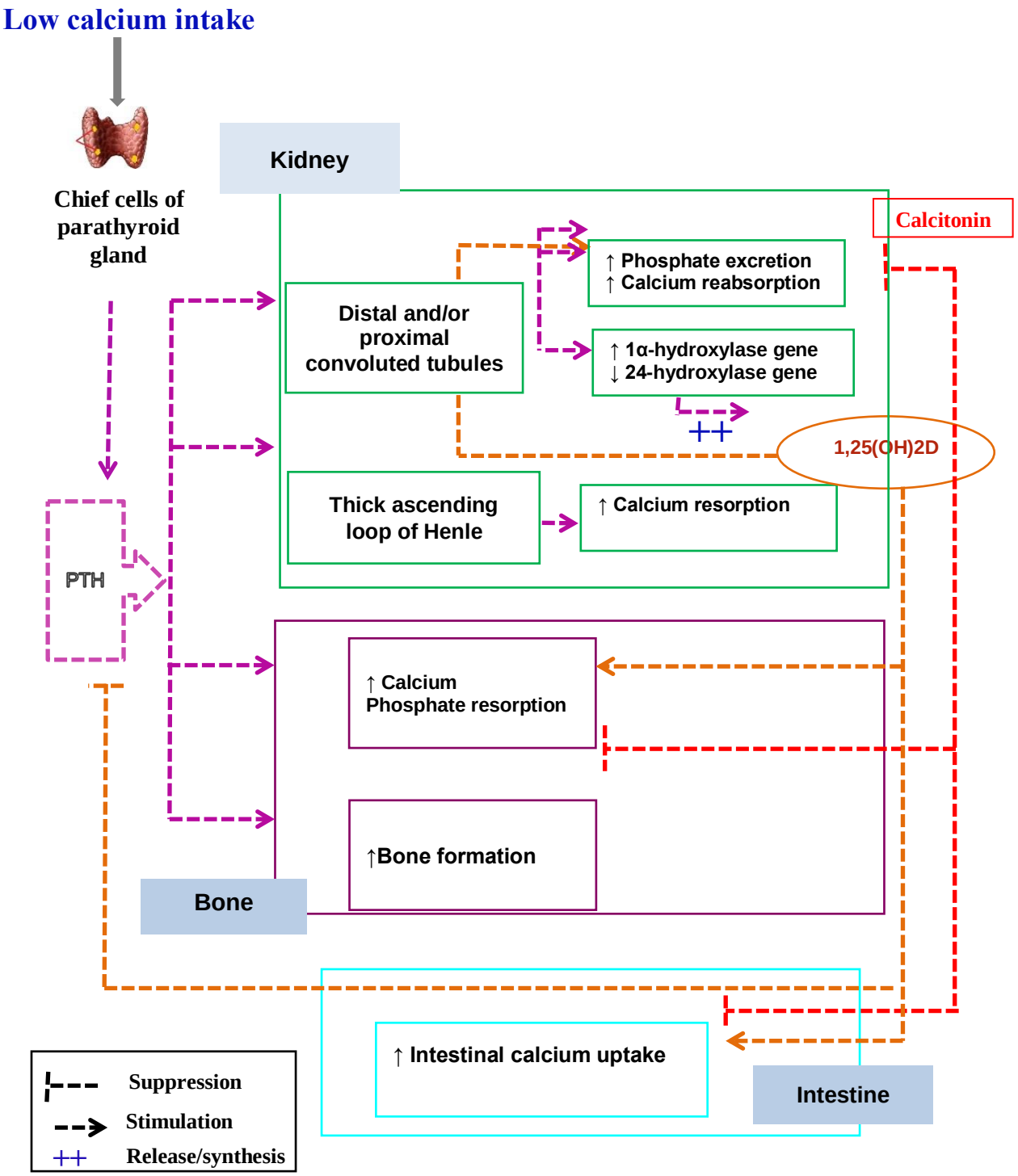


Figure 1-6: Calcium homeostasis.

Low dietary calcium stimulates parathyroid hormone (PTH) release which, in turn, directly stimulates bone resorption, stimulates conversion of vitamin D in the kidney to its active form, 1,25(OH)2D. The active form of vitamin D also works to increase blood concentrations of calcium through facilitating calcium absorption in the small intestine, enhancing fluxes of calcium out of bone and tubular calcium reabsorption, in conjunction with PTH. Calcitonin suppresses the renal tubular reabsorption of calcium and inhibits calcium bone resorption.

1.3.6 Rickets

Rickets, which originates from the Greek word rhakhis, meaning spinal column, has been known since early human history [10, 12]. In the last century, several advances have been made in terms of vitamin D pathophysiology, factor affecting metabolism and mineral homeostasis.

1.3.6.1 Definition of rickets

Rickets is a disease of growing bone caused by lack of vitamin D, calcium, or phosphate and affects children during active growing, prior to fusion of the growth plate. In this disease there is a deficiency or failure in endochondral calcification at the growth plates resulting in distortion of the growth plate and a decrease in longitudinal growth and bone deformities [172]. Several types of rickets have been identified as shown in Table 1-6 and the next section will discuss the most common type of rickets, which is vitamin D deficiency rickets.

1.3.6.2 Aetiology of vitamin D deficiency rickets

As mentioned earlier in this chapter, limited sun exposure, high latitudes, seasons, clothing, increased indoor activity during daylight hours, skin pigmentation, sunscreen and inadequate dietary vitamin D intake in older children and exclusively breast fed infants are the main predisposing factors of nutritional vitamin D deficiency [173-175]. In addition to the above natural factors, co-existence of chronic systemic illnesses and chronic medications such as anticonvulsant drugs and glucocorticoids have a significant impact on vitamin D metabolism leading to vitamin D deficiency. Chronic liver disease and hepatic failure may reduce the circulatory levels of 25(OH)D due to impaired synthesis and/or decreased vitamin D absorption due to portal hypertension, leading to intestinal edema. Steatorrhea (fat malabsorption) and other malabsorption diseases such as celiac disease and cystic fibrosis lead to vitamin D excretion in the stool due to decreased vitamin D absorption. Renal disease/failure may also affect the circulatoring vitamin D levels. For example, decreased renal function secondary to chronic renal failure may affect the 1-α-hydroxylase availability, and decreased glomerular filtration rate may limit the delivery of substrate to the 1-α-hydroxylase enzyme and limit the ability of the kidney to produce 1,25(OH)2D. Finally, medications such as Phenobarbitone may increase the risk of vitamin D deficiency due to direct effects of CYP27A1 and CYP2D25 expression on hepatocytes [23, 176, 177].

Table 1-6: Suggested classification of rickets.

Non-hereditary vitamin D deficiency rickets		
Type	**Biochemical changes**	**Main cause**
Nutritional rickets	↓ 25(OH)D, normal or ↓ calcium and phosphate, normal or ↑ PTH and ALP	Low vit D and/or calcium intake, lack of sun exposure, sun screen and dark skin
Congenital rickets (congenital vitamin D deficiency)		Maternal vit D deficiency during pregnancy
Secondary vitamin D deficiency		Hepatic diseases (↓25 hydroxylase production)
		Malabsorption (End-organ resistance to vit D)
		Renal disease (↓ renal 1,25 hydroxylase)
		Iatrogenic due to medication such as anti-convulsion drugs; ↑degradation of /↓ synthesis of vit D
Hereditary rickets		
Type	**Biochemical changes**	**Main cause**
Vitamin D-dependent rickets (Autosomal recessive)[178]	**Vitamin D-dependent rickets, type I** No change in serum 25(OH)D; ↓↓circulating 1,25(OH)2D, ↓calcium, ↑ PTH, ↑ALP	Mutations in the gene codes for 1α-hydroxylase in kidney
	Vitamin D-dependent rickets, type II (Vitamin D resistant rickets) No change in serum 25(OH)D; ↑ circulating 1,25(OH)2D, normal or ↓calcium, ↓ phosphate, ↑PTH, ↑ ALP	Mutations in the gene codes for VDR. No response to vitamin D treatment
Familial (X-linked dominant) hypophosphataemic rickets[179]	No change in serum 25(OH)D, PTH and calcium; ↓ or normal circulating 1,25(OH)2D, ↑ renal phosphate loss, ↓ circulating phosphate associated with FGF-23 abnormalities, ↑ ALP	Mutations in the gene codes for PHEX causing renal tubular defect in phosphate transport
Autosomal Dominant hypophosphataemic rickets [180]		Mutations in the gene codes for FGF-23
Autosomal recessive hypophosphataemic rickets [180]		Mutations in the gene codes for DMP1
Other (renal) rickets		
Type	**Biochemical changes**	**Main cause**
Proximal (type 2) renal tubular acidosis (Fanconi syndrome) [181]	No change in serum PTH, ↑ renal phosphate loss. Not associated with FGF-23 (a circulating hormone that causes renal phosphate wasting)	**Secondary to dysfunction of the proximal renal tubules due to;** Genetic disorders such as Wilson disease Acquired disorders due to drugs such as aminoglycosides
Distal (type 1) renal tubular acidosis [182]		**Secondary to dysfunction of the distal renal tubules due to;** Genetic due to mutation in Band 3 protein Diseases such as SLE and sickle cell anaemia Drugs such as lithium carbonate

*VDR: Vitamin D receptor. PHEX: Phosphate-regulating gene homologous to endopeptidases on the X chromosome. FGF23: Fibroblast growth factor 23. DMP1: Dentin Matrix Acidic Phosphoprotein 1. SLE: Systemic lupus erythematosus.

1.3.6.3 Growth plate and pathophysiology of rickets

The main function of vitamin D is to promote calcium absorption through the small intestine. In a vitamin D sufficient state, intestinal calcium absorption reaches as high as 80% of the intake whereas in a state of vitamin D deficiency, calcium absorption from the gut is severely affected and reduced to become as low as 10-15% of dietary calcium intake [24]. This reduction in intestinal calcium absorption leads to hypocalcaemia which in turn stimulates production of PTH, leading to an increase in urinary phosphorus excretion. In bone, there is an increase in the rate of phosphorus and calcium resorption in an effort to maintain serum calcium levels accompanied by defective mineralization of the growth plate [134, 183, 184]. However, the direct effect of vitamin D on the growth plate cannot be excluded as shown by the healing of rachitic chick plates following 24,25(OH)D3 injection directly into rachitic chick growth plates [185].

The growth plate, which is also known as the epiphyseal plate, refers to the growing area of immature bone tissue near the end of the long bones (metaphysis) in children and adolescents. Longitudinal bone growth takes place at the growth plate by a process known as endochondral ossification in which the chondrocytes are produced and then progressively replaced by bone tissue. Vitamin D receptors have been identified on growth plate chondrocytes suggesting a direct role for the vitamin D in chondrocyte metabolism [186].

The anatomical structure of growth plate is composed of matrix (non-cellular components) through which the cell columns organized. The growth plate consists of four zones:

The germinal zone (the resting or reserve zone): Consists of small and irregularly scattered cartilage cells. These cells do not have an active function in bone growth. They connect the growth plate to the bone of the epiphysis, serve as storage for nutrients and a place for supplies for the developing cartilage cells. Injury to this layer results in interruption of growth [187].

The proliferative and columnar zones: These layers are where the cell division and active growth of cartilage cells takes place. Chondrocytes divide to replace those that die at the diaphyseal surface of the epiphyseal plate. Before division, the chondrocytes have a flattened appearance. Once they begin to divide, they arrange into columns [187].

The hypertrophic zone: Also known as the maturing zone. No active growth takes place at this layer. The cells begin to differentiate, and are involved in the process of maturation, where they display higher metabolic activity leading to apoptosis. In rickets, the failure of calcification causes accumulation of cells in this layer and the width of this zone is increased, leading to mineralization defects (see Figure 1-7) [188].

The zone of provisional calcification: Death of cartilage cells, high production of ALP and calcification of the cartilage matrix occurs in this zone. The calcified matrix then became a template for osteoblastic bone formation. Invasion from metaphyseal blood vessels also takes place [187].

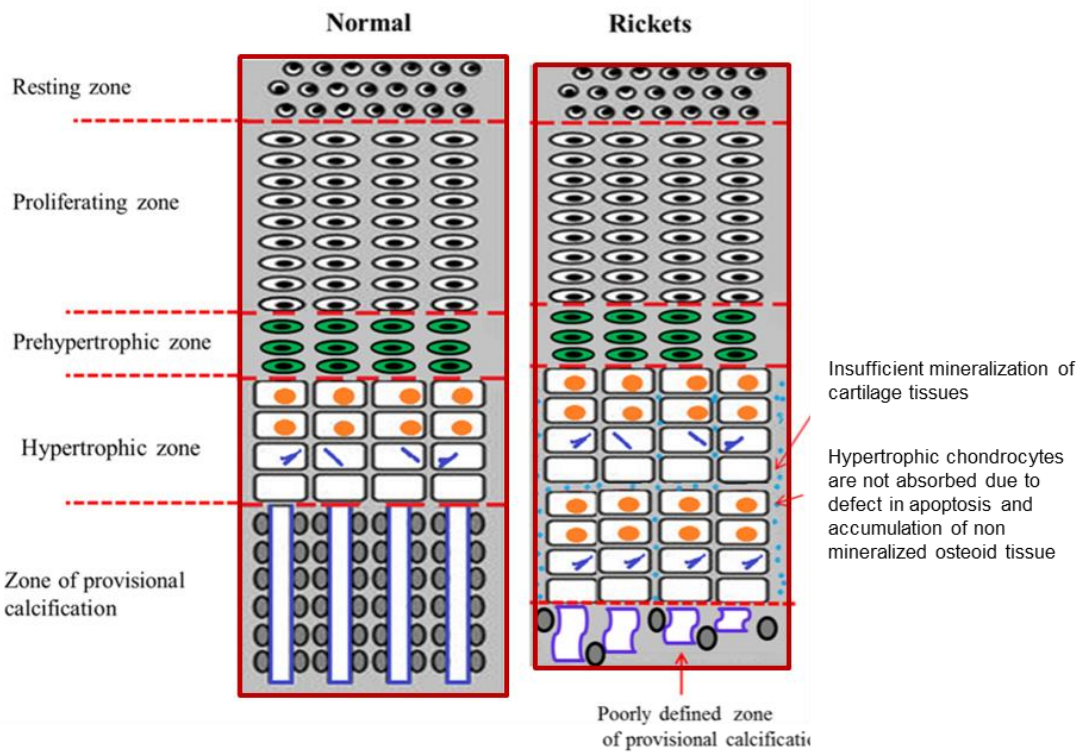


Figure 1-7: The anatomy of the growth plate in normal and rickets conditions.

In rickets, the growth plate becomes more thickened due to growth plate cartilage accumulation. The chondrocytes of the growth plate become disorganized, losing their columnar orientation. In bone tissue below the growth plate (metaphysis), there will be mineralization defect, increased width, cortical thinning and bowing.

1.3.6.4 Clinical presentation

Three main clinical symptoms can be seen in children presenting with vitamin D deficiency rickets: delayed growth, bone pain, and bone deformity, depending on the age of the child and the biomechanical situation at the time of presentation. Clinical presentation of rickets in infants mainly reflects the consequence of hypocalcaemia in relation to the muscular skeletal system. Rickets is manifested as part of three stages. The first stage is characterized by initial temporary hypocalcaemia. The child may present with irritability, excessive crying, apnoeic episodes, hypocalcaemia seizure, tetany, stridor, or even heart failure. No bone abnormalities are obvious during this stage. In the case of congenital rickets, the child will exhibit hypocalcaemia during the neonatal period, which is also reflected in the maternal vitamin D status. In stage II, skeletal abnormalities become evident and deteriorate in stage III. Older infants and younger children may present with delayed motor development, slow growth, abnormal gait, muscular weakness and bone pain [172, 176].

1.3.6.5 Clinical examination

Generalized muscular weakness and craniotabes (soft skull bones) may be seen in young infants. Thickening and enlargement of the skull may occur if the rickets presented at a later age (manifested as frontal bossing due to delays in the closure of the anterior fontanelle). Other skeletal deformities including bow legs, forward projection of the breastbone (pigeon chest or pectus carinatum), funnel chest (pectus excavatum) and rachitic rosary. The weakened lower ribs may also be strained inferiorly toward the inside by the attachment of the diaphragm which is called Harrison's sulcus. At the ankle, palpation of the tibial malleolus may look like a double epiphysis which is called Marfan sign. Spine deformities such as scoliosis or kyphosis occur in chronic cases of profound vitamin D deficiency. The bone deformities such as Genu Varum (bow legs) can be seen clinically and radiologically and mostly in toddlers when they start walking whereas older children may have valgus or windswept deformities [172, 176].

1.3.6.6 Osteomalacia

In childhood rickets, the primary defective organ is the growth plate with its chondrocytes and chondrocyte-derived extracellular matrix. Osteomalacia developed following the closure of the growth plate and generally refers to bone disease due to vitamin D deficiency in adults which leads to accumulation of unmineralized or poorly mineralized bone matrix.

The main clinical presentation of osteomalacia is bone pain and tenderness, and sometimes bone fracture. The main definitive diagnosis is made through laboratory investigation. The biochemical characteristics of osteomalacia are similar to those of rickets. Due to growth plates being closed in adults, the distinctive radiographic features of childhood rickets is absent [189, 190].

1.3.6.7 Radiographic findings in rickets and osteomalacia

Clinically, assessment of changes in bone modelling can be carried out using several densitometry techniques such as the gold standard dual-energy x-ray absorptiometry (DXA). More dynamic assessment can be provided by using biochemical markers of bone turnover in both circulation and urine [191, 192]. In young children, due to an active growing skeleton, the effect of vitamin D deficiency on the normal mineralization process can be easily recognized in X-rays. This becomes further manifested at the growth plate, at the distal ulna and radiud, and at the metaphysis around the knee joint.

Normally, the growth plate appears is a radiolucent gap between the epiphyseal/ metaphyseal interface in X-ray. At the initial stages the epiphyseal part of the growth plate widens with some degree of irregularity. As the disease progresses, the fraying and widening becomes more prominent and can be recognised by the formation of cortical spurs and stippling. This will be more evident at the metaphyseal end of the femur, tibia, or radius. Thin tubular bones such as the ulna and fibula show cupping at metaphysis. Due to this expansion in the width of the metaphysis, soft tissue swelling will be apparent around the ends of the long bones. This can be seen more clearly at the anterior rib ends at the costochondral junctions and is known as ‘rachitic rosary’.

In both rickets in children and osteomalacia in adults, the diaphysis of the long bones is weak (osteopenic) with cortical thinning. Trabeculae of the long bone may have a coarse shape and in some cases may have a ground glass appearance. In severe rickets and osteomalacia, there will be pathological fractures and looser zones. Looser zones, which are also known as pseudofractures or Milkman's fractures are narrow radiolucent lines with irregular sclerotic margins and are not true fractures or stress fractures. The lesions typically consist of poorly mineralized osteoid matrix and are specifically found in osteomalacia. Adults with prolonged vitamin D deficiency may have reduced bone density leading to osteopenia and osteoporosis. A dual-energy x-ray absorptiometry (DXA) scan should therefore be performed to assess bone density (see Figure 1-8, Figure 1-9 and Figure 1-10) [172, 176, 193].

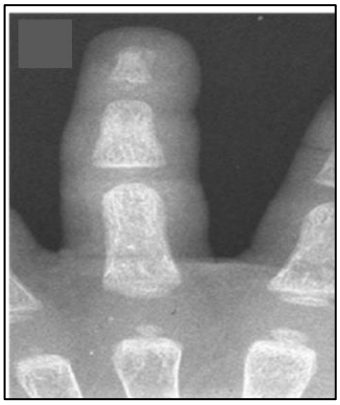


Figure 1-8: Radiological signs of rickets.

Radiograph of finger in the infant showing decreased bone density, coarsening of trabecular pattern, and cortical erosion indicating hyperparathyroidism (taken with permission from [193]).

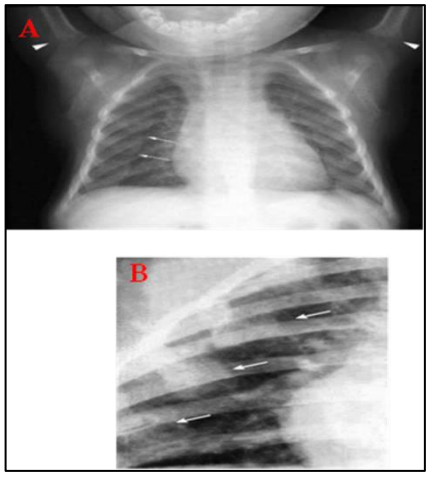


Figure 1-9: Radiological signs of rickets.

A: Chest radiograph in an infant showing expansion of the anterior rib ends (arrows), marked reduction in bone density, cortical tunnelling and fraying, and splaying of the proximal humeral growth plate (arrow heads). **B:** closer chest radiograph illustrates the expansion of the anterior rib ends (arrows), known as a “rachitic rosary” (taken with permission from [193]).

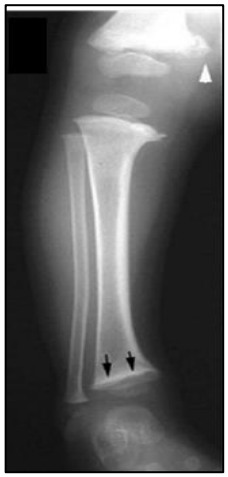


Figure 1-10: Radiological signs of rickets.

Genu Varum, fraying and splaying of the metaphyses with fragmentation of the medial aspect of the lower femoral metaphysis (white arrowhead). Dense zone of provisional ossification (black arrows) indicates that this is intermittent rickets currently in a healing phase (taken with permission from [193]).

1.3.6.8 Laboratory findings

The confirming diagnosis of vitamin D deficiency and insufficiency in both adults and children is completed by determining the level of serum 25(OH)D. In addition, measuring plasma concentrations of calcium, phosphorus, PTH and ALP may further confirm the diagnosis and be assessed to identify the degree of underlying pathology. The classical laboratory finding, in vitamin D deficiency rickets is a reduction in serum calcium and phosphate. Parathyroid hormone, ALP and urinary phosphorus levels is expected to be elevated. However, in some individuals, a longer time may pass before the clinical and radiological signs appear. During this period hypo, normo or hypercalcemia, high levels of PTH, normo or hypophosphatemia, high ALP, high, normal or low levels of 1,25(OH)2D may be identified [172, 176, 193].

1.3.6.9 Treatment of vitamin D deficiency

The most widely used treatment for vitamin D deficiency consists of vitamin D2 (ergocalciferol) or vitamin D3 (cholecalciferol). However, evidence from clinical trials has shown that vitamin D3 is more effective in treating and maintaining serum 25(OH)D levels than vitamin D2 [26, 194]. In a parallel, single-blind trial involving 33 healthy adults randomized to take 50,000 IU/week for 12 weeks of either form of calciferols, Heaney et al. (2011) demonstrated that vitamin D3 is nearly 87% more potent than Vitamin D2 in raising and maintaining serum 25(OH)D concentrations [194]. However, there are several conflicts regarding this issue as other studies have reported that both vitamin D2 and vitamin D3 are effective in treating and maintaining serum 25(OH)D concentrations [195, 196]. In a six-week randomized controlled trial involving 40 infants and toddlers aged between eight and 24 months, Gordon et al. (2008) showed that short-term doses of 2,000 IU vitamin D2 daily, 50,000 IU of vitamin D2 weekly, or 2,000 IU of vitamin D3 daily had an equal result in the treatment of vitamin D deficiency [196].

Table 1-7 summarises the Endocrine Society's recommendation for treatment of vitamin D deficiency. Monitoring of the treatment progression is very important. Obtaining serum calcium, phosphorus, 25(OH)D and ALP levels four weeks after beginning therapy is strongly recommended. The first biochemical change, an increase in serum phosphate levels, may occur within one or two weeks. High ALP levels before the treatment is an indication of a transient rise in the bone formation rate with an initial rise in 1,25(OH)2D levels. Complete radiologic may take months, although changes started as early as one week. Investigations should be repeated after three months [40, 42].

Table 1-7: Treatment of vitamin D defiency according to age groups.

Age	Therapeutic		Maintenance
	Per day (Vitamin D2 or vitamin D3)	Per week (Vitamin D2 or vitamin D3)	
0-12 months	2000 IU for six weeks	50,000 IU for 6 weeks	400-1000 IU/day
1-18 years	2000 IU for six weeks	50,000 IU for 6 weeks	600- 1000 IU/day
19-50 years	6000 IU for six weeks	50,000 IU for 8 weeks	1500-2000 IU/day
51- >70 years	6000 IU for six weeks	50 000 IU for 8 weeks	1500-2000 IU/day
Obese patients/on medication e.g. glucocorticoids & ketoconazole/ malabsorption	6000-1000 IU for six weeks	-	3000-6000 IU/day

Information adapted and edited from [42].

1.4 Extra-skeletal effects of vitamin D deficiency

Over the past decades, there has been a great deal of interest in vitamin D as a potential modulator of several non-skeletal systems and related diseases. It has been suggested that vitamin D deficiency may influence the development of diabetes and autoimmune diseases [197, 198]. Although there is good evidence to show that there is an association between vitamin D deficiency and an abnormality of the above systems, there is little evidence to show that vitamin D supplementation leads to an improvement in function, especially in childhood.

1.4.1 Link between vitamin D and glucose homeostasis

1.4.1.1 Glucose metabolism - an overview

Glucose is one of the vital metabolic fuels required for cellular function, and the process of maintaining blood glucose at a steady state is known as glucose homeostasis. Maintaining euglycemia involves a complex network of mechanisms and requires coordination and communication between several organs under strict neuronal and hormonal control [199]. Any defect in the glucose homeostasis pathway may result in insulin resistance and hyperglycaemia which are characteristic features of metabolic syndrome. Metabolic syndrome can be defined as a cluster of cardiovascular risk factors that have been shown to predict the development of cardiovascular disease and type 2 diabetes, and include obesity, impaired insulin resistance, hyperglycaemia, hyperlipidemia and hypertension. In children aged between 10-16 years the diagnostic criteria for this condition include: obesity (age and sex specific BMI –Z score ≥ 2.0), triglycerides ≥ 1.7 mmol/l, HDL < 1.03 mmol/l, fasting glucose ≥ 5.6 mmol/l and systolic blood pressure ≥ 130. However, metabolic syndrome cannot be diagnosed in children younger than 10 years, but further close follow-up is needed if there is a family history of metabolic syndrome, type 2 diabetes mellitus or hypertension [200, 201].

The liver plays a critical role in maintaining steady glucose levels by switching between glucose uptake and storage through glycogenesis and glucose release through a process of glycogenolysis and gluconeogenesis. Additionally, the pancreas is known as a key coordinator of glucose homeostasis by releasing hormones that target glucose production in the liver [202].

There are two major ways in which insulin can affect glucose homeostasis during food intake. Firstly, in skeletal muscle and adipose tissue, insulin enables glucose uptake through the cell membrane by stimulating mobilization of a transporter called GLUT 4 from intracellular vesicles into the cellular surface [203]. In liver and pancreatic β cells, glucose uptake depends on transporters called GLUT1 and GLUT 2 but does not depend on insulin [204].

Depending on the target tissue, insulin affects glucose metabolism. For example, in skeletal muscle glucose will increase the rate of glycogen synthesis, while in adipose tissues insulin suppresses lipolysis and encourages lipogenesis. Secondly, insulin is a potent inhibitor of hepatic glucose production. Therefore, in the liver, the insulin promotes glycogen synthesis and lipogenesis and inhibits gluconeogenesis. On the other hand, during starvation, insulin secretion is decreased and another pancreatic hormone, glucagon, is increased, leading to an increase in hepatic gluconeogenesis and glycogenolysis. Subsequently, glucose is released into the circulation and the blood sugar level is maintained. Additionally, the rate of lipogenesis in the liver is reduced while lipolysis in adipose tissue increases (see Figure 1-11) [202].

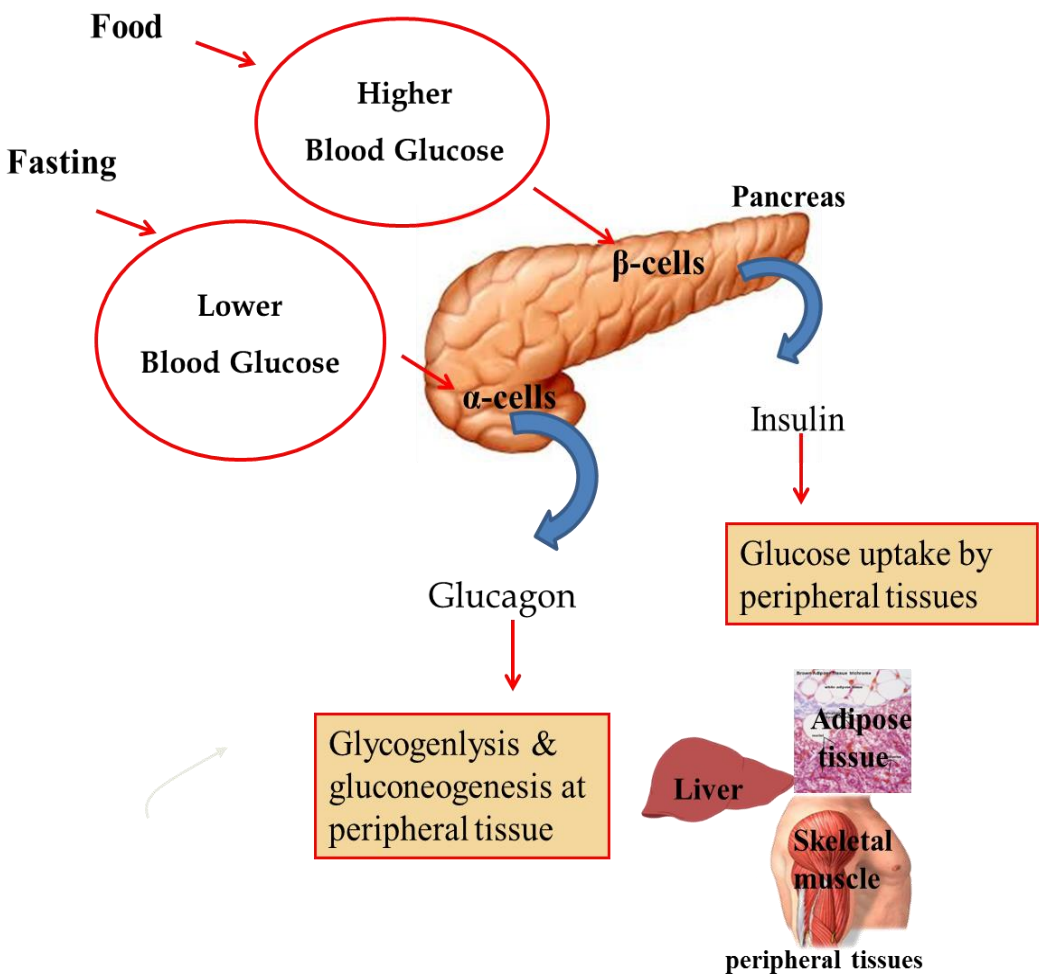


Figure 1-11: Glucose homeostasis.

During food intake, blood glucose concentration increases, stimulating insulin secretion from β cells of pancreas, and eventual glucose absorption by peripheral tissues. During fasting or between the meals, in order to maintain the normal concentration of blood sugar, the α-cells of the pancreas release another hormone called glucagon to encourage glycogenolysis and gluconeogenesis, leading to elevation of blood glucose. Blue arrows: stimulation and release.

1.4.1.2 Insulin synthesis, secretion and mechanism of action

Beta cells (β cells) which constitute around 65-80% of the cells in the pancreatic islets (islets of Langerhans) are the site of insulin synthesis. Insulin synthesis begins with the translation of its respective mRNA on the ribosome into inactive preproinsulin, followed by insertion into rough endoplasmic reticulum. In rough endoplasmic reticulum, preproinsulin is cleaved into proinsulin which consists of three domains: an amino-terminal B chain, a carboxy-terminal A chain and a linking peptide known as the C peptide. Proinsulins are then further cleaved into insulin and free C-peptides before being transported to the Golgi apparatus where it is packaged into secretory granules located close to the cell membrane [205, 206]. Insulin is released from the secretory granules by fusion of the granules with the cell membrane and exocytosis of insulin and C-peptides [207].

Insulin secretion from filled vesicles in β cells follows a biphasic pattern. The first phase, known as acute insulin release occurs in the first 5–10 minutes subsequent to glucose stimulation. Insulin secretion from the 'readily released' pool which is situated near, or even docked to, the plasma membrane accounts for only about 5% of the total amount released after a meal. After the acute insulin response, there is a second insulin secretion in which insulin is released more gradually, but is longer lasting as a result of the secretion from granules in the 'reserve pool'. Here, granules are translocated from the intracellular 'reserve pool' into the 'readily released' pool at the cell membrane [207].

Insulin release leads to activation of insulin receptors in the target tissues followed by phosphorylation and recruitment of a variety of substrate adaptors such as insulin-receptor substrate (IRS) proteins. Insulin receptor substrate (IRS) proteins are docking proteins that act as key mediators triggering a complex signalling network involving second messenger systems that regulate distinct biological effects such as alterations in glucose and lipid metabolism [208, 209] (see Figure 1-12).

Energy is essential for maintaining life, and glucose metabolism is a primary source of energy. As mentioned earlier, glucose enters the β cells via facilitated diffusion mediated by glucose transporters such as GLUT1 and GLUT2 [204]. In the first step of glucose metabolism, glucokinase on the mitochondria is responsible for the conversion of glucose into glucose-6-phosphate. Glucose-6-phosphate lies at the beginning of the two major metabolic pathways, the glycolytic pathway, which generates NADH, ATP, and pyruvate

(generating ATP through the Tricarboxylic acid cycle (TCA) and oxidative phosphorylation in the mitochondria) or the pentose phosphate pathway (PPP) [210]. Increasing the ATP/ADP ratio from the glycolytic pathway closes ATP- gated potassium channels, leading to cellular depolarization and opens voltage-gated calcium channels. Opening voltage-gated calcium channels then leads to a high calcium concentration inside the cell, triggering the first phase of insulin release through the translocation of storage vesicles toward the cell membrane and insulin exocytosis [208]. The pentose phosphate pathway is an antioxidant cellular defence pathway which plays a key role in the synthesis of nucleic acids for DNA and RNA, as well as the generation of NADPH for the synthesis of lipids and intracellular redox homeostasis maintenance (reduction-oxidation reaction) [211].

Finally, it has been suggested that the liver is responsible for most circulatory insulin clearance, compared with peripheral tissue, and both insulin clearance and sensitivity have been shown to be closely correlated. Therefore, decreased hepatic sensitivity may represent the initial stage of insulin resistance instead of increased peripheral resistance .

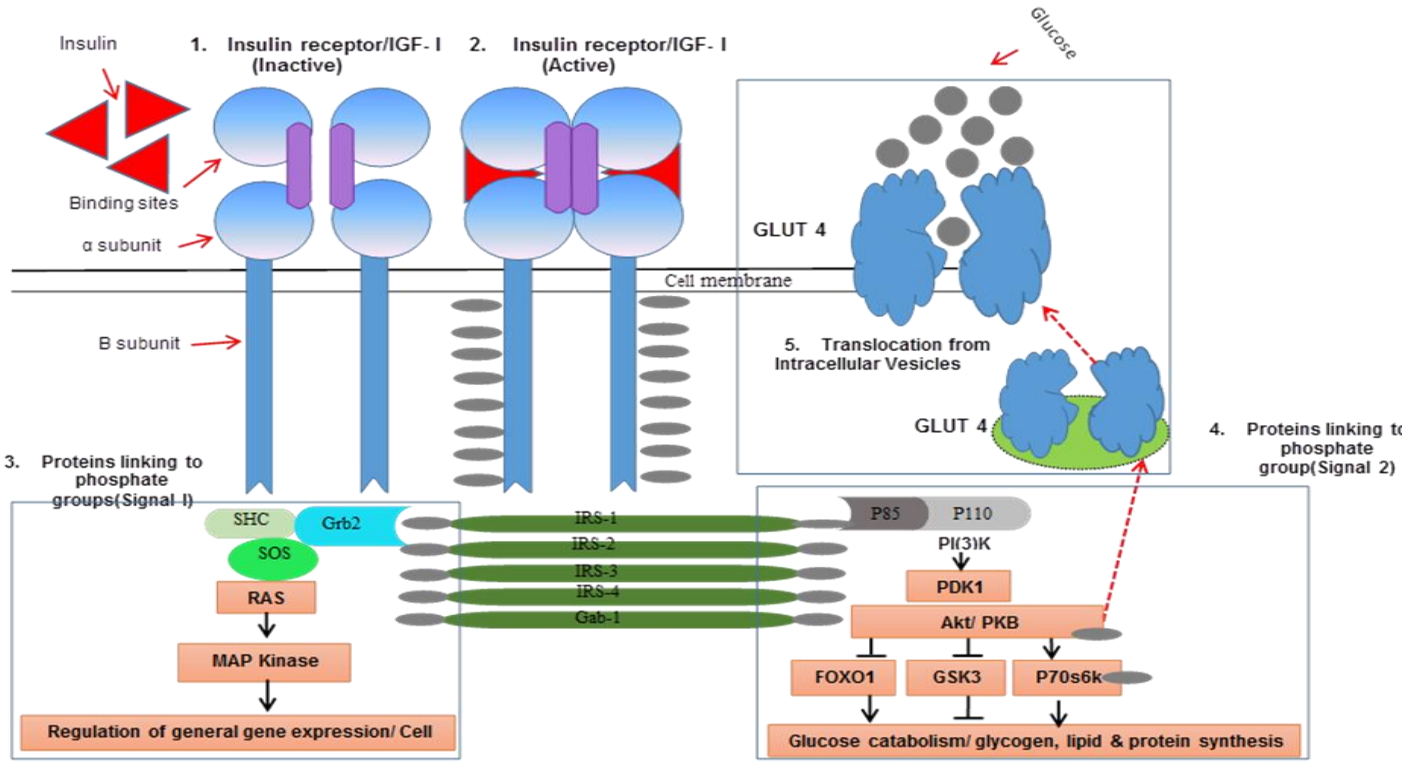


Figure 1-12: Simplified diagram shows insulin receptors and main signalling pathways.

1: Binding of insulin to the insulin receptor causing them to dimerise, 2: Dimerised receptor undergoes trans-auto-phosphorylation (grey ovals), 3: Proteins linking to phosphate groups/signal 1; IRS proteins can lead to recruitment of Grb2 and SHC , initiating of RAS pathway and generating a signalling cascade that is involved in cell differentiation, survival and growth. **4: Proteins linking to phosphate groups/signal 2**; Following phosphorylation, IRS proteins can also recruit P85 and P110 subunits of PI(3)K which in turn activates and localizes protein kinases such as PDK1 followed by activation of members of other kinases such as aPKC and Akt and initiating a cascade of events playing a key role in modulation of glucose, glycogen, lipid and protein metabolism**, 5: Activated Akt substrate P70s6k also stimulates GLUT 4 translocation to the cell membrane and enhances glucose uptake in peripheral tissues.**
IGF-I: insulin-like growth factor. IRS: insulin receptor substrate. SHC: Src Homology 2 domain containing. Grb2: growth factor receptor-bound protein 2. SOS: Ras guanine nucleotide exchange factor, Son of Sevenless. MAP kinases: Mitogen-activated protein kinases. PI(3)K: phosphoinositide 3-kinase. Akt/ PKB: protein kinase B pathway. FOXO1: Fork head box protein O1. GSK3: Glycogen synthase kinase 3. P70s6k: serine/threonine kinase.

1.4.1.3 Insulin resistance

Insulin resistance can be defined as the failure of proper response to insulin action. Generally, there will be a profound dysregulation in glucose homeostasis due to a reduction in glucose oxidation and glycogen synthesis leading to decrease energy production, while glucose production continues unsuppressed. At the cellular level, there will be a decreased sensitivity to insulin action, with an increase in insulin release to compensate for the high glucose level. At the molecular level, there will be a reduction in the strength of insulin signalling via the IRS-1/PI(3)K pathway, resulting in diminished glucose uptake and insulin utilization in target tissues. In this context, two mechanisms have been suggested. The first mechanism is triggered by mitochondrial dysfunction, which causes phosphorylation of IRS-1 by other serine kinases, which in turn decreases the ability of IRS-1 to attract and activate PI(3)K . The second mechanism is an upregulation of the P85 subunit of PI(3)K that affects PI(3)K signalling pathway activation. However, it has been indicated that both increased expression of p85 and increased serine phosphorylation of IRS-1 are required in order for signs of insulin resistance to be displayed in a clinical setting [208, 212].

When insulin resistance occurs in healthy individuals, the pancreatic β cells secrete more insulin in order to overcome the decreased effectiveness of insulin. However, in genetically susceptible individuals, the β cells are eventually unable to overcome this increased load, leading to hyperglycemia as in type 2 diabetes. Accordingly, insulin resistance has been suggested as an early sign and a significant player in the pathogenesis of this disorder [213].

A number of methods are commonly used in the clinical setting to assess insulin resistance and β cell dysfunction as described below.

The hyperinsulinaemic-euglycaemic clamp is widely considered as the ‘gold standard’ method for assessing insulin sensitivity by measuring the rate of whole-body glucose disposal during steady state hyperinsulinaemia, while maintaining a steady blood glucose level by continuous infusion of glucose at 5-10 minute intervals [214]. This technique cannot assess β cell function and is often combined with the hyperglycaemic clamp to quantify the adequacy of β cell response (insulin secretion) to glucose infusion [215]. Although clamp technology has been applied to the study of insulin sensitivity and insulin secretion during childhood, it is

time-consuming (taking between 2-4 hours), invasive, and is technically difficult to perform on very young children [216].

Because no intravenous access is needed, the oral glucose tolerance test (OGTT) has been suggested as a reliable minimal-risk procedure suitable for assessment of large populations. There are several discrepancies in this test in terms of the oral dose of glucose and sampling. A standard oral glucose load of 75g is often used in adults, following an overnight fast. If the glucose is administered intravenously, the test is called an intravenous glucose tolerance test (IVGTT). Although the OGTT does not require IV access, it does require several blood draws over the two- or three-hour period following oral glucose administration [206, 217].

The homeostasis model assessment (HOMA) for insulin resistance (HOMA IR) and the quantitative insulin-sensitivity check index (QUICKI) are simple methods to assess insulin sensitivity and β cell function [218]. Two versions of the HOMA method are currently available. The original HOMA1 was developed in 1985 based on a simple formula using fasting serum insulin (FSI) (mIU/l) and fasting serum glucose (FSG) (mmol/l) measurements to assess insulin resistance with the formula: HOMA1 IR = (FSI×FSG)/22.5. The updated computer model HOMA2 can be calculated using specific software and is employed to determine insulin sensitivity (HOMA2%S), and HOMA IR from fasting glucose (mmol/l) and insulin (mIU/l), and β cell function from fasting glucose (mmol/l) and C-peptide concentrations (a marker of insulin secretion). The quantitative insulin-sensitivity check index (QUICKI) is employed to measure fasting insulin and fasting glucose to assess insulin sensitivity.

In children, the accuracy and precision of the basal fasting methods have been assessed and found to correlate reasonably well with clamp techniques [219, 220]. The fact that these tests require only a single venepuncture in the fasting state and do not call for concomitant intravenous access makes them particularly attractive for using in the paediatric population. Additionally, childhood assessment of basal fasting glucose homeostasis variables such as fasting glucose, fasting insulin and HOMA IR has been suggested as significant predictors of future adulthood pre-diabetes, type 2 diabetes and cardiometabolic risk factors [221]. However, it is important to note that the interpretation of HOMA IR values is particularly challenging in children and adolescents due to the pre-existence of multiple HOMA IR

thresholds to describe IR. Furthermore, HOMA measures are a reflection of the basal status which represent a single point, whereas OGTT and clamp measures represent a wider picture of the dynamic of β cells in response to rising and falling glucose concentrations, which may not be suitable for children at risk of developing impaired glucose homeostasis [222].

Sex hormone binding globulin (SHBG) is a glycoprotein known traditionally to binds, regulates, and affects the bioavailability of the sex steroids, testosterone and oestradiol. Evidence from observational studies links low serum testosterone level in men and prepubertal children with the tendency towards future development of central obesity, insulin resistance, metabolic syndrome, and diabetes [223, 224]. Evidence also links lower SHBG status with an increasing incidence of type 2 diabetes mellitus, independent of sex hormone levels . One genetic study showed that three single nucleotide polymorphisms of the SHBG gene might have been related to an increased risk of developing type 2 diabetes mellitus; suggesting an association between SHBG and glucose homeostasis parameters prior to the onset of diabetes mellitus [225]. Accordingly, SHBG can be used as a biomarker to identify children at risk of developing obesity-related chronic diseases such as diabetes mellitus.

1.4.1.4 Link between Vitamin D & glucose homeostasis – putative mechanisms

Evidence from in vitro and in vivo studies has supported the role of vitamin D in glucose metabolism and β cell function. Schneider et al.(1977) provided a direct in vitro indication for the involvement of vitamin D in glucose homeostasis by observing abnormal serum 1,25(OH)2D levels in diabetic rats compared with healthy controls, and the restoration of 1,25(OH)2D to a normal level after insulin treatment [226]. Coexisting hypovitaminosis D and abnormal glucose metabolism has also been reported in patients with type 2 diabetes mellitus (T2DM) compared with healthy controls [227]. A possible link with vitamin D was also raised when seasonal variation in plasma glucose and insulin was observed in normal individuals [228].

There have been several biological mechanisms suggested by which vitamin D may contribute to the development of T2DM, mainly through its direct/indirect contribution to the abnormality in β cell function and glucose metabolism, insulin resistance and systemic inflammation, which are the main characteristics of the pathophysiology of T2DM. Table 1-8 summarises some of the current plausible biological evidence for mechanisms by which vitamin D may contributes to the abnormality in glucose homeostasis and development of T2DM. In accordance with these findings, a significant reduction in insulin secretion in VDR mutant mice has been observed [229] and the human insulin gene has been shown to be transcriptionally activated by 1,25(OH)2D [230].

Vitamin D supplementation also appears to have a beneficial effect on glucose-dependent insulin secretion in the case of vitamin D deficiency [231]. Vitamin D may influence insulin secretion indirectly through its role in the regulation of calcium flux through the cell membrane and ensuring a sufficient cytoplasmic pool of Ca+2, combined with its role in the synthesis and regulation of calbindin (a vitamin D-dependent Ca-binding protein in pancreatic β cells) [232]. Treatment of hypocalcaemic wild type-mice with food rich in calcium results in a significant reduction in blood sugar. However, this is not evident in mice that express a functionally inactive mutant VDR [229].

Vitamin D may also protect β cells from fatal immune attacks, or programmed cell death, either directly or through its effect on various components of the innate and adaptive immune system at different levels (as discussed later). It has been reported that 1,25(OH)2D may counteract apoptotic pathways and the inflammatory effect induced by cytokines through inactivation of NF-kappa-B anti apoptotic protein and suppression of Fas receptor expression [233, 234].

Another possible mechanism explaining the involvement of vitamin D in abnormalities of glucose homeostasis and pathogenesis of T2DM is the role of hypovitaminosis D in enhancing insulin resistance at the target tissues [235, 236]. The presence of the VDR in extra-skeletal target sites, such as skeletal muscle and adipose tissues, together with the up-regulation of insulin receptors (INS-R) after 1,25(OH)2D treatment appears to support this hypothesis [237].

Elevated PTH has also been suggested as a direct modulator for both insulin sensitivity and secretion by affecting glucose uptake and inhibiting insulin transport signalling in the target tissues, primarily by increasing intracellular calcium concentration [238]. Chronic inflammation may increase insulin resistance and vitamin D may influence the inflammatory reaction through several mechanisms, including modulation of the release of inflammatory cytokines such as TNFα, regulation of the activity of NF-kappa-B, regulation of genes encoding pro-inflammatory cytokines and down-regulation of Toll-like receptors two (TLR2) and Toll-like receptors four (TLR4) expression [239-241]. Figure 1-13 illustrates the putative role of vitamin D on insulin synthesis, release from β cells, stimulation of insulin receptors by 1,25-dihydroxyvitamin D3 and expression and responsiveness of target tissues to insulin stimulation.

Finally, Experimental evidence suggests that vitamin D may play a role in the sex hormone co-regulation and low vitamin D status has been suggested as a risk factor for conditions linked to sex hormone levels, such as insulin resistance and adiposity [242, 243].

Table 1-8: Some evidence supporting the putative role of vitamin D in beta (β) cell function and insulin action in peripheral tissues.

Evidence from pancreatic β cells
High local expression of VDR and the presence of VDR in insulin gene promoter [244, 245]. Impairment of glucose-mediated insulin secretion can be normalized with vitamin D supplementation in cases of vitamin D deficiency [231, 246]. *Conflicting evidence [232]. Vitamin D can induce glucose-mediated insulin release [247]. Vitamin D deficiency may alter islet cells' mitochondrial metabolism, mainly the oxidative events [248].
Evidence from systemic circulation
Counteract apoptotic pathways via suppression of the cytokine-induced Fas expression at mRNA and protein levels [234]. * Conflicting evidence [197]. Vitamin D can upregulate and maintain high levels of A20, an antiapoptotic protein which is known to block NF-κB activation [233]. Reduce susceptibility of β cells to cytotoxic T lymphocytes and protect cells from auto-destruction [249].
Evidence from peripheral tissues
Identification of VDR in skeletal muscle [250]. Vitamin D can enhance insulin receptor capacity and stimulate insulin response in the control of glucose transport, *in vitro* [237]. Vitamin D may indirectly participate in the process of extracellular calcium normalization and normal calcium influx through cell membranes, ensuring a sufficient intracellular calcium pool which is vital for insulin function in peripheral tissues such as skeletal muscle and adipose tissue [251-253] . High PTH accompanied by vitamin D deficiency may decrease glucose uptake by the liver, muscle and adipose cells [254].

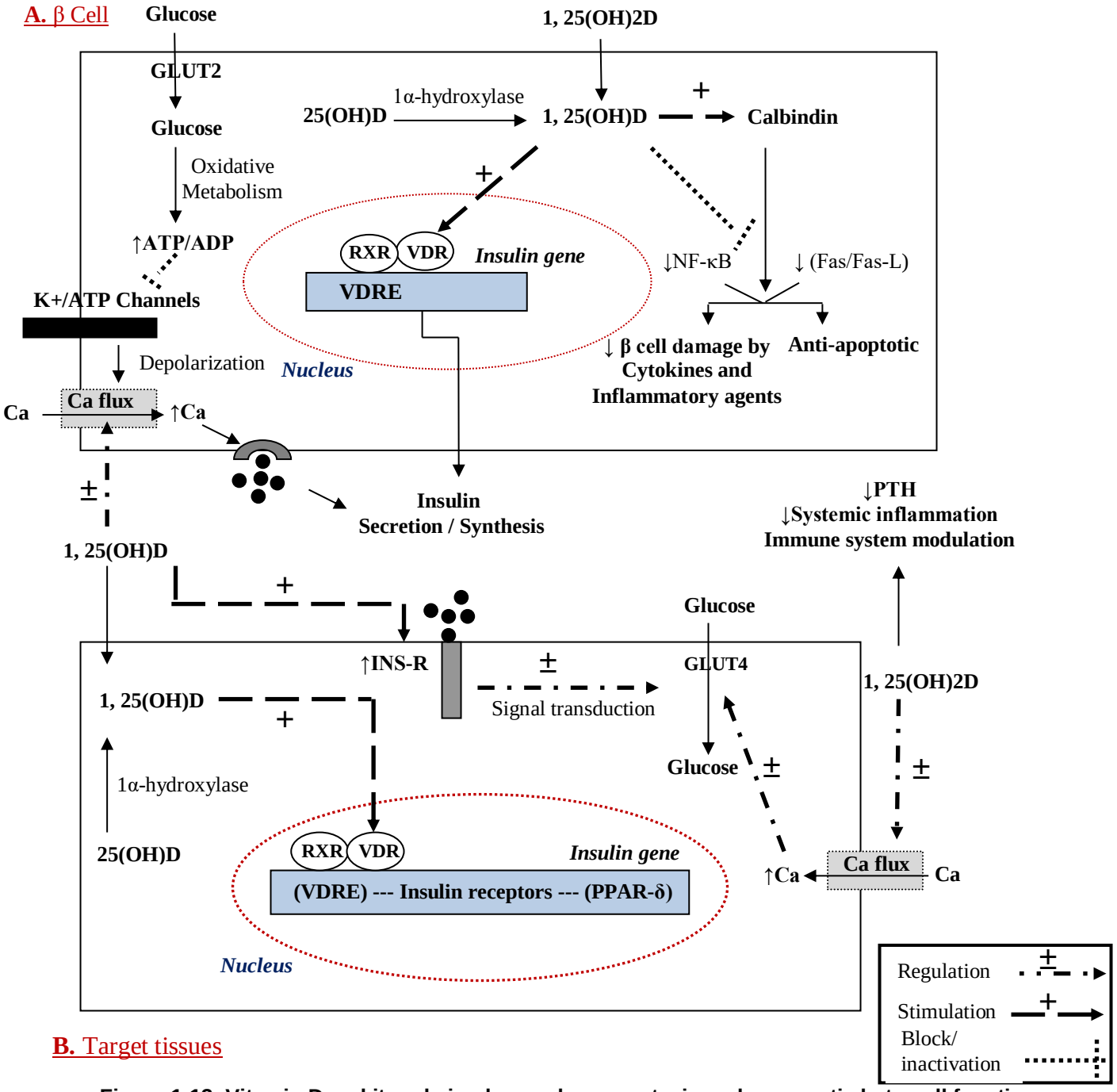


Figure 1-13: Vitamin D and its role in glucose homoeostasis and pancreatic beta-cell function.

A. Beta cells (β cells): 1,25(OH)2 D can come directly from the circulation or is synthesised within pancreatic beta cells. **I.** 1,25(OH)2 D causes upregulation of the insulin gene via VDR binding which leads to more insulin synthesis. **II.** Vitamin D also improves insulin secretion and glucose tolerance through indirect regulation of intracellular calcium through increasing the ATP/ADP ratio resulting in closure of the plasma membrane ATP-gated channels and depolarisation of the cell leading to exocytosis of insulin-containing secretory granules. **III.** Modulation of cytokine secretion and apoptotic pathways occurs through interaction with VDRE in cytokine genes, inactivation of NF-κB and suppression of the Fas receptor. **IV**. Upregulation of calbindin may also protect against cytokine-induced apoptosis by increasing intracellular free calcium. **B. Target tissues: I.** Vitamin D stimulates the expression of insulin receptors and signalling transduction, resulting in translocation of GLUT4 to the membrane and glucose transport in peripheral tissues**. II.** Vitamin D causes activation of peroxisome proliferator-activated receptor (PPAR-δ), a transcription factor implicated in the regulation of fatty acid metabolism.

1.4.1.5 Link between vitamin D & glucose homeostasis – evidence from observational studies

In a cross-sectional study, Orwoll et al.(1994) reported no relationship between serum 25(OH)D, fasting or post-challenge glucose and insulin secretion [255]. However, the indirect method for measuring insulin level and unadjusted results for confounders may have had an impact on the study outcome. A positive association between 25(OH)D and insulin secretion has been reported in both glucose intolerant East London women of South Asian origin [256] and healthy Caucasian elderly men [257]. These studies were performed using an oral glucose tolerance test and have been further supported by other studies which used a hyperglycaemic clamp technique [258, 259]. Indeed, most of the available cross-sectional studies, including a large dataset from the National Health and Nutrition Examination Survey (NHANES), support an inverse relationship between serum 25(OH)D and glycaemia and insulin resistance. However, the results from this survey were applied only to particular ethnic groups, non-Hispanic whites and Mexican–Americans but not non-Hispanic blacks [260].

Prospective studies assessing the association between 25(OH)D and diabetes risks are very limited. A negative association between basal serum concentration of 25(OH)D and future hyperglycaemia and insulin resistance has been found in one longitudinal study conducted over a 10 year period [261]. A recent systematic review and meta-analysis showed that each 10 nmol/l increase in 25(OH)D level was associated with a 4% decrease in risk of type 2 diabetes. In addition, the relative risk of type 2 diabetes was 0.62 (95% CI 0.54-0.70) when compared with the highest to the lowest category of 25(OH)D levels [262].

A number of observational studies have explored the association between vitamin D status and markers of glucose homeostasis in both normal weight and obese adolescents. An association between vitamin D deficiency and insulin resistance has been reported in overweight and obese adolescents. Most of these studies have shown the impact of pubertal status, vitamin D status, influence of PTH status, and the presence of non-alcoholic fatty liver disease (NAFLD) on their study results. Three studies have failed to find any association between vitamin D and insulin resistance in obese children [85, 263, 264]. Other studies report an inverse association between hypovitaminosis D and measures of insulin resistance and glycaemia in obese children [265-267]. Table 1-9 summarises some recent studies that have investigated the association of 25(OH)D with glucose homeostasis in children.

In summary, observational studies investigating an association between vitamin D status (as indicated by 25(OH)D levels) and glycaemia, diabetic risk and β cell function have been performed in multiple cohorts. Despite the overall view in favour of a negative impact of hypovitaminosis D on glucose homeostasis and β cell function, the available literature demonstrates inconsistent results. There are still also inconsistencies regarding the sufficient dose required to improve vitamin D status. There are several possible explanations for this variation, including differences in study design, subject characteristics, study population, confounders and techniques used to assess glucose homeostasis and β cell function.

Table 1-9: Summary of studies reporting an association between 25(OH)D and glucose homeostasis in fasted state (boys and girls).

Ref	Age (Years)	BMI	N	Method	25(OH)D (nmol/l)	Association with glucose homeostasis	Adjusted for variable	Comment
Reinehr et al [263]	12.1 ± 2.4	27.7 ± 3.8	133	Glucose, insulin	50	No	BMI	Longitudinal study (1 year)
Smotkin et al [85]	12.9 ± 5.5	32.3 ± 6.4	217	Glucose, insulin	55.2% < 50, 44.8% ≥ 50	No	None	Retrospective study
Delvin et al [268]	9, 13, 16	20.1 ± 4.2 (Boys), 20.4 ± 4.5 (Girls)	1745	Glucose, insulin	45.9 ± 13 (Boys), 45.9 ± 12.2(Girls)	Yes	Age, sex, BMI, smoking, income, activity	-
Ford et al [269]	12 - 17 (Range)	-	1941	Glucose, insulin, HbA1c	59	Yes	Age, sex, BMI, ethnicity, activity vitamin intake, HDL	Association with insulin resistance in selected sub-groups.
Kelly et al [270]	11 ± 4	2.1 (-1.2,4.1) (Median (min, max))	85	Glucose, insulin, HbAIc	59 ± 32	Yes	Age, sex, season, ethnicity, BMI, puberty	No correlation between HOMA and race, sex, season after adjustment for puberty and BMI-Z.
Olson et al [271]	6 - 16 (Range)	99.2 (Percentile for age)	411	Glucose, insulin, OGTT, HbA1c	49 ± 17.8	Yes	Age, BMI	Correlation exists only after adjustment. No correlation with HbA1c.
Roth et al [266]	11.9 ± 2.7	SDS-BMI; 20% (Non obese) 80% (Obese)	156	Glucose, insulin, HbA1c	39.4 ± 6.6	Yes	Age, sex, BMI	No impact of adiposity on study result.
Poomthavron et al [264]	11.2 ± 2.6	28.6 ± 4.8	150	OGTT(Obese), Glucose (Non obese), insulin	70.4	No	None	Subjected to selection bias as recruited from obesity clinic.
Nsiah-Kumi et al [267]	10.8 ± 0.3	77. ± 1.7 (Percentile for age & sex)	198	Glucose, insulin, 2hr blood glucose	42.4 (16.9, 99.8) Median/range	Yes	BMI	-

*OGTT: Oral glucose tolerance test. QUICKI: Quantitative insulin sensitivity check index. HbA1c: Glycosylated haemoglobin. BMI: Body mass index (kg/m2). BMI-SDS: Body mass index – standred deviation score. Data are shown as mean (SD), unless otherwise specified.

1.4.1.6 Link between Vitamin D & glucose homeostasis – evidence from interventional studies

A possible association between vitamin D and insulin secretion was raised in some interventional studies over three decades ago [256, 272, 273]. However, other studies did not find any effect of vitamin D supplementation [274-276]. Grimnes et al. 2011 administered 20,000 IU vitamin D3 orally twice a week for six months to a healthy Caucasian cohort who had vitamin D levels lower than 40.3 ± 12.8 nmol/l, but could not find any effect on insulin sensitivity or secretion [277]. This was consistent with another study which showed that the injection of two doses of 100,000 IU vitamin D3 did not lower fasting glucose or insulin sensitivity in a Caucasian cohort [278]. However, these results could not be applied to other ethnic populations as shown by a randomised, controlled trial which involved South Asian participants and demonstrated a significant effect of the administration of 4000 IU vitamin D3 for six months; fasting glucose decreased and insulin sensitivity increased in vitamin D-deficient and insulin-resistant South Asian women [279].

Mitri et al. (2011) designed the short-term Calcium and Vitamin D for Diabetes Mellitus (CaDDM) 2 x 2 factorial, double-masked, placebo-controlled trial, which looked at the effect of vitamin D and calcium supplementation alone or in combination on pancreatic β cell function, insulin sensitivity and glucose tolerance in 92 mainly Caucasian, obese and glucose tolerant adults. Study participants were divided into four groups to receive either 2000 IU Cholecalciferol or 400 mg Calcium carbonate, or matching placebo once a day for 16 weeks. The deposition index, which was used as an indication for pancreatic β cell function was measured. The results from this study showed a significant increase in the deposition index in the vitamin D supplement group and improvement in pancreatic β cell function by 15-30% with a tendency to decrease the rise in the measure of glycemia [280].

A more recent randomized, double-blind, placebo-controlled trial conducted by Krul-Poel et al. (2015) examined the effect of vitamin D3 supplementation (50 000 IU/month) on HbA1c, fasting glucose, fasting insulin and insulin resistance in a cohort of patients with type 2 diabetes mellitus (n = 275). After six months of vitamin D3 supplementation, serum 25(OH)D concentration was significantly improved in the vitamin D group compared to the placebo, $p < 0.01$ (from 60.6 ± 23.3 to 101.4 ± 27.6 nmol/l and 59.1 ± 23.2 to 59.8 ± 23.2 nmol/l in the

vitamin D and placebo group, respectively). However, there was no observed effect of vitamin D supplementation on markers of glycemic control [281].

Based on the available data, little is known about the association between insulin sensitivity/resistance, glucose intolerance and vitamin D deficiency in the paediatric population. A recent double-blind, placebo-controlled trial of obese vitamin D-deficient adolescents aged 9-19 years failed to find any significant effect of six months supplementation of 4000 IU vitamin D on BMI, inflammatory markers, fasting glucose and insulin [282]. In a further two 12-week trials, administration of either 4000 IU/day or of 300,000 IU/week of Cholecalciferol demonstrated favourable effects on glucose homeostasis and/or metabolic syndrome outcomes on obese adolescents [283]. Although larger doses had been used in the weekly vitamin D trial, it seems that daily supplementation was more efficient in raising serum 25(OH)D concentrations (increase of ~47 nmol/l in the former versus 35 nmol/l in the latter). A further non-randomized trial did not find any significant effects of 8 weeks of 50,000 IU/week supplementation on glucose homeostasis of obese adolescents [9].

In conclusion, results from interventional studies show some degree of inconsistency with a heterogeneity of techniques, study designs, subject characteristics and therapeutic regimen. Most of the available studies used indirect methods to measure the effect of vitamin D on insulin sensitivity/resistance. Basal vitamin D levels were not available for most of the studies and there was no universal threshold to define vitamin D deficiency, insufficiency or sufficiency. There is a need for more interventional studies, involving larger sample sizes, focusing on both normal weight and obese children with careful selection of the dose, treatment regimen, and achievement of target 25(OH)D concentrations.

1.4.2 Link between vitamin D and immune function

1.4.2.1 Immune response - an overview

The principle roles of the immune system are providing protection and recognition and responding with subsequent destruction of foreign and harmful events such as diseases and infections. This is done by maintaining self-tolerance (discriminate self from non-self-molecules), and developing immunologic memory against non-self-molecules. The ability of the immune system to recognize self and respond to non-self-harmful events is known as immune response, which has been divided into innate and adaptive immune responses. The adaptive immune response is further divided into humoral and cell-mediated adaptive immune responses [284].

1.4.2.1.1 Innate immune responses

The main function of the innate immune response is to provide a first line of protection during the time between pathogen exposure and early adaptive response. The significance of the innate immune responses can be appreciated by considering its initiation time, which may takes a few minutes, compared with the development of a specific adaptive immunity which may takes days to weeks [284].

The first component of the innate immune system consists of a combination of physical and chemical barriers. Physical barriers are not generally considered a proper part of the immune system; when an infection overwhelms the physical barriers the role of the immune system starts [285]. The second level of the innate immune system is the production of antimicrobial peptides (AMPs) such as α defensins (HNP1-4 and HD-5, 6), β defensins (Hbd-1 to 4) and cathelicidin (hCAP18) [285]. Activation of the innate immune response is mediated partly by a family of receptors called pattern-recognition receptors (PRRs) which sense and recognize the conserved pathogen-associated molecular patterns (PAMPs), such as microbes' cell-wall components and viral nucleic acids [286]. The recognition of microbes by PRRs direct the immune response accordingly through subsequent recruitment of innate and adaptive immune cells [287]. In mammals, toll-like receptors (TLRs) have been recognized as a family of PRRs and suggested to induce the innate inflammatory reaction causing a release of various inflammatory mediators and AMPs [288, 289]. The third and most specific level of innate immune system is phagocytosis, promotion of healing and initiation of inflammatory

responses though phagocytic leukocytes, dendritic cells and natural killer (NK) cells. T helper type 2 (Th2) inflammatory responses is a specific type of innate immune response against helminths invading at cutaneous or mucosal sites and characterized by recruitment and activation of mast cells, basophils and eosinophils, and the production of Th2 cytokines such as interleukin-4 (IL-4), IL-5 and IL-13 by naive T cells before stimulation of the adaptive immune response (discussed in the next section) [290].

1.4.2.1.2 Adaptive immune responses

Unlike the innate immune responses, the adaptive responses are more specific and stimulated when a particular pathogen is ingested by immature dendritic cells in the infection site. There are two broad classes of adaptive immune responses - humoral (antibody response), and cell-mediated immune responses. Lymphocyte populations are key components for the adaptive immune responses, including thymus-derived lymphocytes (T-lymphocytes), CD8+ (cytotoxic) and CD4+ (helper) T cells, bone-marrow-derived (B-lymphocytes) and NK cells [284].

The humoral immune response refers to the generation of antibodies by the B cell population known as plasmablasts (immature plasma cells) and plasma cells, leading to the destruction of invading microbes, providing both short and long term protection to pathogenic challenge [291]. Binding of antibodies to microbes inactivates them by blocking their ability to bind to corresponding receptors on targets cells, and marks them for phagocytic cells of the innate immune system to ingest them. This type of immune response may be stimulated directly by microbes or through collaboration with T cells which recognize antigens presented through MHC (major histocompatibility complex) class II molecules on the B-cell surface or through interaction between CD40 ligands (CD40L) on the T cell and CD40 on the B cell. T helper 2 (Th2) cells which secrete cytokines IL-4 have been suggested to play a key role in B cell differentiation and IGg/IgE response [292, 293].

Cell-mediated immunity involves the activation of macrophages and NK cells, the production of antigen-specific cytotoxic T-lymphocytes, and the release of various cytokines in response to an antigen. Three major effector T cell classes have been identified which play essential roles in adaptive immune response: CD8 +T cells, cytotoxic T cells (antigens presented to its

surface by MHC class I molecules) and CD4+ T cells (differentiated further into several effectors as discussed below).

1.4.2.1.3 CD4-positive T cells (CD4+ T cells)

Following their development in the thymus, naive T cells are conveyed through systemic circulation to peripheral lymphoid organs and remain in circulation between blood and lymphoid organs until they encounter their antigens. Following antigen stimulation, the role of naive T cells in adaptive immune response begins by proliferation and differentiation into cells capable of contributing to the removal of the antigen known as effector T cells or CD4+ T cells.

Accumulating evidence has suggested that CD4+ T cells are key players in the immune system response to harmful events. Firstly, CD4+ T cells have been shown to assist B cells switch class to produce antibodies [274]. Secondly, activation of macrophages promotes microbial killing [275]. Thirdly, they stimulate and maintain responses of CD8+ T cells [276]. Furthermore, CD4+ T cells have been postulated to play a role in the process of recruitment of inflammatory cells such as eosinophils at sites of infection and inflammation [277]. Finally, through their ability to differentiate and to produce a wide range of cytokines and chemokines, CD4+ T cells can be considered as a key coordinator of the immune response [278].

During the process of adaptive immune response, the naive CD4+ T cell encounters an antigen presented to its surface by MHC proteins that protrude from the surface of APCs. The MCH binds to peptide fragments (epitopes) derived from pathogens and presents them on the cell surface for recognition by T-cells. After the antigen presentation, the naive CD4+ T cells then start to proliferate and differentiate into several T cell effectors/CD4+ Th cell subtypes including T helper 1 (Th1), T helper 2 (Th2), T helper 17 (Th17) and induced regulatory T (iTreg) cells [294, 295] (see Figure 1-14).

One of the most crucial factors determining the naive CD4+ Th cell differentiation/ polarization process is the effector cytokine environment produced by APCs [294, 296]. For instance, activation of naive CD4+ T cells in the presence of IL-12 or IL-4 produced by dendritic cells (DCs) lead to production of Th1 or Th2 phenotypes. Transcription factors such as T-bet (T-box) and signal transducers and activators of transcription (Stat 4) are required for Th1 cell differentiation, and transcription factors such as GATA-binding protein 3 (GATA3) and signal transducers and activators of transcription (STAT 6) are required for Th2 cell differentiation (see Figure 1-14).

Transcription factors play a crucial role in CD4 Th cell differentiation/polarization and cytokine production by providing a key link between cytokine receptors and cytokine-induced gene transcription [294]. For example, the STAT 4 signalling pathway can be activated by IL-12, inducing IFN-γ transcription and initiating Th1 differentiation, while the presence of IL-4 activates the Stat6 signalling pathway, resulting in IL-4 transcription and Th2 differentiation [297]. T-box transcription factor (T-bet), a master regulator for Th1 differentiation, is able to activate a panel of genes to promote Th1 phenotype differentiation of a particular phenotype, and is a powerful suppresser of Th2 differentiation [298]. Transcription factor GATA-binding protein 3 (GATA3) known as a master regulator needed for Th2 differentiation [299, 300].

The two main classical types of effector CD4+ T helper cells, Th1 and Th2 have been characterised based on their distinct cytokine secretion patterns Type 1 helper T (Th1) response, the production of interleukin (IL)-2, gamma-interferon (IFN-γ) and tumour necrosis factor-α (TNF-α), whereas Th2 expresses IL-4, IL-5, IL-6 and IL-13. The diversity in cytokine patterns lead to different functions of these two types of effector T cell subset [294, 301]. Ideally, humans should produce well-balanced Th1/Th2 cell responses, and any dysregulation of the effector cells' balance may cause a disruption in immune responses to infections (immunodeficiency) or excessive and incorrect immune responses to harmless environmental antigens (atopy) or self-antigens (autoimmunity). Th1 cells are mainly involved in the development of cellular immune responses (stimulating phagocytosis, activating antigen-specific cytotoxic T-lymphocytes and cytokine release). Th2 cells are generally related to humoral immune response (an antibody-mediated system) [302].

The status of Th1/Th2 differentiation determines the type of immune response and how the body reacts to foreign antigens, and both Treg and Th17 can play a role in maintaining either Th1 or Th2 balance or both, which has been implicated in the pathogenesis of immune system-related disorders [303-305]. For example, over-stimulation of the Th1 master gene, T-bet, shifts the differentiation into the Th1 phenotype, exacerbating colitis in experimental animals, whereas suppression of T-bet encourages differentiation to Th2 and Th17 lineages, resulting in decreased Th1 immunity and spontaneous asthma [306, 307]. Most of the CD4+ helper cells undergo apoptosis on resolution of infection, with a few remaining as CD4+ memory cells. Th17 cells were first characterized in 2005 as independent Th1 and Th2 cell linages [308]. The main Th17-secreted cytokines are IL-17A, IL-21 and IL-22 and differentiation of naive

CD4+ Th cells into Th17 has been suggested to be reliant on TGF-β, IL-6, or IL-21 [309].

Retinoic acid -related orphan receptor gamma t (RORγ t) has been suggested as a specific transcription factor stimulating Th17 cell differentiation although it also depends on other transcription factors such as STAT 3 [310, 311].

It has been shown that Th17 can be stimulated in several immune cells found in mucosal sites. For example, at mucosal epithelial sites, IL-17 induces granulocyte colony-stimulating factor (G-CSF) and CXC chemokine (CXCL-8 and CXCL-2) production, leading to neutrophil activation that contributes to microorganism clearance at mucosal sites [312-314]. Moreover, both IL-22 and IL-17 stimulate antimicrobial peptides such as β-defensin production and epithelial repair functions important for the control of extracellular fungal pathogens [315]. On the other hand, improper Th17 stimulation and/or balance has been linked with the development of several inflammatory and autoimmune diseases, such as multiple sclerosis, systemic lupus erythematosus, inflammatory bowel disease and asthma [305, 316-319].

Regulatory T cells (Tregs) have been suggested as significant players in maintaining immune system homeostasis, prevention of autoimmunity and protection against infectious and allergic diseases [320, 321]. Evidence have shown that Tregs can be categorized into two main subtypes, natural CD4+ CD25+ FoxP3+ Treg cells (nTregs) and inducible/adaptive Treg cells (iTregs), which may further acquire CD25+ FoxP3+ expression [322]. Natural Treg cells (nTregs) have been suggested as a key player in several physiological and pathological conditions, such as pregnancy, autoimmunity and cancers [323-325]. Natural Treg cells (nTregs) differentiate in the thymus and migrate to peripheral tissues where they constitute, along with iTregs, approximately 5-10% of the total peripheral CD4+ T cells.The second type of Tregs are known as inducible Treg cells (iTregs) and include T regulatory 1 cells (CD4+CD25- FoxP3- Tr1), which secrete IL-10 , and T regulatory 3 cells (CD4+CD25+ FoxP3- Tr3), which secrete TGF-β, and may be converted to FoxP3+ Tregs following stimulation by TGF-β, in addition to various subsets of CD8+ Treg cells [326-328].

Adaptive/inducible CD4+ Treg cells (iTregs), may generate from naive T cells and acquire CD25+FoxP3+ expression outside the thymus under the influence of TGF-β, and therefore suppress activation and expansion of naive T cells and their effector cells, Th1, Th2 and Th17. In addition to several pathological inducers for iTreg, such as autoimmune diseases and cancer, evidence has also shown that CD4+ iTregs may play a critical role in maintaining normal gut homeostasis [329-331].

Transcription factor expression fork head box P3 (FoxP3) and the maintenance of the Treg cell-specific DNA hypomethylation pattern have been identified as key features of the Treg cell lineage which are necessary for maintaining Treg stability and required for their development. Additionally, Tregs are able to express the high-affinity IL-2 receptor (IL-2R), also known as CD25, constitutively regardless of their functional status, and CD25 has been suggested as an essential element required for Treg survival and maintenance [323, 332, 333]. Studies have shown that both nTregs and iTregs (CD4+ FoxP3+) share one substantial feature: they regulate and suppress naive CD4+ T cells and their effector cells in order to maintain the immune tolerance to self-antigens, and to prevent excessive responses to foreign antigens. It has been suggested that Tregs may be able to suppress naive CD4+ T cells and their effector cell functions and activities through contact-dependent and independent mechanisms. The first mechanism is via expression of negative regulatory receptors such as CTLA4, or destruction of associated dendritic cells (DCs) through secretion of perforin and granzyme B. The contact independent mechanisms involved are secretion of immunosuppressive cytokines IL-10 and TGF-β and consumption of IL-2 [322, 325, 330]. On the other hand, the main differences between nTregs and iTregs may be related to their origin and cell fate and functional stability of the master regulator transcriptional factor FoxP3. For example, some studies have shown that nTreg cells are able to maintain stable FoxP3 expression regardless of extracellular conditions, Foxp3-stimulating signals, or cell proliferation. In contrast, FoxP3+ iTreg cells induced in vitro by TGF-β and/or retinoic acids are less stable in the expression of functional FoxP3 and CD4 as reviewed by Ohkura et al. 2013 [334].

Evidence has suggested that the migratory capacity (homing) of Treg cells to other tissue sites such as sites of infections or inflammations are controlled by a variety of adhesion molecules and chemokine receptors expressed by FoxP3+ Treg cells [335]. For example, it has been shown that FoxP3+ Treg cells are able to express chemokine receptors (CCR) 4, 5, 6, 7 and 8

at a relatively higher level than effector T cells. CCR7 is expressed by the vast majority of FoxP3+ Treg cells, which enables these cells to migrate towards chemokines CCL19 and CCL21. Furthermore, it has been demonstrated that IL-10-producing nTreg cells can be recruited into the site of infection when they express CCR5, and inhibit the development of effector T cells and the production of IFN-γ, thereby promoting the survival of pathogens for long periods [336-338].

Due to some limitation of using CD25 as a specific surface markers for Tregs related to its co-expression by activated effector T cells, FoxP3 has been identified as a better marker for CD4+Tregs. However, there is some functional heterogeneity between different CD4+FoxP3+Tregs populations and several studies has been conducted to look at any cell surface antigens that distinguish Tregs from naive T cells or differentiate between different Treg populations [339, 340].

Experimental studies on the synovial fluid of patients with juvenile arthritis showed that activated CD4+CD25+ Treg cells could be recognized by the expression of cell surface marker CD127 [341]. Other studies have tried to further characterise surface markers of FoxP3+ Tregs using antigens reviewed by Chen and Oppenheim 2011 [340], and several studies has been conducted to look at any cell surface antigens that distinctively distinguish Tregs from naive T cells or differentiate between different Treg populations [339]. Despite extensive research, however, permeabilisation of cells, which is necessary for intracellular antigen detection when the antibody is required for intracellular access, is still required to identify Tregs population by their Foxp3 expression. To date, there is still no consensus about whether any particular markers can be used for identification of Tregs without permeabilising the cells to stain for FoxP3 [342].

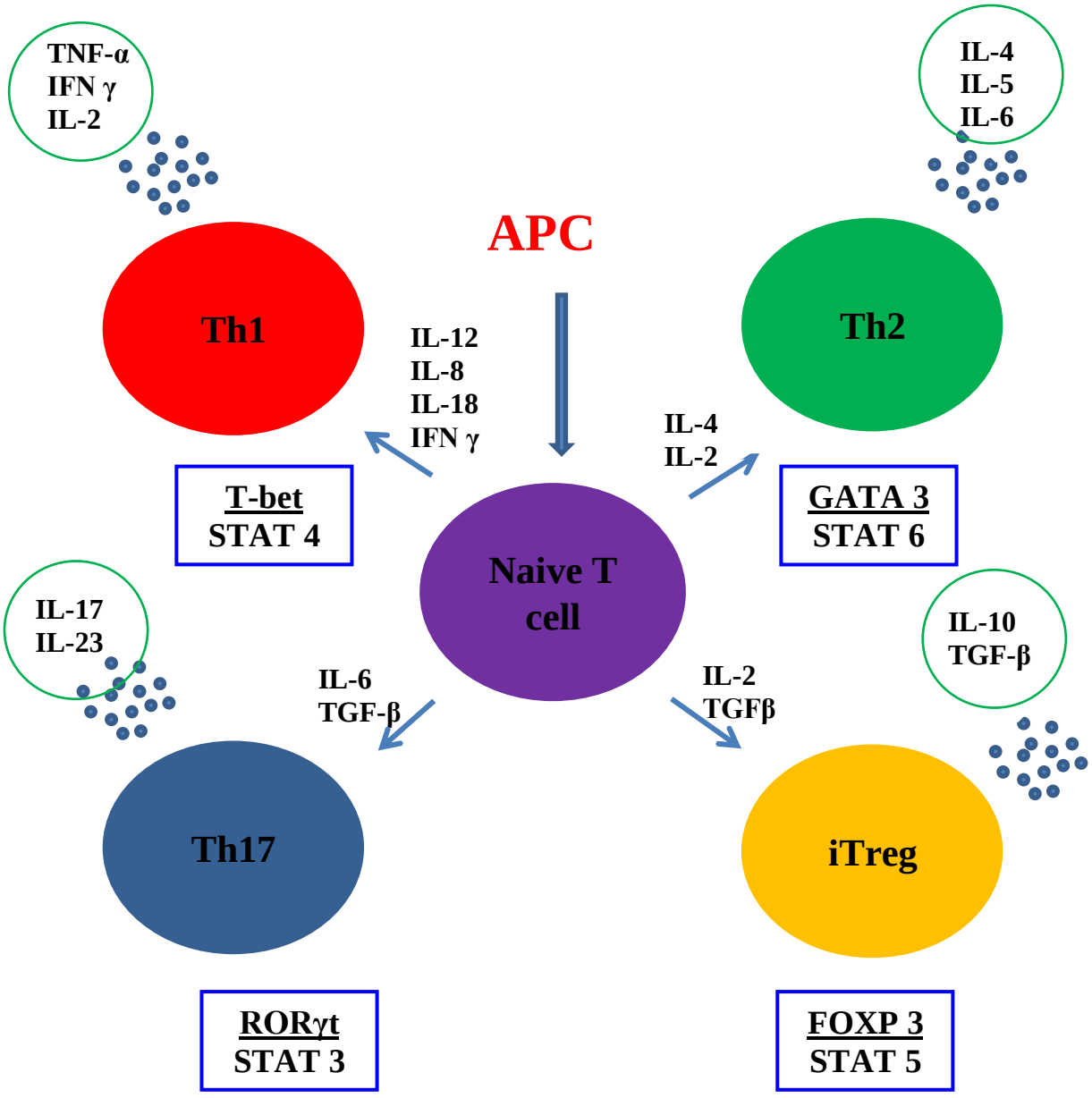


Figure 1-14: Naive CD4+ T cell differentiation (adapted and modified from[343]).

When naive CD4+ T cells are stimulated by antigen-presenting cells (APCs), they may proliferate and differentiate into one of several effectors of CD4+ Th cells, including T helper 1 (Th1), T helper 2 (Th2), T helper 17 (Th17) and regulatory T (iTreg) cells, as defined by their pattern of cytokine production and function. Several transcription factors have been suggested to coordinate this process (Blue boxes; the master regulators are underlined). Cytokines listed over the blue arrows represent major signalling cytokines which stimulate CD4+ T subset differentiations. Cytokines in green circles represent main Th subset-secreting cytokines. T-bet: T-box expressed in T cells. GATA-3: GATA-binding protein 3. STATs 3, 4, 5 and 6: signal transducers and activators of transcription 3, 4, 5 and 6). RORγt: Retinoic acid - related orphan receptor gamma.

Table 1-10: Selected CD4 T helper cell-associated cytokines and their main role in immune system.

Cytokines	Main producing cell	Target cells	Functions
Th1 secreting cytokines			
IL-2 [344]	Mainly from CD4+ Th cells. Others include: CD8+ T, dendritic & mast cells	Activated T cells	Skew naive CD4+T cells towards Th1 & Treg. ↑ CD8+ T cytotoxic activity.
		B cells	↑ Proliferation & antibody production.
		NK cells	↑ Proliferation & cytolytic activity.
TNF-α [345-347]	Th1cells, macrophages, mast & NK cells	Macrophages	↑ Cytokine expression.
		Cancer cells	Cell death.
INF-γ [346, 348, 349]	Th1& NK cells	Immune system	Modulate immune response Antiviral effects.
		APCs	↑ MHC expression & pathogen elimination.
		Activated B cells	Ig class switch to IgG2a.
		Th2 cells	Skew T cell proliferation towards Th1.
Th2 secreting cytokines			
IL-4 [301, 350, 351]	Th2 cells, Naive T cells, APC, mast cells, eosinophils & basophils	T cells	Skew naive CD4+ T cells towards Th2 & inhibiting Th1 differentiation. Regulating cell growth and apoptosis.
		Macrophages	MHC Class II expression.
		Activated B cells	IgG_1 and IgE synthesis
IL-5 [301, 352]	Th2 cells	Activated B cells & eosinophils	Proliferation differentiation
IL-6 [353]	Various, including monocytes, macrophages, endothelial cells, fibroblasts	Activated B cells	Differentiation into plasma cells
		Plasma cells	Antibody production.
		T cells	Skew CD4+ T towards Th2 & Th17 Inhibiting Treg differentiation Anti-apoptosis.
Main Treg and Th17 secreting cytokines			
IL-10 [354, 355]	Various, including Treg, Th2 and APCs cells	Monocytes & macrophages	Inhibits MHC class II expression, limiting pro-inflammatory cytokine (IL-6, IL-12, IL-18, and TNF-α) & chemokine (MCP1, RANTES, IL-8 and IP-10) production.
		Naive T cells	Differentiation into Treg.
IL-17 [356, 357]	Various, including Th17 T cells, macrophages and NK cells	APCs & neutrophils	Recruitment.
		T cells	↑T cell proliferation.
		B cells	↑antibody production.
Other cytokines			
RANTES [358, 359]	Various, including endothelial cells, fibroblasts, T cells & macrophages	Eosinophils, basophils & monocytes	Chemotaxis.
		NK & Naive cells	Chemotaxis, proliferation & activation. INF γ & IL2 production by T cells (skew naive T CD4 towards Th1).
MCP-1 [360]	Various, including endothelial cells, epithelial cells, monocytes & macrophages.	Monocytes, basophils, NK cells, memory &T cells	Chemotaxis., ↑ IL-4 production and differentiation towards Th2.
MIP-1β [358]	Lymphocytes	Monocytes &T cells	Chemotaxis.
IP-10 [361]	Secretion stimulated by INF-γ from various cells such as monocytes, endothelial cells, neutrophils & eosinophils	Monocytes, macrophages, dendritic cells, NK & T cells	Chemotaxis. Growth & proliferation. Angiogenesis at site of infection.
EOTAXIN [359, 362-365]	Various, including eosinophils, epithelial cells & B cells	Eosinophils & basophils Th2 cells	Chemotaxis & degranulation. ↑ IL-4 & IL-5 production from Th2.
IL-8 [366, 367]	Macrophages & endothelial cells	Neutrophils	Chemotaxis & degranulation.
		Th1	TNF α production.
IL-12 [354, 368]	Macrophages & B cells	NK cells	Activation.
		Th1	INF γ production.

1.4.2.2 Link between Vitamin D & immune system – putative mechanisms

The immuno-regulatory role of vitamin D was suggested several decades ago when initial observations pointed to the effect of sunshine on increased resistance to infections in experimental animals [267]. The effect of seasons and/or latitude on vitamin D status has also been discussed extensively [20]. The role of vitamin D and/or ultraviolet radiation in curing some health-related illnesses and infections has also been reported [369]. Indeed, significant progress has been made in this area after the identification of the expression of vitamin D receptors (VDR) and of 1α-hydroxylase in most cells of the immune system including monocytes/macrophages, dendritic cells, and stimulated T & B lymphocytes, which indicated the potential for local synthesis of vitamin D by these cells [370-372]. This local production of vitamin D, in addition to the expression of VDR, also suggests that intracrine actions of vitamin D may modulate immune function and help fight external challenges [373, 374]. Recent advances have shown that active vitamin D metabolites are involved in the regulation and expression of several genes implicated in innate and adaptive immune defence mechanisms [375, 376]. The evidence for vitamin D as a modulator of immune responses is reviewed below.

1.4.2.2.1 Physical barrier and innate immunity

Expression of VDR and 1 α-hydroxylase by epithelial cells in the respiratory passages, skin, gut and urogenital system suggests that vitamin D may be involved in the preservation of barrier integrity and maintaining intracellular functions [377, 378]. Its role in maintaining barrier functions is also supported by the observation that vitamin D upregulates genes which encode junctional proteins between epithelial cells, such as tight junctions (e.g. Occludin), gap junctions (e.g. connexion 43) and adherens junctions (e.g. E-cadherin) [379-381]. However, more research is needed to fully understand the role of vitamin D in this area.

Toll-like receptors (TLRs) have been identified in several cell types of the immune system, such as monocytes, macrophages and dendritic cells. Stimulation of TLRs has been found to upregulate CYP27B1 and production of 1,25(OH)2D [382]. Several studies have reported a role for vitamin D in the production of antimicrobial peptides (AMPs). Vitamin D has been suggested to stimulate human AMPs expression such as cathelicidin at different epithelial tissue sites such as bronchial [383], urogenital [384], skin [385] and myeloid cells [386], mainly by the ability of vitamin D to sense the pathogen-associated molecular patterns

(PAMPs) by means of TLRs [387]. Aside from their direct antimicrobial role, AMPs such as cathelicidin and defensins have also been reported to perform many other immune-mediating processes, including induction of chemotaxis, mast cell degranulation, stimulation of cytokine and chemokine production, cell differentiation, vascular permeability, mediators of inflammation, wound healing and the process of antigen presentation [388-391].

Finally, vitamin D has been suggested to play a vital role at the third level of defence, the specific immune response, mainly through its effect on the recruitment of phagocytic cells, promotion of healing and initiation of inflammatory responses [392, 393] as discussed below.

1.4.2.2.2 Antigen presentation and adaptive immunity

Macrophages and dendritic cells (DCs) express vitamin D receptors (VDR) and are able to present antigens to the immune cells, such as T cells [284]. Dendritic cells (DCs) are key immune system elements that processes and present antigens to T cells, and modified effector T cell responses. There are two different DC subtypes: myeloid DCs, which mainly act on the naive T cells [394] , and plasmacytoid DCs which have a more tolergenic phenotype (prevent autoimmunity) [395].

Vitamin D has been suggested to produce different effects on both DCs subsets to enable more tolergenic responses, rather than immunogenic response and thereby promote the development of regulatory T cells [396, 397]. While leaving plasmacytoid DCs unaffected, 1,25(OH)2D can block DCs myeloid maturation, causing a reduction in fully active MHC class II, CD1a (marker molecule for mature DCs, CD83 (marker molecule for mature DCs) and DCs co-stimulatory molecules (CD40, CD80 and CD86) [398].

Additionally, it has been reported that 1,25(OH)2D is able to inhibit IL-12 production by both activated macrophages and mature DCs and to stimulate IL-10 production, resulting in induction of CD4+FoxP3+ Tregs and upregulation of DC-characterized molecules such as CD152. These vitamin effects lead to inhibition of alloreactive T cell stimulation and decrease T sensitivity to alloantigens (transplant rejection) [398, 399].

Hewison et al. (2004) have shown that both macrophages and DCs are able to express mounting levels of 1α-hydroxylase as they differentiate towards a mature, antigen-presenting

phenotype. In dendritic cells, the outcome of this is that both vitamin D and its metabolite (25(OH)D3) are able to suppress the differentiation and function of these cells. Concomitantly, when the APCs become mature their ability to produce the active form of vitamin D is increased but they expresses less VDRs. The active form of vitamin D which is produced by APCs has been suggested to act in paracrine fashion on the neighbouring immature APCs to trigger more antigen presentation and the ability of mature APCs to decrease the expression of VDR prevents over-stimulation [400].

Early researchers have demonstrated that T cells express VDR and appear to be a direct target of vitamin D [401]. This was followed by several studies which showed that vitamin D is able to modulate naive T cells phenotypes and functions, affecting their differentiation into effector cell subsets and ability to produce more regulatory T cell (Treg) phenotypes [402, 403].

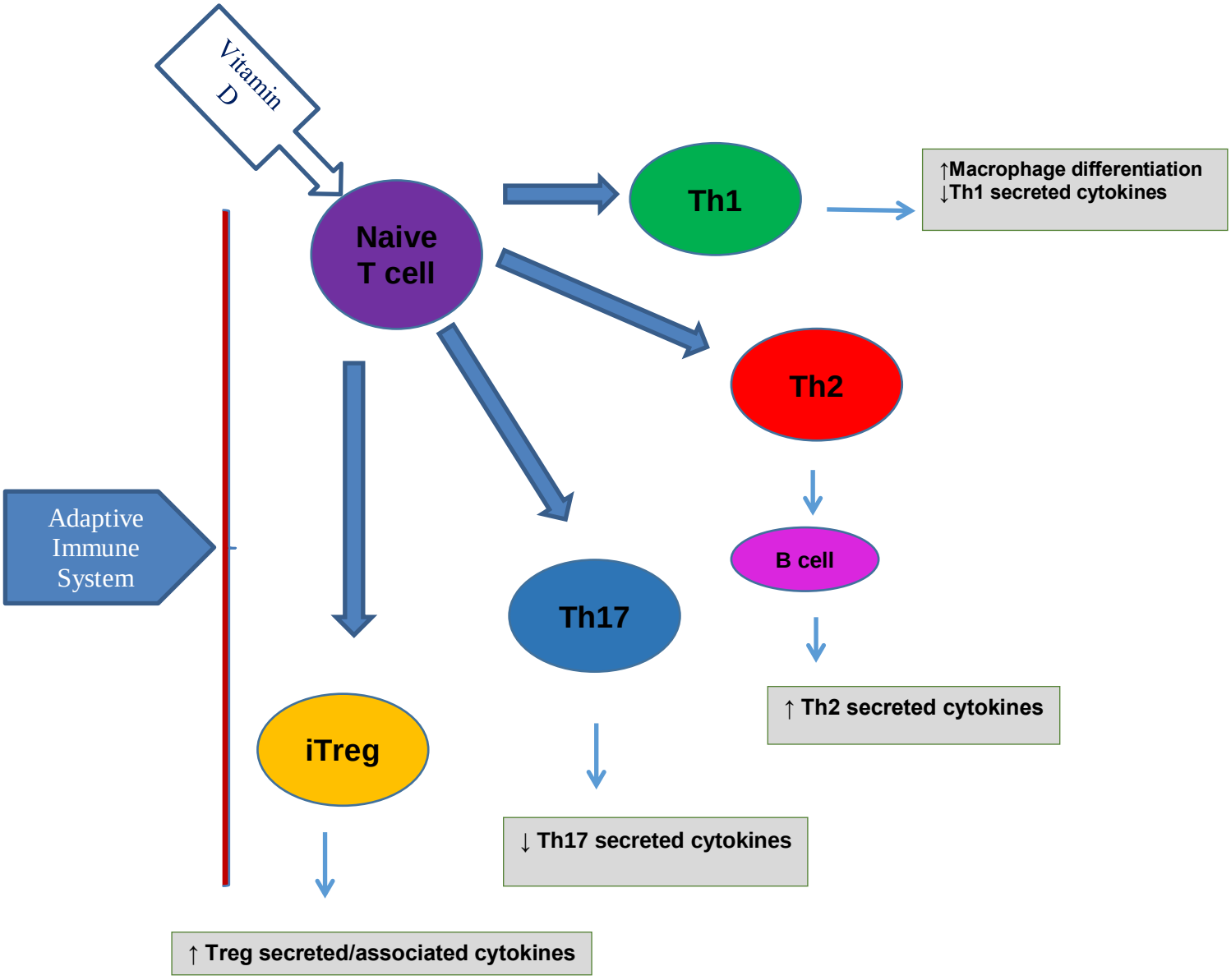
It has been suggested that vitamin D may be able to inhibit the production of several Th1 related cytokines such as TNF-α and INF-γ, whilst promoting production of Th2 cytokines such as IL-4 and IL-5 [404, 405]. However, other studies could not confirm these findings [406]. T helper 17 cells (Th17) are a recently discovered subset of Th cells, which are thought to be important in the pathogenesis of autoimmune diseases such as multiple sclerosis and rheumatoid arthritis. Experimental studies showed that vitamin D loss may lead to an increased Th17 population and vitamin D treatment causing a down-regulation of Th17-secreted cytokines such as IL-17 and IL-21 [406, 407].

Vitamin D was also shown to increase IL-10 production by Treg and induce Treg activity leading to induction of DCs and suppress T cell activation. Therefore, vitamin D deficiency may promote autoimmune diseases through both direct and indirect roles in regulating Th1/Th17 balance and in improving Treg function in addition to skewing cytokine production towards a Th2 phenotype. Figure 1-15 shows the suggested effect of vitamin D on naive T cell differentiation and their effectors cells.

Finally, a number of reports indicate that vitamin D can inhibit the generation of memory B cells from naive B cells, inhibit B cell proliferation, induce apoptosis and suppress immunoglobulin secretion and maintain the development and function of natural killer cells

[408-410]. Figure 1-16 summaries the potential role of vitamin D in the different components of the immune system.

Figure 1-15: Regulation of naive T cell differentiation and function by vitamin D.



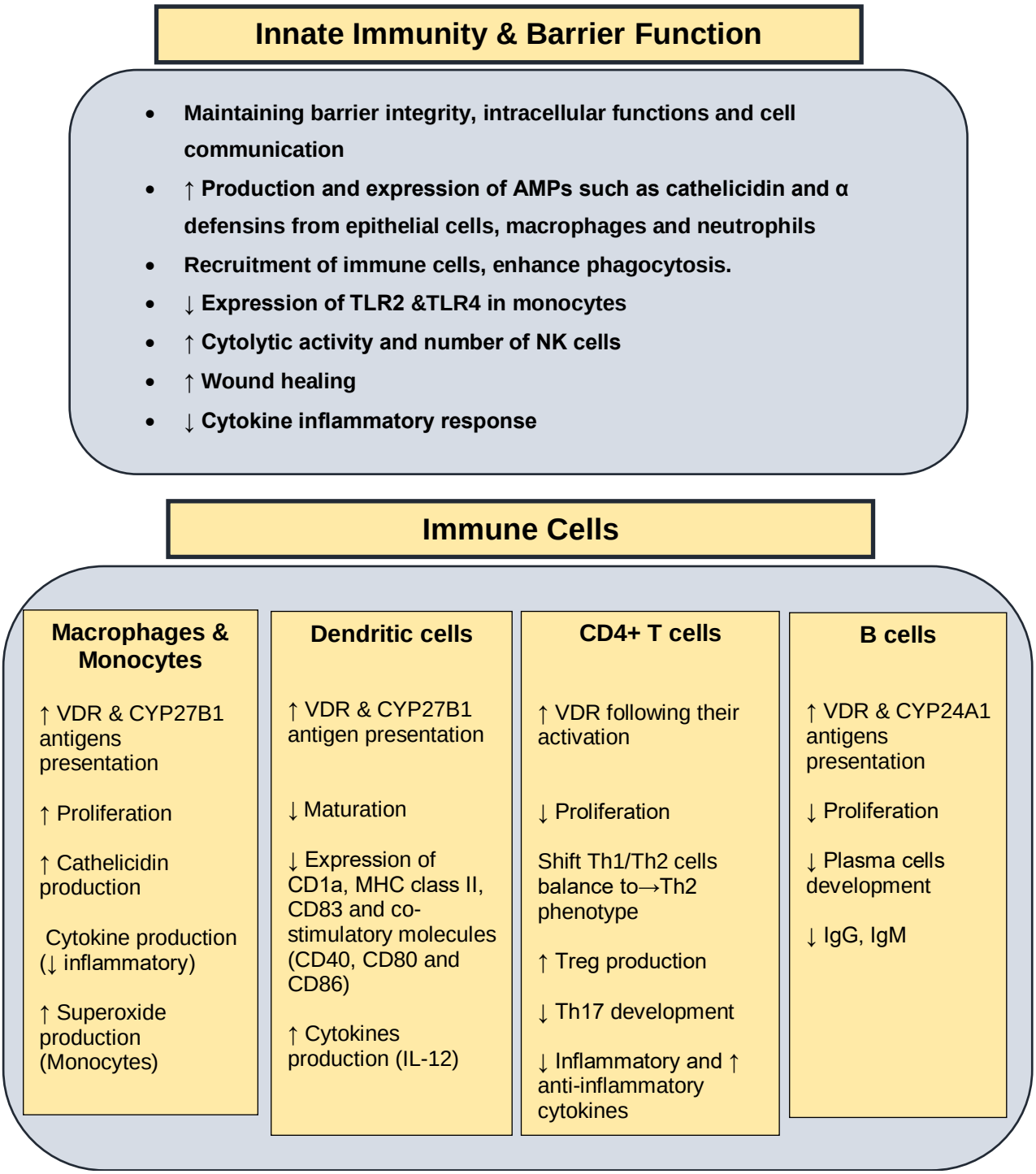


Figure 1-16: Summary of the potential action of systemic and/or locally synthesized vitamin D on different components of the immune system.

AMPs: Antimicrobial peptides. TLR: Toll-like receptors. NK cells: Natural killer cells. VDR: Vitamin D receptors. CYP27B1 (cytochrome p450- one-alpha-hydroxylase): Cytochrome p450 enzyme that catalyses the conversion of 25-hydroxyvitamin D3 (25(OH)D) to 1-alpha,25-dihydroxyvitamin D3 (1,25(OH)2D). MHC class II: Major histocompatibility complex class II molecules. IL-12: Interleukin- 12. Th cells: T helper cells. Treg: Regulatory T cell. IgG: immunoglobulin G. IgM: immunoglobulin M.

1.4.2.2.3 Link between vitamin D & immune function – evidence from observational studies

Clinical associations between the prevalence of immune related illness and vitamin D deficiency have been reported widely in the literature. Results from most observational studies report an inverse relationship between vitamin D status and the risk of acute respiratory tract infection, tuberculosis and pneumonia in both paediatric and adult populations [409, 411-413]. In addition, there is a strong association between vitamin D and the prevalence of gastrointestinal [397], urinary tract [384] and ocular infections [414]. The impact of vitamin D on several dermatological conditions such as wound healing, psoriasis, atopic dermatitis and acne has also been reported [415-418]. Finally, several autoimmune diseases such as inflammatory bowel diseases, multiple sclerosis (MS), systemic lupus erythematosus, type 1 diabetes mellitus and rheumatoid arthritis have been reviewed. Again significant associations between a low vitamin D and the prevalence of autoimmune disorders were reported [419-421].

1.4.2.2.4 Link between Vitamin D & immune function – evidence from interventional studies

As described earlier, the immunomodulatory effects of vitamin D have been well documented in vitro. It is therefore of potential importance to explore the effect of vitamin D supplementation as promising immunomodulatory therapy for infectious diseases associated with chronic inflammation and other immune system related disorders. However, despite the strong observational and theoretical links between vitamin D and immune system disorders, interventional studies looking at the therapeutic effect of vitamin D supplementation show conflicting results. In a randomized double-blind study, 4000 IU of vitamin D3 daily for one year in patients with immune disorders and frequent attacks of acute respiratory tract infection showed a 23% decrease in the number of attacks and 60% reduction of antibiotic use [422]. However, in another trial using healthy adults, administration of vitamin D did not affect the severity or prevalence of acute respiratory tract infection [423, 424]. In one study, a high dose vitamin D3 supplementation was reported to accelerate clinical and radiological improvement in pulmonary tuberculosis patients [425]. However, others reported no effect [426]. A recent systematic review of randomized, placebo-double-blind trials assessing the benefit of vitamin D administration in patients with multiple sclerosis was unable to reach a verdict with no significant effect seen in four out of five available trials [427]. Although there

are limited studies looking at the effect of vitamin D intervention in children, several publications have shown a beneficial effect of vitamin D supplementation on reducing acute respiratory tract infections [428, 429].

A randomized controlled trial looking at the effect of 100,000 IU of vitamin D3 supplementation in South Asian children with pneumonia showed a reduced number of repeat attacks in the vitamin D group (58%; relative risk 0.78; 95% CI 0.64, 0.94; $p = 0.01$) [8]. However, supplementation with the same dose once every three months for a period of 18 months did not reduce the prevalence of pneumonia in high-risk South Asian infants [430]. Table 1-11 summarises some recent clinical studies which have been conducted in children.

Accumulating experimental and animal evidence have indicated the involvement of CD4+ T cells in the pathogenesis of autoimmune diseases and immune system related disorders such as MS and HIV, and the immunomodulatory role of vitamin D on different effector CD4+ T subset balance [431]. Therefore, a number of studies were conducted to further explore these effects and look at the beneficial effects of vitamin D intervention in different CD4+ T cell subsets or related cytokines/chemokines in healthy and in diseased conditions in human subjects. In one interventional study including participants with MS, six months of daily 1000 IU vitamin D3 and 800 mg calcium supplementation significantly increased TGF-β1 levels. However, the results for IFN-γ, IL-2 and TNF-α were variable and inconclusive. The increased TGF-β1 following vitamin D supplementation provides some evidence that vitamin D could potentially improve the symptoms of MS patients. Further significant effects of vitamin D intervention as an anti-inflammatory agent were observed in another trial conducted in patients with congestive heart failure who received 1000 IU vitamin D3 and 500 mg calcium for nine months. This trial showed a remarkable increase in IL-10 in the vitamin D supplemented group and significant increase in Th1 inflammatory cytokines, TNF-α, in non-vitamin D supplemented congestive heart failure patients [404, 432]. On the other hand, weekly doses of 25,000 IU vitamin D3, but not 800 IU/day was effective in influencing Treg cell trafficking in patients infected with HIV with an increased level of CCR10 expression, while a reduction in CCR4 expression (chemokine required for HIV entry into target cell) in HIV infected patients was observed [433].

Clinical trials on vitamin D regulating serum cytokines in younger population are very limited and very little is known about the effect of vitamin D supplementation in healthy children and adults [434, 435]. This will be discussed in more detail in Chapter 7.

In summary, scientific studies support an important role for vitamin D in many aspects of the immune response. Clinical evidence from observational studies shows a strong correlation between vitamin D deficiency and the development of various immune and infectious diseases.

Table 1-11: Summary of studies reporting association between 25(OH)D and immunity in children (boys and girls).

Ref	Age	n	25(OH)D (nmol/l)	Outcome assessed	Association with outcome	Comment
Wright et al. [436]	16 y (median)	38	44.9	Severity of SLE	Yes	25(OH)D lower in SLE patients (African origin & obese) compared with control (36.8% vs 9.2%, $p < 0.001$)
Peroni et al. [437]	5.6 y	37	92.1 ± 39.1(Mild AD), 68.6 ± 21.4 (Moderate), 51.1 ± 14.7 (Severe AD)	Severity of AD	Yes	25(OH)D higher in mild AD compared with moderate and severe AD ($p < 0.05$)
Greer et al. [438]	10.1 ± 0.9 y	56	81.4 (T1DM), 70.5 (Newly diagnosed)	T1DM	Yes	25(OH)D lower in children with T1DM compared with controls [mean (95% CI) = 78.7 nmol/l (71.8-85.6) vs. 91.4 nmol/l (83.5-98.7), $p = 0.02$]
Allen et al. [439]	12.7 ± 0.7 months	928	-	Infantile food allergy	Yes	Infants of Australian parents with 25(OH)D ≤ 50 nmol/L were more likely to be allergic to peanuts (aOR, 11.51; 95% CI, 2.01-65.79; $p = 0.006$) and/or egg (aOR, 3.79; 95% CI, 1.19-12.08; $p = 0.025$)
Interventional studies						
Manaseki-Holland et al. [8]	13.1 ± 9.1 y	224	-	Reduction of pneumonia attack	Yes	Children received 100 000 IU vitamin D3 along with AB: decreased risk of repeat episode in treatment group (92/204; 45%) compared with control (122/211; 58%)
Camargo et al. [429]	10.1 ± 0.9y	143	17.47	ARIs attack in winter	Yes	Children received milk fortified with 300 IU of vitamin D3: reduction in ARIs attack in treatment group compared with control (aOR 0.52 (95% CI: 0.31-0.89)
Manaseki-Holland et al. [430]	1-11 months (Range)	1487	-	Incidence of pneumonia	No	Children received 100 000 IU vitamin D3 every 3 months for 18 months: no effect of vitamin D on the frequency of pneumonia episodes

*SLE: Systemic lupus erythematosus. AD: Atopic dermatitis. TB: Tuberculosis. AB: Antibiotics. ARIs: Respiratory infections. T1DM: Type 1 diabetes mellitus. aOR: Adjusted odds ratio. Data are shown as mean (SD), unless otherwise specified.

1.5 General conclusion

The evidence from experimental and animal studies continues to accumulate and support a link between vitamin D, glucose and immune regulation. The number of publications regarding vitamin D have increased over the last decades, due to widespread vitamin D deficiency and suggested therapeutic potential. Accumulating evidence from observational studies has also pointed to vitamin D deficiency and insufficiency as a clinical problem of global proportions. However, interventional studies have produced inconclusive results particularly among the younger population.

Chapter 2

Study Objectives and Justification

The primary aim of this PhD thesis was to conduct an interventional study of vitamin D supplementation in vitamin D deficient but otherwise healthy children, to assess the association between vitamin D deficiency and bone status, glucose homeostasis and immune function before and after six weeks of receiving vitamin D3 treatment. The research subjects all presented at the endocrine outpatient clinic at the Royal Hospital of Sick Children in Glasgow (RHSC). A second aim of this thesis was to assess the effectiveness of electronic surveillance, developed by the Scottish Paediatric Surveillance Unit (ScotPSU) to determine the current incidence of hospital-based presentation of childhood vitamin D deficiency in Scotland. As the major biological function of vitamin D is to maintain bone health, the third objective was to investigate the relationship between bone biochemical profiles with glucose and immune profiles.

To the best of this author's knowledge, most, if not all, previous interventional studies assessing the effect of vitamin D supplementation on glucose homeostasis involve higher-risk older children and/or adolescents (either obese or pre-diabetes). At the time of the study design there had been no study investigating vitamin D-regulating serum cytokines in healthy individuals. Subsequently, two studies have been conducted on healthy adults, and both have failed to observe consistent changes in circulatory cytokines, or their corresponding transcription factors [435, 440]. There is limited information available concerning the effect of improving vitamin D status on glucose homeostasis and immune function, in vitamin D deficient healthy children or infants. Due to inconsistent evidence, we have also examined the association between serum 25(OH)D levels and bone remodelling markers, and further explored measurable changes in these markers following 5000 IU vitamin D3 treatment. Finally, by linking the results from these studies with clinical management, this work will explore any changes in vitamin D related parameters following routine vitamin D replacement.

The Institutes of Medicine (IOM) defined the vitamin D deficiency as serum 25(OH)D below 50 nmol/l (20 ng/ml), and serum 25(OH)D >14 nmol/l (5.6 ng/ml) considered as severe/ profound deficiency. Vitamin D insufficiency when serum 25(OH)D >75 nmol/l (30 ng/ml) . An expert panel for the IOM recommended that a level of 50 nmol/l was

sufficient to optimize musculoskeletal health and serum 25(OH)D levels between 75-100 nmol/l have been recommended as an optimal range for multiple health outcomes. However, it is not known to what degree improving serum levels from 25(OH)D <50 nmol/l to <75-100 nmol/l is ideal for multiple health outcomes [42, 441, 442].

In Scotland, the incidence of vitamin D deficiency is largely undefined because of continual demographic change and the introduction of new public health measures such as The Healthy Start Scheme. However, evidence continues to accumulate, with resultant warnings that children presenting symptomatic vitamin D deficiency in Scotland might suffer serious consequences, such as seizure and cardiomyopathy in addition to developmental delay and increased risk of fractures [5]. There is also a need to institute a reliable system to collect data and monitor the incidence of this preventable condition. Therefore, this work was designed to estimate the current incidence of vitamin D deficiency in children using electronic surveillance systems, and to validate electronic surveillance.

Four studies were conducted during the course of my PhD, with specific aims outlined in the following section:

2.1 An Electronic Surveillance System for Monitoring Nutritional Vitamin D Deficiency in Children in Scotland (Chapter 3)

Hypothesis: An electronic surveillance system can provide reliable data to study the epidemiology of vitamin D deficiency.
Aim: To assess the effectiveness of an electronic surveillance system and determine the current incidence of hospital based presentation of childhood vitamin D deficiency in Scotland.

2.2 Effect of Vitamin D3 Supplementation on Bone Status (Chapter 5)

Hypothesis: Six weeks of vitamin D3 supplementation will improve vitamin D status, and significantly affect the markers for bone remodelling in vitamin D deficient children aged from 3 months to 10 years.
Aim: To study the baseline and post treatment relationships between serum 25(OH)D and bone remodelling markers.

2.3 Effect of Vitamin D3 Supplementation on Glucose Homeostasis (Chapter 6)

Hypothesis: Vitamin D supplementation will improve vitamin D status, and accordingly will improve the glycaemic status and insulin resistance.

Aim: To investigate the effect of vitamin D3 supplementation on glycaemic status and insulin resistance in vitamin D deficient healthy children.

2.4 Effect of Vitamin D3 Supplementation on Immune Function (Chapter 7)

Hypothesis: Vitamin D3 supplementation will improve vitamin D status, and may promote a shift in the balance between T helper 1 (Th1) and T helper 2 (Th2) cells toward Th2 domination.

Aim: To assess the effect of short-term vitamin D3 supplementation on main Th1 and Th2 cytokines, Th17 cytokine, T regulatory cytokine (Treg) and chemokines, linking the balance between Th1 and Th2 subsets. Additionally the association between these cytokines and bone remodelling markers before and after vitamin D3 supplementation was investigated.

2.5 Research Outcomes

2.5.1 Primary outcomes

- The incidence of cases of nutritional vitamin D deficiency presented to hospital-based pediatricians across Scotland between 2009 and 2011 (Chapter 3).
- The impact of the short term effects of vitamin D3 replacement on insulin resistance in healthy children (Chapter 6).
- The levels of chemokines / cytokines in plasma, pre- and post- treatment with high doses of vitamin D3 (Chapter 7).

2.5.2 Secondary outcomes

- How effective is the electronic surveillance system (Chapter 3).
- The range of clinical presentations of vitamin D deficiency in Scotland (Chapter 3).

- Whether short-term vitamin D3 replacement has an impact on glycaemic status and lipid profile (Chapter 6).
- The peripheral blood lymphocyte subset profile pre and post vitamin D3 replenishment in patients presenting with vitamin D deficiency (Chapter 7).
- Association between peripheral chemokines /cytokines and bone remodelling markers (Chapter 7).
- The impact of short term effects of vitamin D3 replacement on bone remodelling markers in vitamin D deficient healthy children (Chapter 5).

Chapter 3

An Electronic Surveillance System for Monitoring Nutritional Vitamin D Deficiency in Children in Scotland

3.1 Abstract

Background

Given its geographical position and demography, the population of Scotland is at a relatively high risk of vitamin D deficiency. There is a need to institute a reliable system that can monitor the incidence of this preventable condition.

Aim

To assess the effectiveness of an electronic surveillance system to determine the current incidence of hospital-based presentation of childhood vitamin D deficiency in Scotland.

Methods

Active surveillance was performed for two years as part of an electronic web-based surveillance programme by the Scottish Paediatric Surveillance Unit. Notifications from reporting paediatricians were followed by completion of a questionnaire. To further examine the validity of the system, cases with severe vitamin D deficiency in Glasgow and Edinburgh were identified from the regional laboratory and their clinical details were checked against those identified through the surveillance system. Vitamin D deficiency was defined as serum 25(OH)D level >50 nmol/l, severe vitamin D deficiency when serum 25(OH)D >14 nmol/l and vitamin D insufficiency when serum 25(OH)D >75 nmol/l.

Results

Between September 2009 and August 2011, 163 cases of vitamin D deficiency were notified. The majority of cases (n = 82) were reported in Glasgow with an annual incidence of 41 cases per year. Fourteen cases were reported in Edinburgh during the first year of the study and two cases during the second year. At the time of clinical diagnosis,

the median age of the children was 2 years (ranging from 3 months to 16 years). Cross-validation of data showed that amongst symptomatic cases who had a measured serum vitamin D level of less than 14 nmol/l, 89% of eligible cases had been reported in Glasgow and 33% of cases had been reported in Edinburgh.

Conclusion

The incidence of vitamin D deficiency remains high in Scotland. An electronic surveillance system can provide data for studying the epidemiology of vitamin D deficiency and may helpful for assessing the effectiveness of public health measures. However, it may underestimate the number of positive cases.

3.2 Introduction

Vitamin D deficiency, which is associated with nutritional rickets, is a preventable condition that is associated with morbidity in children. There is a considerable amount of emerging data suggesting that low vitamin D levels are associated with a diverse range of pathologies. Children and adults with vitamin D deficiency may be at higher risk of morbidity in a number of systems.

The Scottish Paediatric Surveillance Unit (ScotPSU) has recently been developed as an electronic version of the British Paediatric Surveillance Unit (BPSU) which has been very successful at monitoring the incidence of rare childhood conditions in the UK [443]. A monthly survey of symptomatic vitamin D deficiency was launched as part of the Scottish Paediatric Surveillance Unit in September 2009 (ScotPSU). The aims of the current study were two-fold: firstly, to study the incidence of cases of nutritional vitamin D deficiency that present to hospital-based paediatricians across Scotland and, secondly, to validate the electronic surveillance system by cross-referencing cases identified through the clinical biochemistry service.

3.3 Methods

As part of the Scottish Paediatric Surveillance Unit (ScotPSU) programme, all paediatricians known to the Royal College of Paediatrics and Child Health in Scotland and the Scottish Paediatric Society are sent an email each month containing a list of conditions being surveyed. This email notification is linked to the ScotPSU website (www.scotpsu.co.uk) where definitions and reporting instructions are given for the conditions being surveyed. The reporting paediatrician is asked to check boxes against any of the reportable conditions they have seen in the preceding month, or to check a 'nil return' box if none have been seen, and return the email to the ScotPSU. For this study, paediatricians were asked monthly from September 2009 – August 2011 to report the number of new cases of symptomatic vitamin D deficiency seen in the previous month (see Figure 3-1).

The inclusion criteria were children with symptoms or signs related to vitamin D deficiency or symptoms related to hypocalcaemia. The exclusion criteria were osteopathy of prematurity within the previous six months, rickets due to genetic or drug-induced defects of phosphate or vitamin D metabolism, and coexistent chronic conditions such as chronic malabsorption, liver disease, chronic renal insufficiency and thalassaemia. 'Positive returns' were identified by the ScotPSU's coordinator, and electronically forwarded to the study investigator, who then contacted the reporting clinician directly and sent the study questionnaire. The data collection form did not contain any patient identifiable information.

The questionnaire was sent to the referring paediatrician, and anonymous details about the patient, regarding the presenting features that were ascertained in each case at the time of diagnosis, was requested. The date of birth, as well as centre and mother's maiden name, was requested to allow the identification of duplicate reports (see appendix A-1: ScotPSU/ Case Report Form). If the child had been notified by several paediatricians, one clinician was selected to give the clinical details. Investigators did not make any contact with the patients and the ScotPSU did not receive any identifiable data. For some cases, the researcher visited the hospitals in order to collect some missed information directly from the reporting clinician. The study did not collect any recognisable personal patient data and was approved as a health service evaluation by the National Research Ethics Committee.

To examine the validity of the reporting system, cases of vitamin D deficiency in Glasgow and Edinburgh where the serum 25 hydroxy-vitamin D (25(OH)D) concentration was measured as undetectable at the regional clinical biochemistry laboratory were identified and their clinical details were studied. These cases were then compared to the reporting instructions of the ScotPSU survey to check the percentage of eligible cases that were not notified.

3.4 Statistical analysis

All continuous data were described as medians and ranges and categorical data were compared using Chi-squared tests (Microsoft Excel 2007).

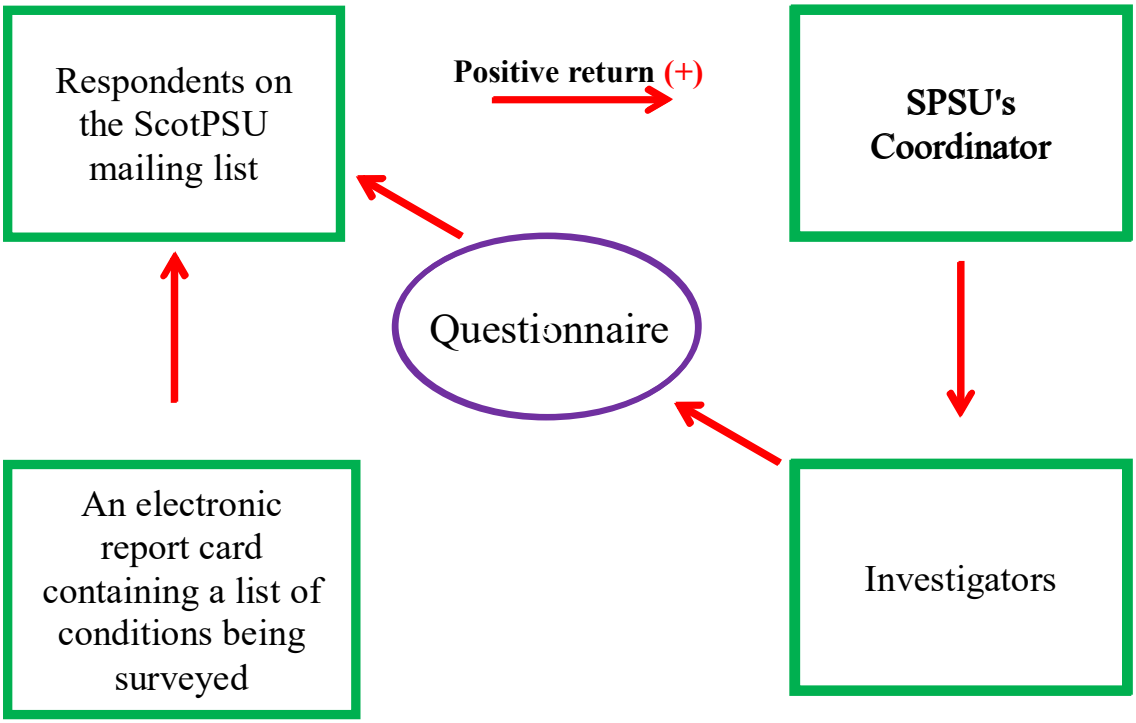


Figure 3-1: Monitoring of nutritional vitamin D deficiency in children in Scotland via the Scottish Paediatric Surveillance Unit reporting system.

3.5 Results

3.5.1 Demography

Over the two-year study period, 163 cases were notified and, of these, 54 were excluded for a range of reasons including duplicate notifications (n = 9), failure to return questionnaire (n = 16), incorrect notification (n = 16) and coexistence of chronic diseases (n = 13) (see figure 3-2). Of the remaining 109 cases, in 103 (94.5%), details of ethnic origin were available: 9 (9%) cases were classed as white whereas the rest were broadly classed as non-white (Table 3-1). Of the 109 cases, 82 (75%) were reported from Glasgow and 16 (15%) were reported from Edinburgh. Based on the total population of white and non-white children under the age of 15 years in Scotland as reported by the General Register Office for Scotland in 2009 (www.gro-scotland.gov.uk), the incidence of hospital presentation of vitamin D deficiency was 0.8/1000 in Glasgow and 0.19/1000 in Edinburgh. The reported incidence in non-white children was greater in Glasgow at 7/1000 compared to 2.6/1000 in Edinburgh (p = 0.009) when calculated over the two years of the study (Table 3-2). When comparing the two regions over the first year, the incidence remained higher in Glasgow, but did not reach statistical significance (p = 0.08).

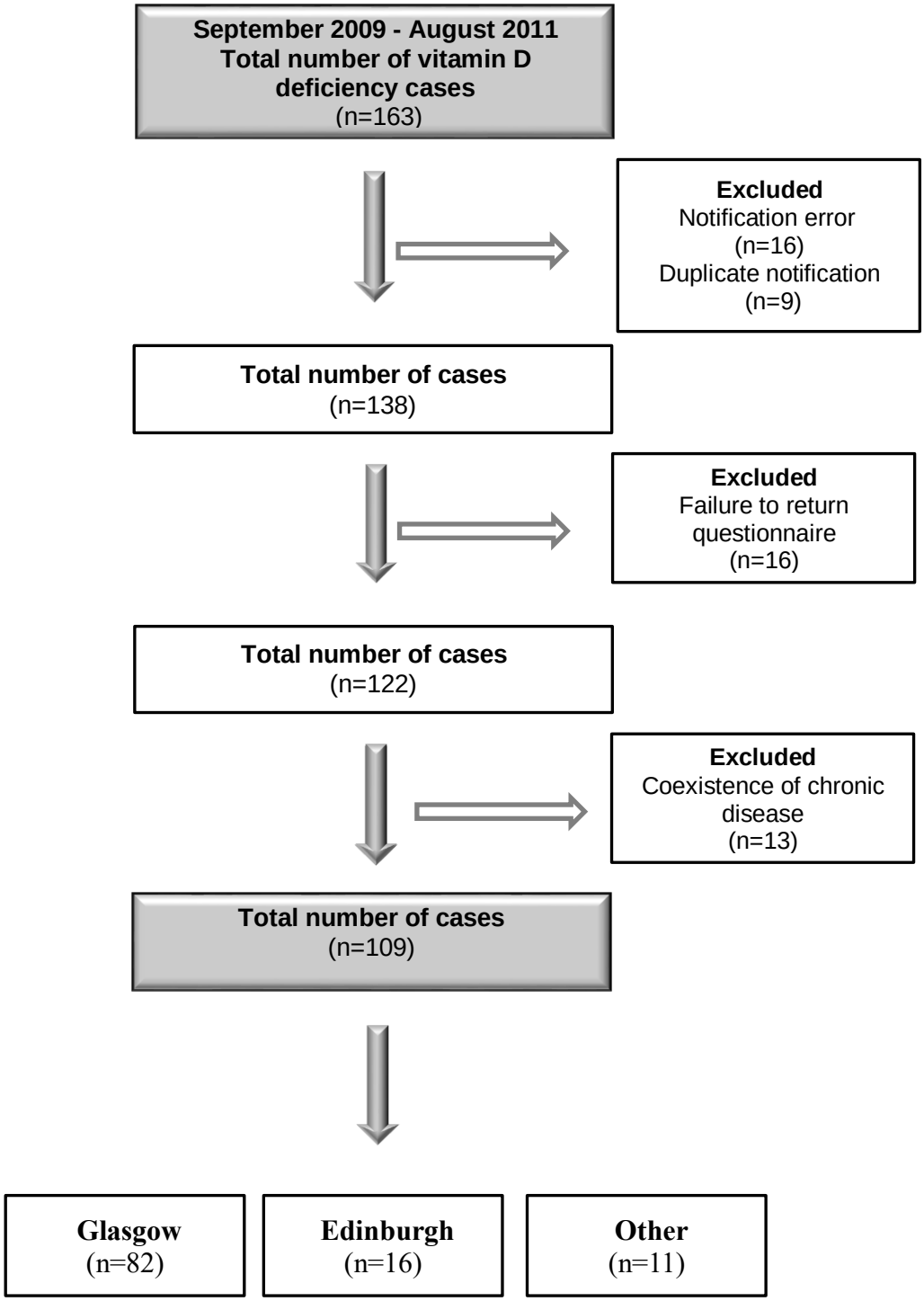


Figure 3-2: The flow diagram of study notifications.

Table 3-1: General characteristics of notified cases.

	N	%
Sex (109)*		
F	49	45
M	60	55
Age (107)*		
0-3Y	67	63
3-6Y	12	11
6-16Y	28	26
Ethnic origin (103)*		
South Asian	55	53
Sub-Saharan African	21	20
Middle Eastern	9	9
Caucasian	9	9
North African	6	6
Other	2	2
Chinese	1	1
Season (103)*		
Autumn	30	29
Winter	30	29
Spring	28	27
Summer	15	15
Referral source (95)*		
GP	45	47.5
Orthopaedic	25	26
Accident and Emergency	13	14
OPD Medical	9	9.5
Inpatient Medical	3	3
Breast Feeding (67)*		
Yes	57	85
No	10	15
Treatment (95)*		
Vitamin D only	**37**	39
Ergocalciferol	15	
Cholecalciferol	14	
Alfacalcidol	2	
Not specified	6	
Vitamin D and Calcium	**25**	26
Ergocalciferol & Calcium	18	
Cholecalciferol & Calcium	7	
Multivitamin Only	**31**	33
Multivitamin and Calcium	**2**	2

*Figures in parentheses denote the number of cases for which information was available.

Table 3-2: Distribution of reported children from Glasgow, Edinburgh and other centres according to year of notification and extent of biochemical vitamin D deficiency.

	25(OH)D (nmol/l) (Sep 2009 - Aug 2010)					25(OH)D (nmol/l) (Sep 2010 - Aug 2011)				
	<14	**14-25**	**26-50**	**>50**	**NK**	**<14**	**14-25**	**26-50**	**>50**	**NK***
Glasgow (n=82)	18	10	5	2	7	10	10	6	-	14
Edinburgh (n=16)	7	6	-	-	1	-	-	-	-	2
Other (n=11)	5	2	1	-	-	1	-	-	-	2

*__NK__, unknown / unavailable serum (25(OH)D) data in 26 cases.

3.5.2 Cross-validation of notification

To examine the completeness of the notifications, childhood cases of sever vitamin D deficiency (serum 25(OH)D level of <14nmol/l) were identified in Glasgow and Edinburgh through the biochemistry laboratory and cross-referenced to those cases notified through ScotPSU over the one year period of the study.

In Glasgow, of the 72 children identified through the laboratory records between September 2009 and August 2010, 38 had chronic diseases which would have predisposed them to vitamin D deficiency and these were excluded. Of the remaining 34 cases, 10 cases had not been notified through the ScotPSU survey. Three of these 10 cases were asymptomatic and one was being investigated for failure to thrive and did not have any symptoms which could be attributed to vitamin D deficiency. In two cases, the reason for checking 25(OH)D could not be clarified because the medical records were not available. In the remaining one case, the test was performed by a general practitioner who would not have been included in the ScotPSU survey. In summary, cross-checking cases of severe biochemical vitamin D deficiency in Glasgow over a one year period of the ScotPSU survey revealed that of the 34 children with a serum 25(OH)D concentration of <14nmol/l who did not have a coexisting chronic disease, only three (11%) cases of clearly symptomatic vitamin D deficiency had been missed by the ScotPSU survey (see Figure 3-3).

In Edinburgh, 66 childhood cases of vitamin D deficiency with a serum 25(OH)D of <14nmol/l were identified through the biochemistry laboratory and cross-referenced to the cases notified through ScotPSU during 2010. Twenty-nine children had chronic diseases which predisposed them to vitamin D deficiency. Of the remaining 37 cases, 29 cases had not been notified to the ScotPSU survey. Nine of these were asymptomatic and were being investigated because of failure to thrive, family concerns and suspected accidental ingestion of vitamin D tablets. In two cases, the medical records were not available. In the remaining two cases, the test was performed by a general practitioner. Thus, of the 37 children in Edinburgh with a serum 25(OH)D of <14 nmol/l who did not have a coexisting chronic disease, 16 (67%) symptomatic cases were not reported through the ScotPSU survey (see Figure 3-4).

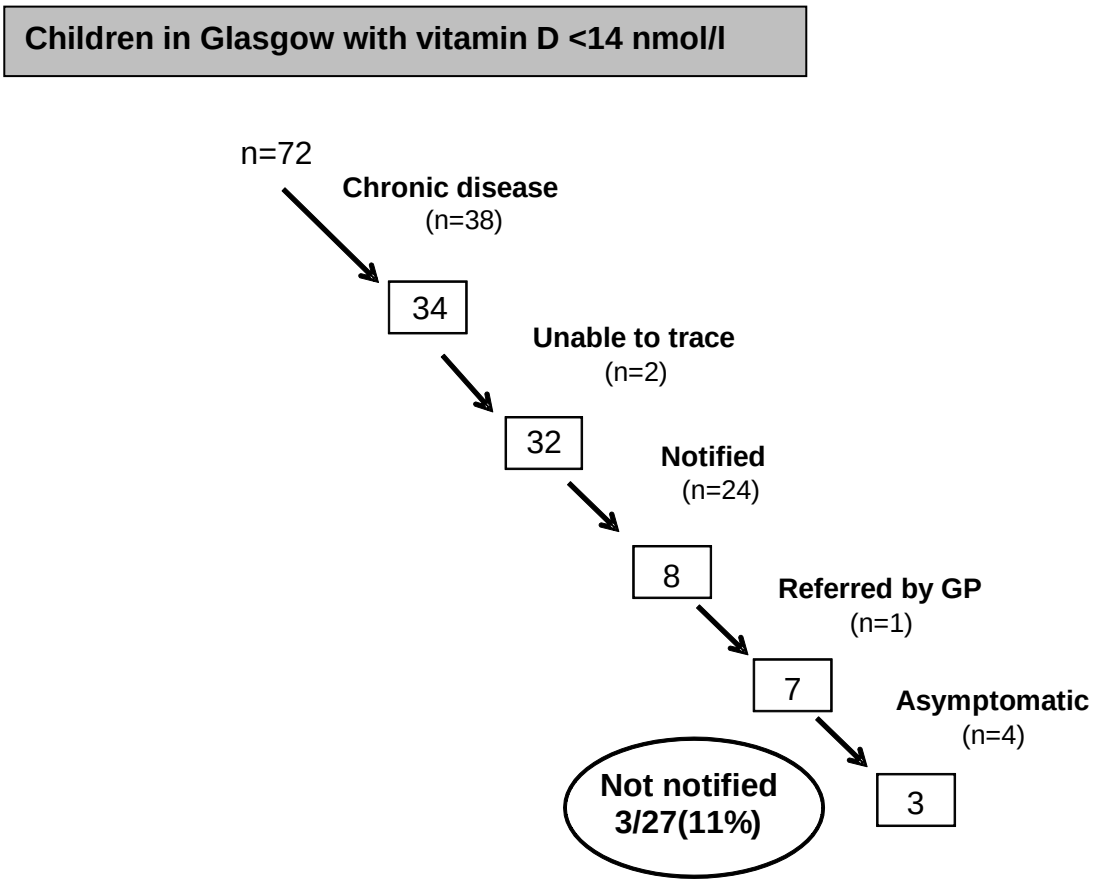


Figure 3-3: An assessment of the reliability of the ScotPSU surveillance system for identifying symptomatic cases of vitamin D deficiency presenting to paediatricians in Glasgow.

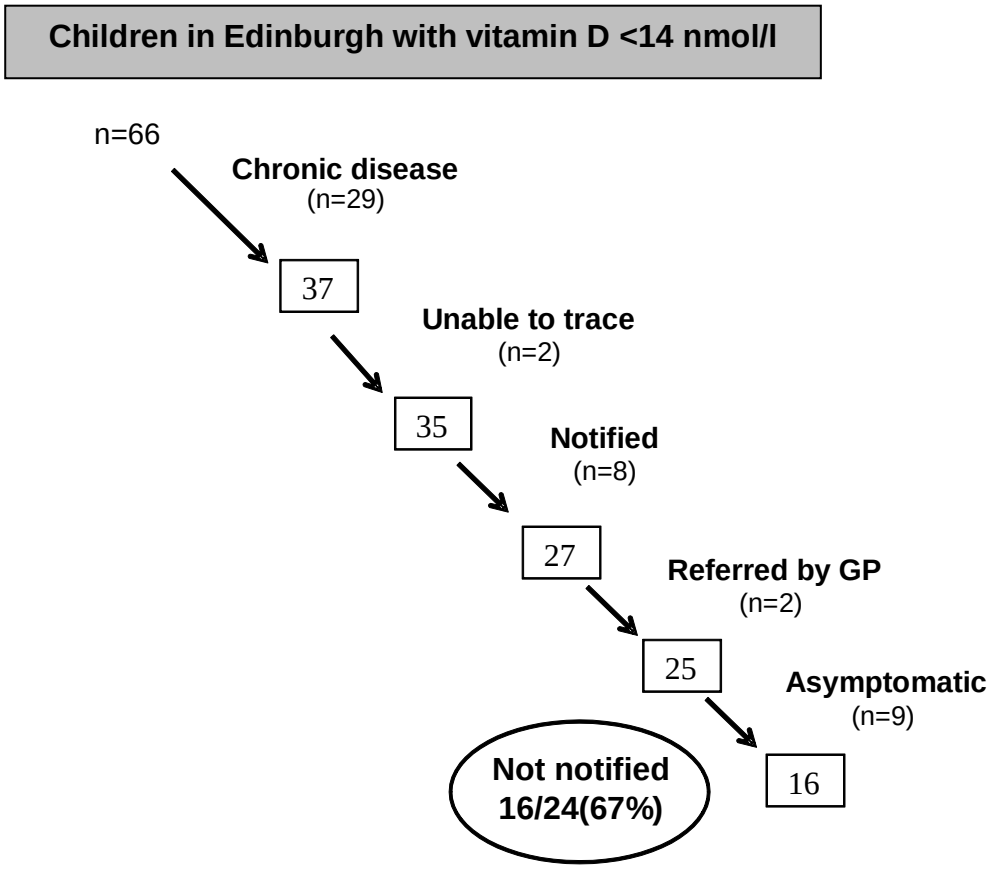


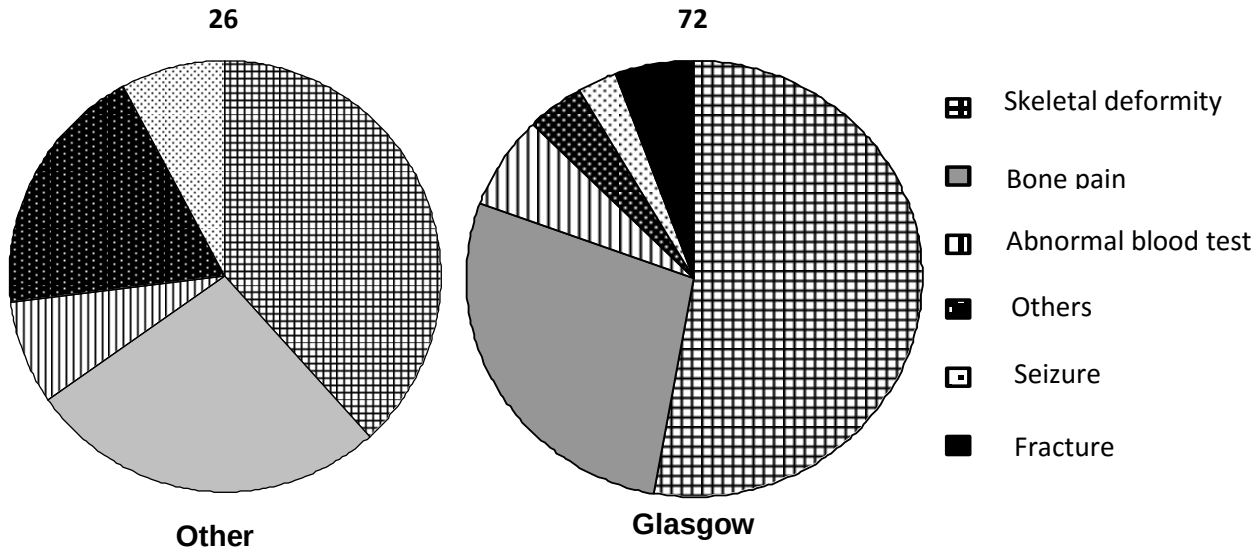
Figure 3-4: An assessment of the reliability of the ScotPSU surveillance system for identifying symptomatic cases of vitamin D deficiency presenting to paediatricians in Edinburgh.

3.5.3 Clinical features

The median age for the 109 children was 2 years (range, 3 months - 16 years). In 83 (76 %) cases, the children had skeletal based clinical suspicion of vitamin D deficiency. This included bowed legs or concerns about gait in 41 (38%) children at a median age of 2 years (7 months - 4 years), bone pain in 27 (25%) children at a median age of 7.7 years (11 months – 9.5 years), wrist swelling or chest wall deformity in 7 (6%) children at median age of 15 months (6 months - 4 years), features of rickets on an x-ray performed at the time of a fracture in 4 (4%) children at a median age of 11 months (4 months - 17 months), and hypocalcaemic seizure in 4 (4%) children at a median age of 8 months (4 months - 9 months). In 11 (10%) children, 10 of whom were from Glasgow and one from elsewhere, information about the clinical presentation was not available. In 7 (6%) children, a family history of symptomatic vitamin D deficiency was the sole reason for investigation. In 8 (7%) children, there were other causes of referral such as concerns about nutritional status, recurrent constipation and general dietary concerns. The proportions of the different categories of presentation were similar between Glasgow and the other centres (see figure 3-5).

Of the 40 cases that had presented with bowed leg and/or gait disturbance, an x-ray had been performed in 32 (80%) children. There was no information available about the radiological image in the remaining eight children. Of the 32 children who had an x ray done, 27 (84%) had radiological signs of rickets. All four cases of fractures were reported from Glasgow and were of South Asian origin, breast fed and had radiological signs of rickets with undetectable serum 25(OH)D levels in three of these cases. Nine children (8%) were reported to be white Scottish in origin, of which six were from Glasgow and three from other locations in Scotland. Of these nine cases, bone pain was the presenting feature in four cases, bowed leg and/or gait disturbance in two cases and concerns about nutritional status in the remainder.

Figure 3-5: Proportion of reported presentations in Glasgow and elsewhere



Values above the chart represent the actual number of cases in each reported centre.

3.5.4 Management of vitamin D deficiency

Clinical details about the treatment of vitamin D deficiency were available in 95 of 109 cases notified to the ScotPSU. Of the 95 children, 34 (36%) received oral vitamin D and 3 (3%) received intramuscular injection as the sole therapy. Oral preparations in the 34 children included ergocalciferol (15) and cholecalciferol (16), with doses ranging from 1,000-12,000 IU/day for four to nine weeks. In three cases the oral preparation of vitamin D was not known. Of the 95 children, 25 (26%) received both vitamin D and calcium supplements. In 2 children (2%), multivitamin and calcium supplements were the reported treatment regimen and 31 (33%) received multivitamins (Abidec: 10, Dalivit: 15, Healthy Start vitamins: 2, unknown: 4). In 4 cases, vitamin D was administered as Calcit D3, Calcichew D3 and 1α-hydroxycholecalcidol. There was no clear relationship between the severity of vitamin D deficiency (clinical or biochemical) and the therapy received (see Figure 3-6).

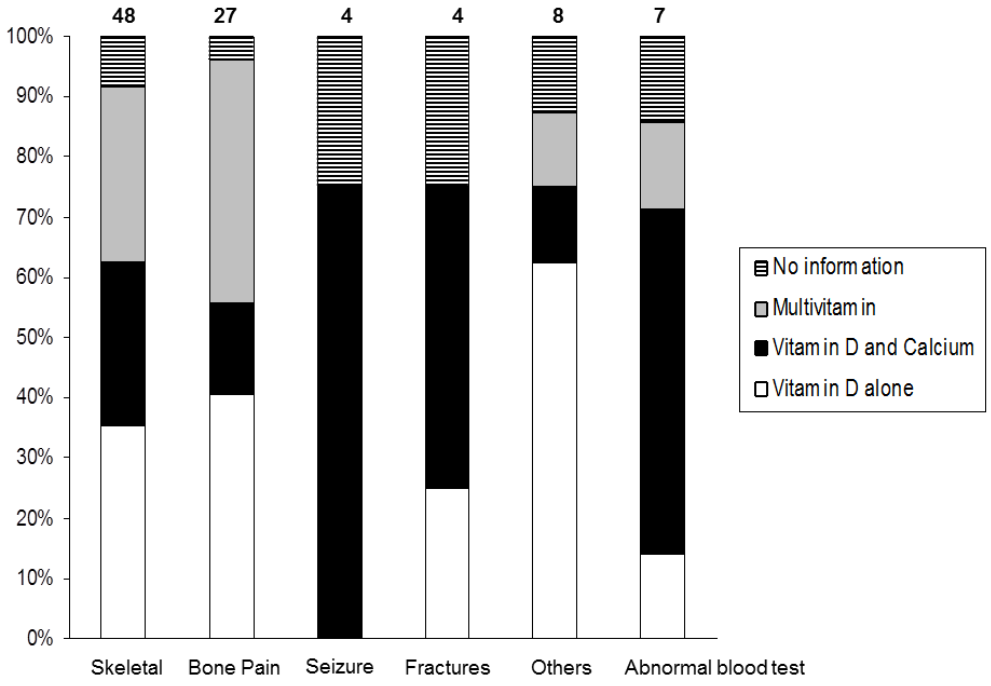


Figure 3-6: Types of vitamin D therapy administered categorised according to type of presentation.

Figures above the bars represent the actual number of cases in each presentation category.

3.6 Discussion

Vitamin D deficiency rickets has been historically recognized as a widespread health problem in the UK [2, 444]. As a consequence, a number of preventative measures have been introduced in an attempt to decrease the prevalence of vitamin D deficiency, especially among vulnerable groups such as ethnic minorities, and pregnant and lactating women. A Greater Glasgow Health Board campaign was launched in 1979 targeting vitamin D supplementation among the Asian community [109, 110]. Although guidelines for vitamin D supplementation were issued in the UK in 1994, a survey carried out in 2001 showed that vitamin D deficiency still exists, raising concern over the ineffectiveness of public health measures [106].

Anecdotal reports suggest that vitamin D deficiency occurs across Scotland but the relative geographical incidence is unclear [193, 445]. The increase in the occurrence of vitamin D deficiency among the population in Scotland has been attributed mainly to its northerly location, insufficient dietary intake and population mix [4]. As a result, the Healthy Start Scheme was implemented in 2006 but recent surveys indicate that symptomatic vitamin D deficiency is still prevalent among children in the UK [5, 446]. However, this survey may have underestimated the true incidence. There is a need for more effective public health measures which particularly target the at-risk population in this region, but most importantly there is a need for a surveillance system that monitors incidence uniformly across Scotland.

The current study also shows that about 10% of symptomatic cases are reported in children who are not of a South Asian, African or Middle-Eastern background. The presenting clinical features in this surveillance were also similar to those previously published [5]. The ScotPSU survey targets paediatricians but it is possible that children with vitamin D deficiency also present to other professionals such as general practitioners and orthopaedic surgeons who are not included in the survey [446]. It is, therefore, likely that the actual incidence of symptomatic vitamin D deficiency is higher than that identified in this survey.

The reported incidence of vitamin D deficiency was higher in Glasgow than Edinburgh. It is possible that the threshold for notification may have been different in the two centres. The children from Glasgow had a varied level of vitamin D deficiency whereas the ones from Edinburgh had more severe biochemical deficiency, suggesting that the threshold for investigating and reporting cases of vitamin D deficiency may be different between the two

centres. However, a higher incidence in Glasgow was found even when comparing regional differences between these groups of severe cases, although it did not reach statistical significance. Furthermore, the proportion of cases with different clinical presentations was also similar when comparing between Glasgow and other centres. There was a marked fall in notifications in cases in Edinburgh compared with Glasgow in the second year of the survey. In the absence of a clear change in public health interventions, this may simply reflect a change in enthusiasm for notification. It is also possible that vitamin D deficiency is more prevalent in the west of Scotland compared with the east, possibly due to differences in the ethnic variation, climate or nutritional behaviour. The adjustment for ethnic background did not account for detailed ethnic variations but simply examined white vs non-white backgrounds and there is a need for further refining of this analysis. Validation of the ScotPSU notification of affected cases by checking a proportion of the notified cases through the clinical biochemistry department was helpful and revealed that only a minority of symptomatic cases with profound biochemical vitamin D deficiency remained unreported in Glasgow. This exercise was not performed for those with milder levels of vitamin D deficiency which would have led to a higher identification of unreported cases. However, amongst similar cases in Edinburgh, there was a much higher number of cases which were unreported. It is, therefore, likely that the lower incidence of cases of symptomatic vitamin D deficiency in Edinburgh may have been partly due to a lower general rate of reporting these cases.

The recommended method to manage vitamin D deficiency is to provide the child with a high dose of vitamin D either as an oral daily dose for 8-12 weeks or as a single oral or intramuscular dose [42]. To maintain a sufficient vitamin D level, children should be provided with a maintenance dose of 400-800 IU vitamin D per day thereafter, usually as part of a multivitamin supplementation [42]. However, this diversity in management of severe vitamin deficiency among different practises could be attributed to the absence of a clear consensus in the literature regarding treatment regimes of vitamin D deficiency[447]. Approximately one third of the children in the current study received suboptimal therapeutic doses of vitamin D. Similar to previous observation; a small number of cases were treated with Calcit D3, Calcichew D3 and 1α-hydroxycholecalcidol.

The main strength of this study is the use of an established active surveillance system, which is further supported with cross-validation. Musculoskeletal clinical manifestations of vitamin D deficiency may present to other professionals such as GPs and orthopaedics, and clinical evaluation may be carried out directly at their local clinics. Therefore, cross-validation would address this issue. Our recommendations for future applications of an electronic surveillance system is to use an additional independent source for reporting in order to make comparisons and to address any lack of reporting, and to assess the epidemiology of symptomatic vitamin D deficiency more broadly in the UK.

In summary, this study represents the first description of an electronic surveillance system which could be used for notification of cases of vitamin D deficiency presenting to hospitals. It demonstrated a similar incidence to that reported recently in Glasgow, confirming the ongoing presence of profound vitamin D deficiency among children in Scotland [5]. Profound vitamin D deficiency, presenting primarily as rickets is a real problem in Scotland. An electronic surveillance system targeted at paediatricians in Scotland has confirmed the continued presence of symptomatic vitamin D deficiency in children. The incidence in Glasgow for two consecutive years is similar to that previously reported and seems to be higher than in the rest of Scotland. However, this may have been due to changes in levels of awareness of referring clinicians. Therefore, some of these differences may be due to ascertainment bias and future use of such systems needs to consider this bias.

Chapter 4

Methodology and Demographics

Effect of vitamin D supplementation on bone status, glucose homeostasis and immune function in children with vitamin D deficiency

4.1 Summary

This chapter includes the research methodology, summary of recruitment procedures, and inclusion/exclusion criteria to be applied to Chapters 5, 6, and 7. It also summarizes information obtained by the Study Case Report Form (CRF).

4.2 Ethics approval

The West of Scotland Research Ethics Service (REC3), REC Reference: 11/AL/0291 approved the studies for the bone (Chapter 5), glucose (Chapter 6), and immune (Chapter 7) components of the thesis.

4.3 Sample size

The studies described were performed as pilot studies for a PhD project, and involved identifying all children with symptomatic vitamin D deficiency at the Royal Hospital of Sick Children in Glasgow (RHSC). When the study was designed, the researcher was unaware of any previous studies examining the impact of high dose of vitamin D supplementation on bone biomarkers, glucose homeostasis and cytokines profile in vitamin D deficient, healthy children. It was therefore difficult to calculate the sample size adequately and we anticipated that this pilot study will provide novel data as a foundation for future studies.

4.4 Inclusion and exclusion criteria

The study included children under 10 years old, referred for evaluation and management of suspected vitamin D deficiency at the endocrine clinic at RHSC. Those individuals taking vitamin D supplementation or with a coexisting chronic disease such as chronic malabsorption, liver disease, chronic renal insufficiency, osteopathy of prematurity, genetic, or drug-induced defects affecting phosphate or vitamin D metabolism or thalassaemia were excluded.

4.5 Study design

Eligible participants were identified and recruited by the researcher (the PhD student) from the Endocrine Outpatient Clinic at the RHSC during their first clinical visit. Detailed information about the study was discussed privately, and the information sheets were handed to potential participants by the researcher at the end of the clinic. Invitation letters with information sheets were posted for those who attended different endocrine clinics at the RHSC, at the same time, or who were refered by a different clinic (orthopedics or outpatient clinic).

A week or two after the first clinical review, a routine clinical biochemical assessment was performed on each patient in a fasting state in the clinical day investigation unit (Ward 1C) at the RHSC. If the child/parent consented, an additional blood sample (5ml) was collected during this visit, at the time of the routine venepuncture on Ward 1C. In addition, the child was X-rayed at the RHSC by the consultant pediatric radiology as a routine clinical procedure to check for rickets. At this visit, the children were prescribed oral vitamin D3 supplements comprising 5000 IU cholecalciferol to be taken once a day. They were then reviewed on Ward 1C six weeks later, when they had a repeat assessment and another blood sample collected (Table 4-1).

Routine clinical information about the cases was collected using similar information to that detailed in the Case Report Forms provided in the Scottish Paediatric Surveillance Unit Study (see appendix B-1). This included capture data, detailing demographics, ethnic background, perinatal history, feeding history, developmental milestones, and previous use of supplements. Additional health information was also gathered, and a full clinical examination was carried out to identify any signs of vitamin D deficiency, as listed in the ScotPSU's CRF.

Anthropometry measurements, i.e. weight and height, were also recorded. Body mass index (BMI) was calculated as weight in kilograms (kg) divided by the square of height in meters (m2). According to The National Institute for Health and Care Excellence (NICE) recommendations, the most suitable measure to define obesity is BMI ≥ 98th BMI centile (>2 SDS above the mean), using the UK 1990 growth chart produced with the LMS method (the distribution of BMI at each age by its median (M) and coefficient of variation (S), in addition to a measure of skewness according to the Box-Cox power (L)).

Therefore, BMI was expressed as a standard deviation score (BMI SDS), and the BMI SDS cut-off for an obese child was > 2.054.

As the BMI is difficult to interpret in infancy and any abnormality in BMI during this period should be considered as clinically significant only if it persists after the second year of life [448, 449]. Therefore, for the children younger than 24 months, the weight and age standard deviation score (Wt SDS) was measured instead of the BMI SDS.

Table 4-1: Timetable for investigations and visits.

Procedures	Visit 1*	Visit 2**	Visit 3***	Visit 4****
Time intervals	1-2 weeks before tests	Week 0 (baseline)	Week 6 (end of the study)	6 months
Routine clinical visits	+	+	+	+
Study visits		+	+	
Information about study	+			
Consent obtained		+		
Clinical blood tests		+	+	
Study blood tests		+	+	
X-ray		+		
High dose Cholecalciferol		5000 IU units orally for 6 weeks		
Low maintenance dose			Daily oral supplements of 400 IU from this visit onwards	

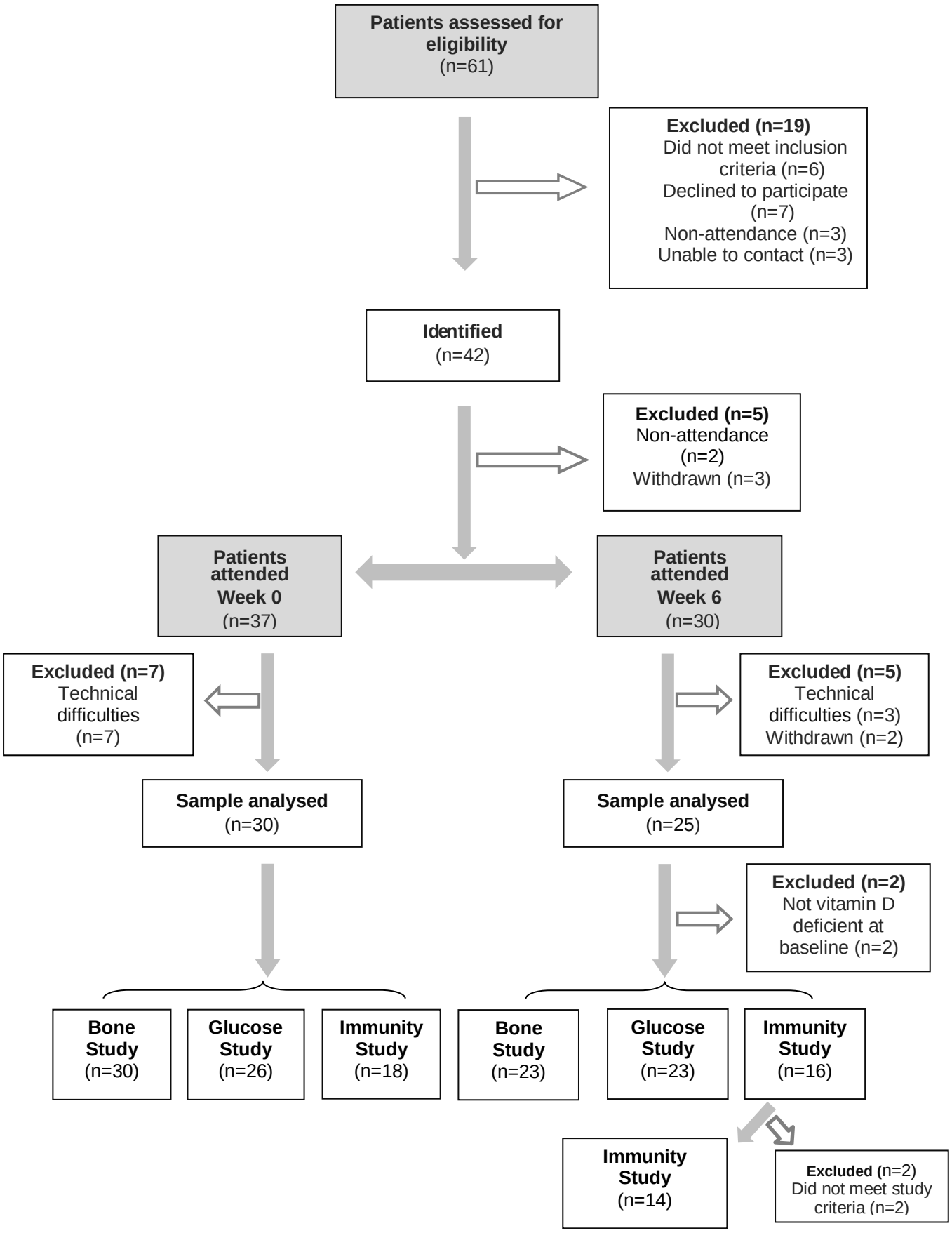
*****Visit 1: routine clinical assessments were carried out by the clinician, and information sheets were handed to the patient by the researcher. **Visit 2: routine clinical samples and study samples (if consent had been granted) were collected. At this visit patients received the vitamin D3 treatment. ***Visit 3: six weeks after visit 2, the routine clinical samples and the study samples were collected and patients received a letter for their general practitioner recommending prescription of a multivitamin supplement. Information about patients' enrolment and treatment, once received, were sent directly to the general practitioner by the researcher (see appendix D-2). ****Visit 4: patients underwent a routine clinical follow-up by the clinician.

4.6 Recruitment summary

Figure 4-1 shows a flow chart summarising the study recruitment. In total, 61 children were identified and screened during the 24 months of the study period between 2011 and 2014. Of these, 42 met the inclusion criteria and agreed to participate. Nineteen children were excluded, and of those 19 children, six did not meet the inclusion criteria (two children were already on high dose vitamin D3 supplementation prescribed by their referring clinician prior to the endocrine clinical appointment, two children were older than 10 years old, one child suffered from a concurrent thyroid illness and one had hypophosphatemic rickets). Of the 42 children who qualified for the study, 37 were recruited and attended the first study visit. Thirty samples were analysed at the first visit, for use at different stages of the project.

Thirty samples were analysed for the bone study at baseline, and 25 samples were analysed at week 6 (see Chapter 5) for the same study. Twenty-five samples were analysed at week 6 for the glucose study, of which only 19 had full glucose homeostasis parameters available at the two time points (see Chapter 6). All the children who participated in the glucose study had an available bone profile and participated in the bone study. Of those 19 children who had their glucose and bone data analysed, 17 children had an available cytokine profile. At the end of the study, during the process of data analysis, two children were excluded as both of them showed vitamin D levels within the normal reference range at the baseline study visit. Therefore, only 23 samples were suitable and participated in bone and glucose studies. Eighteen samples were analysed for the cytokine assay in the immunity study, of which only 14 were suitable and participated in the study (see Chapter 7). Additionally, five samples were available for further lymphocyte subset analysis. With the exemption of one child, all the children who participated in the immune study had an accessible bone and glucose profile.

Figure 4-1: Flow diagram of study recruitment



*Sixty-one children were identified through the endocrine clinic. Forty children were excluded during the study period for a variety of reasons as shown in the diagram.

4.7 Baseline characteristics

4.7.1 Anthropometric and demographic characteristics

Initially, 37 children appeared to meet the inclusion criteria, consented, and attended the first study visit during the 24 months of the study period. Of these, 17 (46%) were boys and 20 (54%) were girls. The median age for all the participants was 36 months (10 months - 115 months). The median age for the boys was 24 months (10 months - 115 months), while the median age for girls was 41 months (16 months - 115 months). This age difference was non-significant ($p = 0.5$). All the study participants had been born in the UK and lived there since birth. The median (range) BMI SDS for the participants aged 24 months or older was 0.49 (-1.45, 2.58), and the Wt SDS for all participants was 0 (-4.5, 2.88) (Table 4-2).

After selection, the participants were subdivided into groups based on ethnicity for further comparison. The participants had a number of different ethnic backgrounds. The majority of participants were of South Asian origin (38%). In addition, it should be noted that the highest percentage of participants was recruited in winter (40.5%). Figure 4-2 shows the average basal 25(OH)D level of recruited participants, according to ethnic origin and season of recruitment. Children of Sub-Saharan African ethnic origin showed more profound vitamin D deficiency throughout the year, compared with other ethnic groups.

Table 4-2: Characteristics of recruited children.

Variables	N	Boys (n = 17)	Girls (n = 20)
Age (months)	**37**		
		24 (10, 115)	41 (16, 115)
Anthropometrics			
BMI SDS	25/37	0.73 (-0.68, 2.29)	-0.49 (-1.45, 2.42)
Wt SDS	37/37	-0.16 (-1, 2.88)	-0.4 (-4.5, 2.65)
Ethnic origin	**37**		
South Asian	14	7	7
Middle Eastern	7	5	2
Sub-Saharan African	7	3	4
Caucasian	8	1	7
Mixed	1	-	1
Season of enrolment	**37**		
Autumn	9	5	4
Spring	5	2	3
Summer	8	5	3
Winter	15	6	9

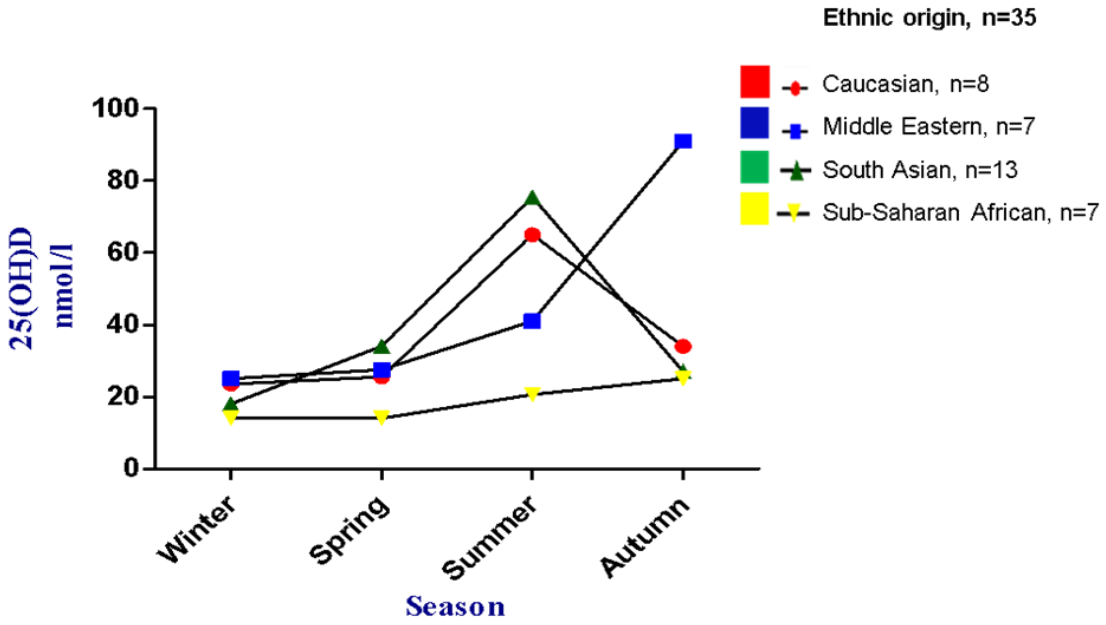


Figure 4-2: Average baseline serum 25(OH)D levels for 35 children, according to ethnic origin and season of recruitment.

Two children were excluded from this graph, and both were recruited in winter (one child's basal 25(OH)D level was missing (South Asian) and another child had mixed ethnic origin (25(OH)D) < 14 nmol/l).

4.7.2 Dietary data and health information

The majority (89%) of the participants reported positive for milk intake (cow's milk or breast milk). No children in the study were taking formula milk. Out of the 37 children recruited, 24 (65%) children had never taken a high vitamin D dose or multivitamin supplement. Five children (three boys and two girls) reportedly had been on high doses of either vitamin D2 or vitamin D3 supplementation, followed by a low vitamin D dose in the form of multivitamins. Thirteen (35%) children had taken low dose vitamin D as a multivitamin prescription (including those who had a high vitamin D dose). Many parents reported that the multivitamin taken was Dalivit and/or Abidec, which provides 400 IU vitamin D3, and most of the parents reported their child's intake as intermittent. It was difficult to obtain information concerning the types of medicines taken from some of the parents. Additionally, 65% of the recruited children were free of any concurrent medical condition, and most of the study participants had been either directly or indirectly referred from the GP or from Orthopaedics (Table 4-3)

Table 4-3: General health information of study participants.

Variables	N	Boys	Girls
Dietary information	**37**	**(17)**	**(20)**
Milk intake			
Breast feeding		5	4
Cow's milk		12	12
No dairy intake		-	4
Multivitamin supplement			
Yes, previous supplement		4	4
Yes, current supplement		3	2
No supplement		10	14
Prior high dose vitamin D supplement			
Yes		3	2
No		14	18
Health information	**37**	**(17)**	**(20)**
No diseases		11	13
Haematological (iron and B12 deficiency anaemia)		2	3
Respiratory (chronic bronchitis, asthma)		2	1
Allergic (eczema, atopic conditions)		-	3
Other		2	-
Referral	**37**	**(17)**	**(20)**
General practitioner (GP)		10	4
General paediatrician		-	3
OPD medical		1	2
Inpatients medical		2	2
Orthopaedics		4	9

4.7.3 Parental health information

Maternal ages ranged from 20 to 42 years (median 32 years), and 14 (38%) of the mothers reported their ethnicity as South Asian. Other ethnic origins were: Caucasian (UK/other European) (9; 24%), Middle Eastern (7; 19%) and Sub-Saharan African (7; 19%). Only four (11%) mothers reported having vitamin D deficiency (two of these were having vitamin D injections and calcium supplements as treatment), and 78% of mothers did not take any vitamin D supplements. Limited knowledge about vitamin D deficiency affected around 46% of the study cohort. Additionally, 54% of mothers wore clothing that limits sun exposure for cultural and/or religious reasons (Table 4-4).

Table 4-4: Parental information.

Variables		
General Information	**Yes**	**No**
Postnatal vitamin D supplementation	8	29
Known maternal vitamin D deficiency	4	33
Maternal clothing limiting sun exposure	17	20
Parental understanding of vitamin D deficiency	17	20
Parental health problems	12	25
Parental health information (n=37)		
Asthma		
Mother	2	35
Father	3	34
Skin disease		
Mother	1	36
Father	2	35
Allergy		
Mother	-	-
Father	-	-
Hypertension		
Mother	-	37
Father	2	35
Diabetes		
Mother	2	35
Father	2	35

4.7.4 Clinical and radiological data

The patients' clinical and radiological findings are summarized in Table 4-5. Their clinicians as well as the research student saw all patients. Around 54% of the children had clinical signs of rickets at the first clinical evaluation, and all of the children with more than one clinical sign of rickets were suffering from profound vitamin D deficiency of < 14 nmol/l, and an elevated serum PTH level.

In older children, clinical presentation of vitamin D deficiency noticeably differs from younger children. They presented with vague manifestations including pain in weight bearing joints, thighs and/or calves and muscle cramps. Children younger than 36 months mainly presented with skeletal deformities/ abnormalities including bow legs, wrists swelling and fractures (see figure 4-3). On the basis of concentrations of 25(OH) D alone, it was difficult to assess the clinical biochemical consequences of vitamin D deficiency and / or insufficiency (see figure 4-4). However , all children with abnormal bone biomarkers (PTH, ALP, calcium and phosphate) had concurrent clinical and/or radiological signs of rickets (Table 4-6).

Table 4-5: Clinical and radiological findings.

Variable	N	Boy	Girls
Clinical sign of rickets (n = 37)			
Yes	20	11	9
No	17	6	11
Clinical sign of rickets on examination (n = 20)			
Bowed legs	13	7	6
Abnormal gait	2	2	-
Wrist swelling	14	9	5
Patent anterior fontanelle	2	1	1
Rachitic rosary	1	1	-
Weakness	1	1	-
Prominent chest cage	1	1	-
Abnormal dentition	-	-	-
Frontal bossing	5	3	2
X-ray performed (n = 37)			
Yes	30	15	15
No	2	1	1
No information	5	4	1
Main X- ray sites (n = 30)			
Wrist	25	15	10
Ulna and radius	3	3	-
Hip and tibia	2	2	-
Clear rickets on X- ray (n = 30)			
Yes	12	6	6
No	18	9	9

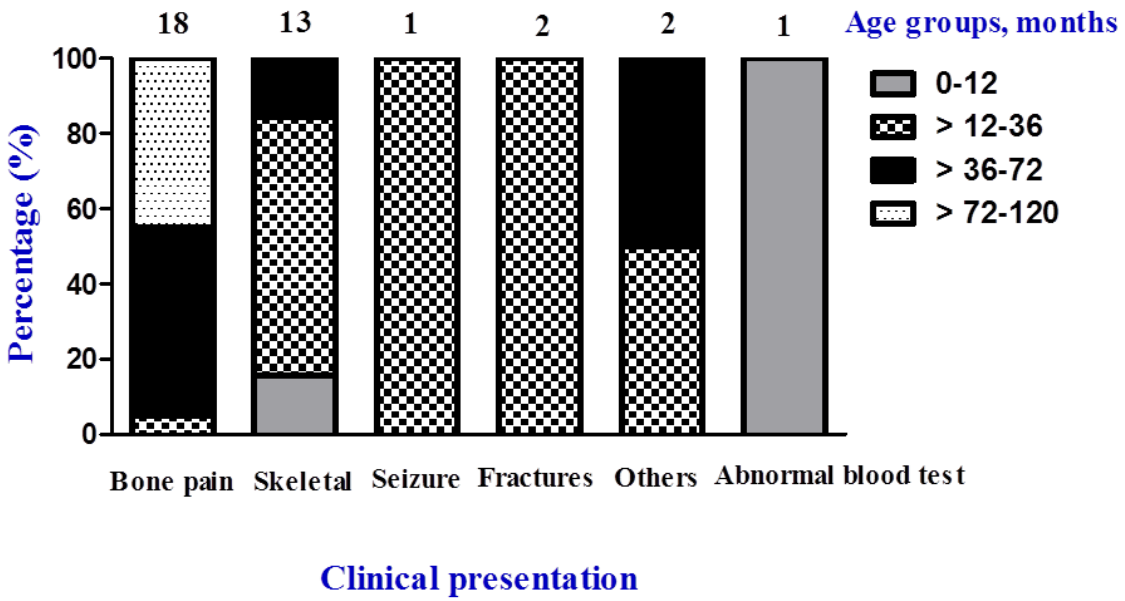


Figure 4-3: Clinical presentation of vitamin D deficiency based on age groups.

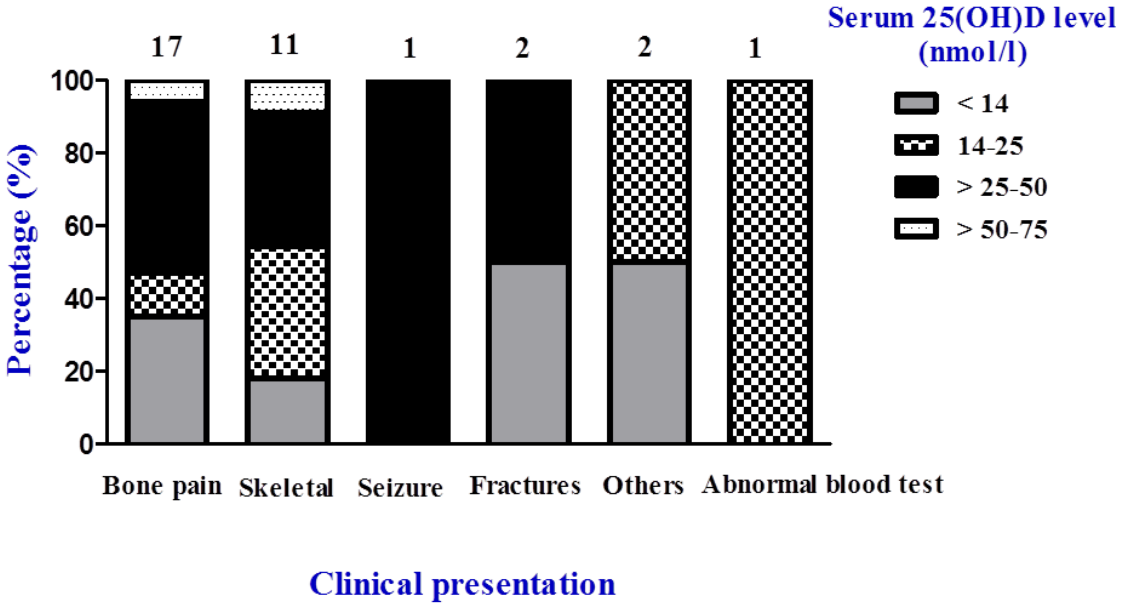


Figure 4-4: Clinical presentation of vitamin D deficiency in 34 children with basal serum 25(OH)D <75 nmol/l.

4.7.5 Laboratory data

Table 4-6 summarizes the available clinical baseline laboratory data for all 37 children who were recruited for the vitamin D study. However, due to technical problems there were insufficient blood samples collected, and many children were later excluded, as described earlier in this chapter. By the end of week six of the study period, the number of samples available for the bone, glucose and immunity studies were n = 25, n = 25 and n = 18 respectively.

At baseline, serum 25(OH)D data was available for 36 (97%) children. The basal serum 25(OH)D level was absent for one child, due to technical difficulties (Patient ID: 25). A high dose of vitamin D3 supplementation was given to this child on the basis of clear clinical signs of rickets (bowed legs and wrist swelling), and a family history of nutritional rickets (mother and two siblings), and because the patient had not taken any previous supplements. Of the 36 children with available 25(OH)D data at baseline, 18 (50%) children had very low serum 25(OH)D levels of < 25 nmol/l, 14 (39%) children had low serum 25(OH)D levels between 25-50 nmol/l and two (5.5%) children had insufficient serum 25(OH)D levels, between 50-75 nmol/l. In a further two (5.5%) children, the blood results showed normal vitamin D levels at the baseline, and they were excluded from further statistical analysis. These two children were originally referred from a general paediatrician, and during the first clinical visit their parents had given imprecise information about previous vitamin D supplements.

For the 37 children, baseline serum PTH levels were available for 30 (81%) with high basal serum PTH levels reported in 15 (50%) cases with available PTH data. Alkaline phosphatase levels were available in 28 children; of these, the serum ALP was abnormally high in just seven (25%) children. Serum calcium and phosphate were available in 32 (86.5%) and 28 (76%) children respectively, and abnormally low calcium levels were reported in three (9%) children, with low phosphate levels in only one child (3%).

Table 4-6: Baseline laboratory data for the 37 children who attended the first study visit.

Patient ID	Age (months)	Sex	25(OH)D (nmol/l)	PTH (pmol/l)	ALP (u/l)	Calcium (mmol/l)	Phosphate (mmol/l)	*Study
1	43	M	**33**	5.3	99.7	2.36	1.41	1,2,3
2	23	F	125	**6.5**	180	2.29	1.15	-
3	102	M	**19**	4.3	153	2.51	1.5	1,2
4	12	M	91	5.4	422	2.44	1.57	-
5	113	F	**44**	5.4	270	2.42	1.18	1,2
6	36	F	**29**	**8.4**	219	2.35	1.55	1,2,3
7	115	F	**33**	5.5	161	2.37	1.38	1,2,3
8	46	F	**34**	4.1	**450**	2.36	1.43	1,2
9	78	M	**<14**	4.9	236	2.51	1.27	1,2
10	79	F	**<14**	**6.8**	-	2.33	1.43	1,2,3
11	115	M	**<14**	4.3	251	2.41	1.38	1,2,3
12	33	F	**<23**	4.1	224	2.52	1.79	1,2,3
13	24	M	**<14**	**6.8**	-	2.47	1.62	-
14	48	M	**<14**	**18**	-	-	-	-
15	51	F	**33**	**7.1**	-	3.37	1.33	1,2,3
16	24	M	**17**	5.1	403	2.43	1.45	-
17	71	F	**20**	**7.3**	102	2.49	-	1,2,3
18	54	F	**<14**	-	-	2.41	1.6	-
19	24	F	**<14**	**19.3**	261	2.39	1.81	-
20	30	F	60	-	**2128**	2.44	1.24	-
21	23	F	**28**	-	-	-	-	-
22	63	F	65	4.1	185	2.48	-	1,2,3
23	22	F	**<14**	-	194	-	-	-
24	84	M	**<14**	-	-	2.48	1.68	-
25	11	M	**-**	-	-	-	-	-
26	63	F	**34**	5.5	178	2.45	1.51	1,2,3
27	16	M	**27**	**29.8**	434	2.44	1.59	1,2,3
28	16	F	**23**	**36.2**	**679**	2.53	1.57	1,2,3
29	19	M	**26**	**134.1**	**2834**	**2.17**	**0.81**	1,2,3
30	96	M	**49**	3.6	226	2.47	1.56	1,2,3
31	24	M	**23**	**40.4**	**733**	**1.82**	-	-
32	10	M	**18**	**14.1**	**864**	2.38	1.07	1,2,3
33	18	M	**19**	**9.7**	338	2.52	1.62	1,2,3
34	66	M	**33**	5	285	2.25	1.47	1,2
35	20	F	**37**	-	-	-	-	-
36	22	F	**<14**	**23.9**	**2551**	**1.38**	1.64	1,2
37	72	F	**31**	4.4	181	2.48	1.41	1,2

Numbers in bold represent abnormal laboratory values. *****Study 1: bone (see Chapter 5); Study 2: glucose (see Chapter 6); Study 3: immune function (see Chapter 7).

4.8 Discussion

Of the participants in the present study, 39% had low vitamin D levels with serum 25(OH)D between 25-50 nmol/l, and 50% had more profound vitamin D deficiencies, with serum 25(OH)D levels of < 25 nmol/l (total prevalence of vitamin D deficiency was 89%). The prevalence of vitamin D deficient status was evenly distributed between boys and girls. In terms of age, children younger than 36 months (3 years) had more profound symptomatic vitamin D deficiency, associated with a high rate of bone metabolism compared with older children. Additionally, around 54% of study participants were found to have clear signs of rickets upon clinical examination, and 40% who underwent a radiological examination showed clear signs of rickets on X-ray.

A number of factors that appear to influence poor vitamin D status were mentioned in our study. Since darker skin pigmentation reduces vitamin D synthesis by the sun, due to enhanced melanin production, certain ethnicities, such as South Asian and Sub-Saharan Africa are more likely to develop vitamin D deficiency. In our cohort, prevalence rates of deficiency were highest among South Asian (38%, n=14). Due to the fluctuating weather conditions in Scotland, evaluating data by isolating individual factors contributing to sun exposure was extremely difficult. However, examination of the distribution of study recruitment throughout the year (mainly in winter and autumn), suggested decreased sun exposure is an important contributor to lower vitamin D status. In non-breastfed infants and young children, UK dietary surveys show the main contributor to dietary vitamin D intake is formula milk, providing an average of 85% of intake for children aged 4-6 months, 72-80% for those aged 7-11 months, and 29% for those aged 12-18 months [450]. Additionally, because plain cow's milk is not fortified in the UK and breast milk is low in vitamin D, dairy intake is likely to be one of the contributors to low vitamin D status in our study cohort. However, full dietary intake data was not collected and so for future studies we recommend well-designed questionnaires to assess vitamin D intake.

Around 35% of our study participants had chronic and/or recurrent medical conditions, such as asthma, recurrent hospital admissions due to chest infections, atopic dermatitis, eczema, and autism. All of the above-mentioned conditions have been correlated with low vitamin D status (as reviewed in the introduction to this thesis). Parental awareness of vitamin D deficiency is relatively low; 54% of parents had no basic understanding about vitamin D deficiency. Covering the skin is known to be an important risk factor for vitamin D deficiency, and in our cohort, 46% of mothers were wearing clothing that limits

sun exposure for cultural and/or religious reasons. As 78% of mothers do not take any vitamin D supplements, we would expect them to have lower vitamin D levels, possibly contributing to the lower vitamin D status in younger breast feeding children.

In conclusion, a considerable number of children with vitamin D deficiency have been identified through the endocrine clinic at RHSC. This deficiency appears to result from poor dietary intake of vitamin D, ethnicity and cultural behaviours. These findings imply a requirement for improved levels of public education, to ensure children have a greater opportunity to maintain adequate levels of vitamin D.

Chapter 5

Effect of Vitamin D3 Supplementation on Bone Status

5.1 Abstract

Introduction

Evidence from epidemiological studies on the association between vitamin D status and bone remodelling markers were inconsistent.

Objective

To study the effect of six weeks of vitamin D supplementation on bone remodelling markers in vitamin D deficient children. We also aimed to study the baseline and post treatment relationships between serum 25(OH)D and these markers.

Method

Twenty-three prepubertal children aged 10 months to 9.5 years (10 boys) were diagnosed with vitamin D deficiency and recruited during 24 months of the study period. Treatment consisted of 5000 IU vitamin D3 (cholecalciferol) given orally once a day for six weeks. At baseline and completion of treatment the following serum levels were measured: 25 hydroxyvitamin D (25(OH)D), parathyroid hormone (PTH), Alkaline phosphatase (ALP), serum osteocalcin (OCN), and bone resorption markers include the C-terminal telopeptide of type I collagen (CTX).

Results

The median (range) of serum of 25(OH)D level in 23 children increased significantly in response to supplementation, from 27 (14, 65) nmol/l at baseline to 106 (37, 202) nmol/l at week 6, $p<0.0001$. The median (range) of PTH in 21 children decreased significantly from 5.5 (3.6, 134.1) pmol/l at baseline to 3.8 (1.9, 6.8) pmol/l at week 6, $p<0.0001$. The median (range) of ALP in 21 children decreased significantly from 236 (99, 2834) u/l at

baseline to 195 (64, 1364) u/l at week 6, p=0.001. No significant changes were observed in the medians of OCN and CTX from baseline to week 6, p=0.5 and p=0.2, respectively.

Conclusion

Short term improvement of vitamin D deficient status significantly increases serum 25(OH)D levels, decreases serum PTH levels and decreases marker the of bone formation ALP.

5.2 Introduction

Vitamin D and the parathyroid hormone (PTH) have long been recognized as significant variables required for the maintenance of calcium homeostasis and bone health [451].

The active form of vitamin D, 1,25(OH)2D can promote intestinal calcium absorption, and renal resorption by assisting in the upregulation of various proteins within the intestinal and renal epithelial cells [167, 280]. The best-known skeletal action for the active form of vitamin D involves regulating both bone formation and resorption, as enacted through osteoclastic and osteoblastic cell lineages. Both 1,25(OH)2D and PTH can induce the expression of osteoclast differentiation factor (ODF) or stromal osteoclast forming activity (SOFA) by osteoblasts stromal cells which in turn, through cell-to-cell interaction stimulate the osteoclast precursors of the monocyte-macrophage lineage to recognize ODF/SOFA and further differentiate into osteoclasts, thereby enhancing bone resorption [452]. In addition, vitamin D can stimulate bone formation and mineralization either indirectly via intestinal and renal regulation of calcium homeostasis or through acting directly on osteoblasts, by adjusting the gene expression of various osteoblast differentiation and mineralization-associated genes [135, 143].

In case of serum calcium deficiency, it is suggested that vitamin D exerts a synergistic role with PTH, encouraging osteoclast proliferation and bone resorption, while simultaneously inhibiting bone mineralization, leading to an increase in calcium bone loss. When vitamin D is also low, the compensatory increase in PTH secretion (secondary hyperparathyroidism) can enhance vitamin D synthesis and maintain calcium homeostasis. Therefore, serum calcium level may be normal in a vitamin D–deficient infant or child, whereas serum phosphate may be normal or reduced, due to the direct influence of PTH on the phosphorus reabsorption in the kidney, causing phosphorus to be lost into the urine [451, 452].

Although the association between vitamin D status and bone remodelling has been studied extensively, studies continue to report inconsistent results, particularly regarding measurable changes in the bone remodelling markers following improvement of vitamin D status, and the duration of vitamin D treatment necessary to return these markers to the normal reference range. Additionally, results remain inconsistent regarding the association between serum 25(OH)D level and bone remodelling markers [453-457].

The radiological assessment of the functional consequences of vitamin D deficiency such as bone fracture and bone mass require a study designed over a relatively long duration with a big sample size. Therefore, the assessment of biochemical markers for bone remodelling can be considered a more practical way to measure the effects of vitamin D supplementation on bone. This part of our project was undertaken to test the hypothesis that six weeks of vitamin D supplementation would treat vitamin D deficiency and affect the bone remodelling markers in vitamin D deficient children aged between three months and 10 years. We also aimed to study the baseline and post treatment relationships between serum 25(OH)D and the bone remodelling markers. Bone formation markers include serum alkaline phosphatase (ALP) and serum osteocalcin (OCN), and bone resorption markers include the C-terminal telopeptide of type I collagen (CTX).

5.3 Subjects and methods

5.3.1 Subjects

Chapter 4 of this thesis described in detail subject characteristics and inclusion/exclusion criteria.

5.3.2 Study methods

The study protocol is detailed in Chapter 4 of this thesis. Briefly, the study design involved six weeks of 5000 IU vitamin D3 (cholecalciferol) given orally once a day to children referred to the endocrine department at RHSC for vitamin D deficiency.

5.3.3 Blood collection

Blood samples were collected in Ward 1C at RHSC, in the morning after a six-hour overnight fast. At baseline and completion of treatment, serum 25 hydroxyvitamin D (25(OH)D), parathyroid hormone (PTH), alkaline phosphatase (ALP), serum collagen type 1 cross-linked C-telopeptide (CTX), serum Osteocalcin (OCN), serum calcium and serum phosphate were measured.

5.3.4 Biochemical assays

All blood sampling was performed at the time of routine clinical visits; samples were immediately centrifuged at 2600-2800 rev/minute for 10 minutes, and the serum was subsequently stored at -80°C until the assays were performed. Serum 25(OH)D levels were assayed using the Abbott Architect Chemiluminescent Microparticle Immunoassay (CMIA). Serum PTH measurements were assayed using intact PTH CMIA (Abbott Architect). Serum ALP was measured by Ostase® BAP immunoenzymetric assay immunodiagnostic systems Ltd (IDS Ltd, Boldon, Tyne and Wear, UK) with an intra-assay CV of 0% to 2.3%. Serum osteocalcin (OCN) was measured using N-MID® osteocalcin ELISA (IDS Ltd, Boldon, Tyne and Wear, UK) with an intra-assay CV of 0.8% to 3.1%. Serum CTX was determined using serum crossLaps® ELISA (IDS Ltd, Boldon, Tyne and Wear, UK) with an intra-assay CV of 2.05% to 1.61%. Dr. McNeilly performed the assays for serum 25(OH)D in the Biochemistry Department at Southern General Hospital, Glasgow. The remaining samples were sent directly to the biochemistry laboratory at RHSC to test for calcium and phosphate.

5.3.5 Statistical analysis

All statistical analyses were performed using GraphPad Prism 5 (San Diego California, USA) or Mini Tab 17 (Minitab, Coventry, UK) statistical software. The results were presented as median and ranges, unless otherwise specified. The significance of the changes in the measurements between baseline and week six for all study participants were assessed using the Wilcoxon signed-rank test. Mann-Whitney U tests were performed to analyse the two independent subgroups. Kruskal-Wallis tests were performed to compare three independent subgroups. A Spearman correlation analysis was also performed, to reveal the relationship between vitamin D, PTH and calcium, and changes in the relationship between vitamin D, PTH and the bone remodelling markers.

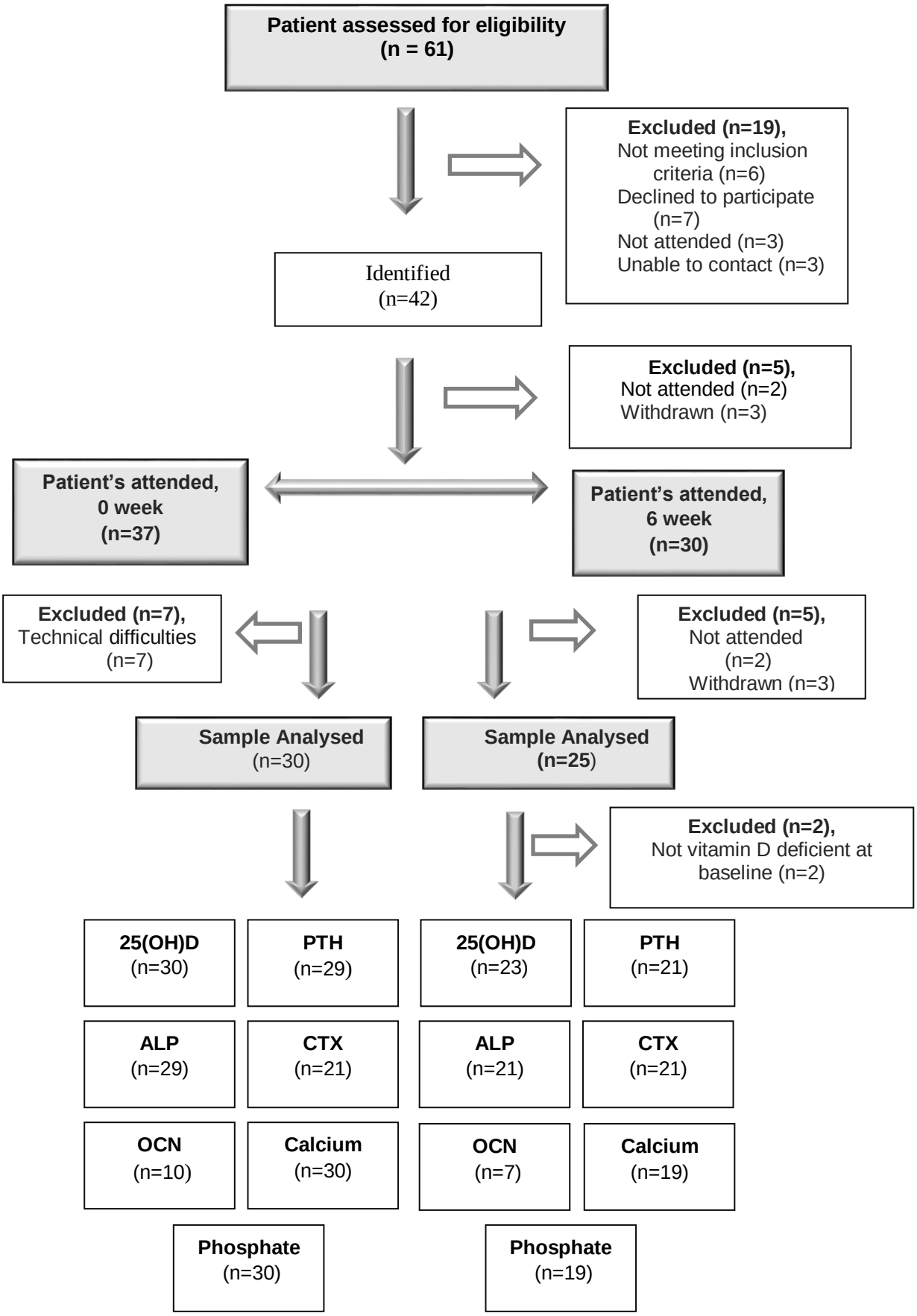
Using SPSS 22 statistical software (USA), a Mixed Effects Model (MEM) analysis was employed where appropriate to further explore the confounding effects of independent variables. For the MEM analysis, the log transformed values for 25(OH)D, PTH and ALP were used to satisfy the assumption of normal distribution, whereas calcium and CTX remained in their original units. We were unable to perform the MEM for OCN, due to the low sample size. A significance level of $p < 0.05$ was used to evaluate all the study results. Only children with data available at both the baseline and week six of the study period were included in the MEM statistical analysis. Children for whom there was missing data recorded at either point in the study period were included in the univariate association analysis only.

5.4 Results

5.4.1 Recruitment summary

In total between 2011 and 2014, during the 24 months of the study period, 61 children were identified and screened. Of these, 42 met the main project inclusion criteria and agreed to participate. 24 of the children were excluded before the first visit for several reasons (as detailed in chapter 4). Out of the 42 children who qualified for the study, 37 attended the first study visit and 12 children were lost over the course of subsequent study visits. At six weeks, 7 children were excluded. Of those 7 children, 5 children were excluded due to either non-attendance, or because they withdrew from the study. In the remaining 2 children, the blood results showed normal vitamin D levels at baseline, and therefore they were excluded from further analysis. Both the children who showed normal serum 25(OH)D levels at baseline were referred originally from a GP and there was inaccuracies in their records concerning previous vitamin D supplementation. Figure 5-1 shows the flow diagram for the study recruitment.

Figure 5-1: Flow diagram for study recruitment



The total number of children identified through the endocrine clinic was 61 children. Thirty eight children were excluded throughout the study period for a variety of reasons as shown in the diagram.

5.4.2 Demographic characteristics

There were 23 pre pubertal children, 10 boys with a median age of 55 months (10 to 115 months) and 13 girls with a median age of 63 months (16 to 115 months) enrolled in the study at both time points. Body Mass Index (BMI) Standard Deviation Score (SDS) (BMI SDS) were 0.2 (-0.68, 2.29) and 0.67- (-1.45, 2.42) for the boys and girls respectively. Weight Standard Deviation Score (Wt SDS) were 0.05 (-1.02, 2.88) and 0.39 (-4.5, 2.65) for the boys and girls respectively. The main cause of referral was bone pain, with 14 of the children recruited for this reason (61%). Out of those 14 children, 13 were referred from an orthopaedic clinic and one child directly from their GP. Radiological screening was performed in 20/23 study participants and we were unable to trace the report for 2 children referred mainly due to bone pain. There was one reported x-ray appointment. Other causes of referral included bowed legs, seizure, dietary concerns, fracture and family history (Table 5-1).

Table 5-1: Characteristics of children who completed the study.

	N	
Age, years	**(23)**	**%**
0-2	5	22
2-6	9	39
6-10	9	39
Gender	**(23)**	**%**
Boy	10	43.5
Girl	13	56.5
Race	**(23)**	**%**
South Asian	10	43.5
Middle Eastern	4	17.5
Sub-Saharan African	3	13
Caucasian	6	26
Anthropometrics		**Median (range)**
BMI SDS	18/23	-0.22(-1.45, 2.42)
Wt SDS	23/23	-0.25(-4.5, 2.88)
Vitamin D supplementation	**(9)**	**%**
Prior high dose vitamin D supplementation	2	22.2
Prior multivitamin supplementation	4	44.5
Current multivitamin supplementation	3	33.3
Season of enrolment	**(23)**	**%**
Autumn	7	30.5
Spring	6	26
Summer	6	26
Winter	4	17.5
Reason of referral	**(23)**	**%**
Bone pain	14	61
Bowed legs	5	22
Other	4	17

5.4.3 Effects of treatment

Only children with available data at both time points of the study period, week 0 and week 6 were included in this analysis. The median (range) of serum 25(OH)D level in 23 children increased significantly in response to supplementation, from 27 (14, 65) nmol/l at baseline to 106 (37, 202) nmol/l at week 6, $p<0.0001$. The median (range) of PTH in 21 children decreased significantly from 5.5 (3.6, 134.1) pmol/l at baseline to 3.8 (1.9, 6.8) pmol/l at week 6, $p<0.0001$. The markers of bone remodelling showed variability in response to vitamin D3 treatment. There was a significant reduction in ALP value, from 236 (99, 2834) u/l at baseline to 195 (64, 1364) u/l at week 6, $p=0.001$. No significant changes were observed in the medians of OCN and CTX from baseline to week 6, $p=0.5$ and $p=0.2$ respectively. There was a trend towards an increase in the median value of serum calcium level, from 2.38 (2.17, 2.55) mmol/l at baseline to 2.45 (2.33, 2.69) mmol/l at week 6, $p=0.05$ with no change in serum phosphate, $p=0.6$ (Table 5-2).

Table 5-2: Comparison of biochemical measurements of bone remodelling.

	Baseline		Week six		*p*-Value (from baseline to 6 week)	Normal reference range
	n	Median (min, max)	n	Median (min, max)		
25(OH)D (nmol/l)	23	27 (14, 65)	23	106 (37, 202)	**<0.0001**	70-150
PTH (pmol/l)	21	5.5 (3.6, 134)	21	3.8 (1.9, 6.8)	**<0.0001**	0.9-5.5
ALP (u/l)	21	236 (99, 2834)	21	195 (64, 1364)	**0.001**	100-420
CTX (ng/ml)	19	1.80 (0.9, 3.16)	19	1.72 (0.8, 3.2)	0.2	*
OCN (ng/ml)	7	39 (16.5, 79)	7	52.8 (19.9, 76)	0.5	*
Calcium (mmol/l)	19	2.38 (2.17, 2.55)	19	2.45 (2.33, 2.69)	0.05	2.20-2.70
Phosphate (mmol/l)	19	1.43 (1.07, 1.79)	19	1.48 (1.23, 1.89)	0.6	0.9-1.8

25(OH)D; 25. hydroxyvitamin D. PTH; parathyroid hormone. ALP; Alkaline phosphatase. CTX; Serum cross linked C-telopeptide of type I collagen. OCN; Osteocalcin. * Values for CTX and OCN were compared with corresponding age-matched healthy control groups (10th, 90th centiles) in children aged from 1year to 10years (internal data obtained by the same assays used in our study). Only non-missed values were included in this analysis.

5.4.4 Individual changes in bone remodelling markers

5.4.4.1 Vitamin D (25(OH)D) and Parathyroid hormone (PTH)

Figure 5-2 shows individual changes in serum 25(OH)D for the 23 children who had available 25(OH)D data at both baseline and week 6 of the study period. Of those 23 children, 22 children (96%) had basal serum 25(OH)D in the deficiency range (<50 nmol/l) and one child (4%) had serum 25(OH)D in the insufficiency range (51-75 nmol/l). Following vitamin D3 treatment, there was variability in the serum 25(OH)D response among the study participants. Two children (9%) had final serum of 25(OH)D <50 nmol/l, 6 (26%) children had final serum 25(OH)D from >50-75 nmol/l, 12 (52%) reached a final serum of 25(OH)D >75-150 nmol/l and finally 3 (13%) children exceeded the normal reference range with a final 25(OH)D >150 nmol/l. Figure 5-3 summaries the distribution of study participants according to their final serum 25(OH)D level.

Current evidence suggests a difference in growth velocity and rate of bone remodelling between infants, older children and adolescents. For example, there is a higher rate of bone remodelling in early infancy and in pre pubertal children, although remodelling is generally stable from 4 to10 years of age [458-460]. Due to the key role of both vitamin D and PTH in the process of bone turnover, and to further compare the effect of vitamin D3 supplementation between different age groups, the participants were grouped into three age categories; from 10 months to 3 years, from > 3 years to 6 years and from > 6 years to 9.5 years.

Our results showed that serum 25(OH)D levels increased regardless of patient's age group or basal serum 25(OH)D levels. The increase in serum 25(OH)D from baseline to 6 week was significant in all age groups, with *p*-value of 0.01, 0.02 and 0.01 for 10 months to 3 years, > 3 years to 6 years and > 6 years to 9.5 years respectively. There were no observed significant inter-group differences in the levels of 25(OH)D.

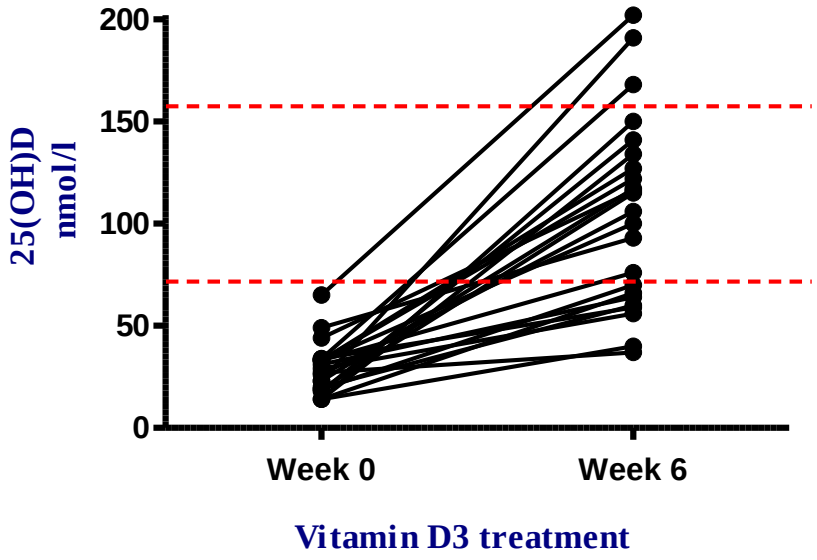
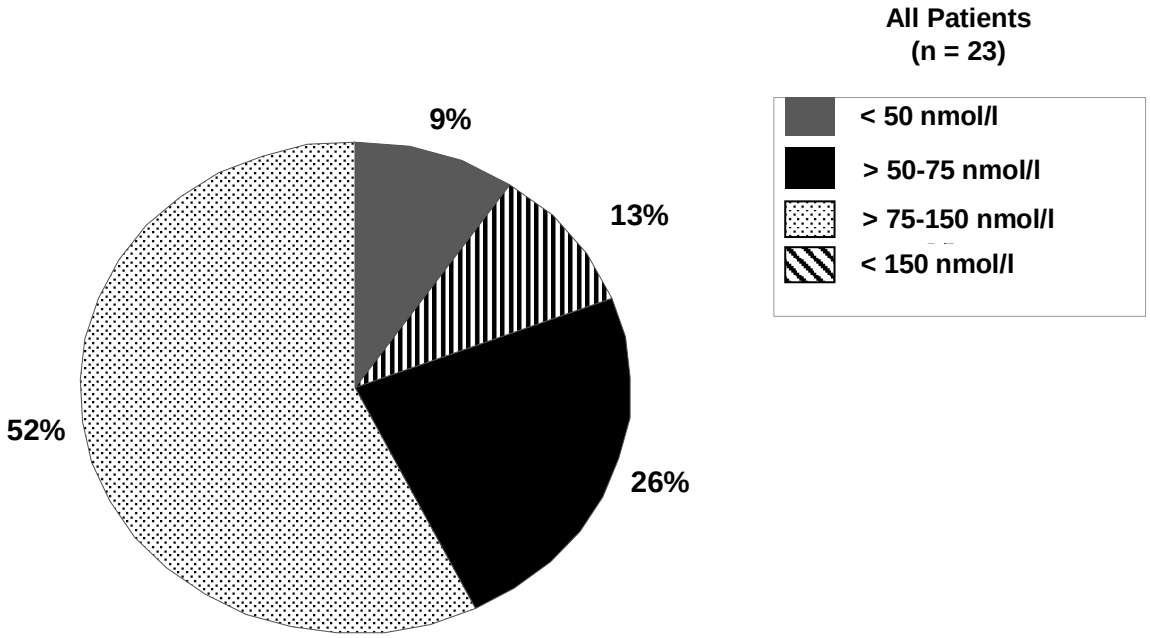


Figure 5-2: Individual changes in serum 25(OH)D levels for 23 children before and after 6 weeks of receiving 5000 IU vitamin D3 treatment.

Significant increase in the median of serum 25(OH)D levels was observed, p <0.0001. Each subject's vitamin D level is represented by a line connecting week 0 with week 6 values. Area between two red lines; normal reference ranges for vitamin D.

Figure 5-3: Pie chart of response to vitamin D3 supplementation by study participants



*The distribution of patients according to their serum 25(OH)D reached at week 6, following vitamin D supplementation. < 50 nmol/l, vitamin D deficiency; >50-75 nmol/l, vitamin D insufficiency; >75-150 nmol/l, vitamin D sufficiency; <150nmol/l, exceeded normal reference range.

Figure 5-4 shows individual changes observed in serum PTH for the 21 children for whom PTH data was available at both time points with a significant reduction in the median of serum PTH levels from baseline to week 6, p <0.0001. Of those 21 children, 12 (57%) children had basal serum PTH levels within the normal reference range and 9 (43 %) children had basal PTH levels, which exceeded the normal reference range. After 6 weeks of vitamin D3 treatment, PTH levels returned to the normal range in almost all study participants, except for two children who remained just above the upper limit of normal.

The highest basal level of serum PTH was observed in the youngest age group (10 months- 3 years) with a median of 14.1(4.1, 134) pmol/l. The basal median PTH for those younger group differed significantly from the both middle 5(4.1, 7.3) pmol/l and oldest 4.9(3.6, 6.8) pmol/l groups, p=0.02. There was no significante difference in basal PTH between the middle and oldest groups, p=0.6. Using a multiple group comparison test, a significant difference was observed between the groups (p = 0.02) (see Figure 5-5).

Within the subgroups, serum PTH decreased significantly from baseline to week 6 in both the younger age group (10 months- 3 years, p=0.01) and the middle age group (> 3- 6 years, p =0.01). No significant reduction in serum PTH was observed in children age > 6- 9.5 years, p=0.1 (see Figure 5-6). Table 5-3 summaries the percentage change in serum 25(OH)D and PTH, in study participants from baseline to week 6 grouped according to their age group.

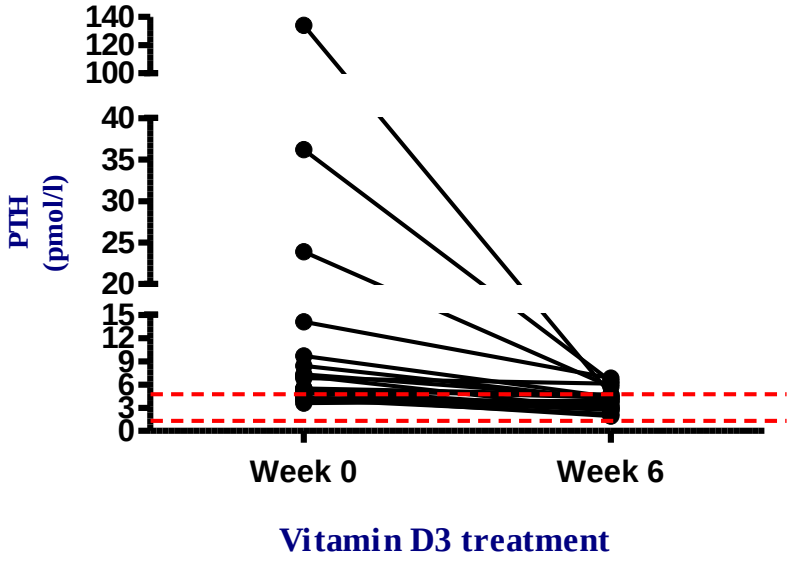


Figure 5-4: Individual changes in serum PTH levels for 21 children before and after 6 weeks of receiving 5000 IU vitamin D3 treatment.

A significant decrease in the median of serum PTH levels was observed, P <0.0001. A line connecting week 0 with week 6 values represented each subject's PTH level. Area between two red lines; normal reference ranges for PTH.

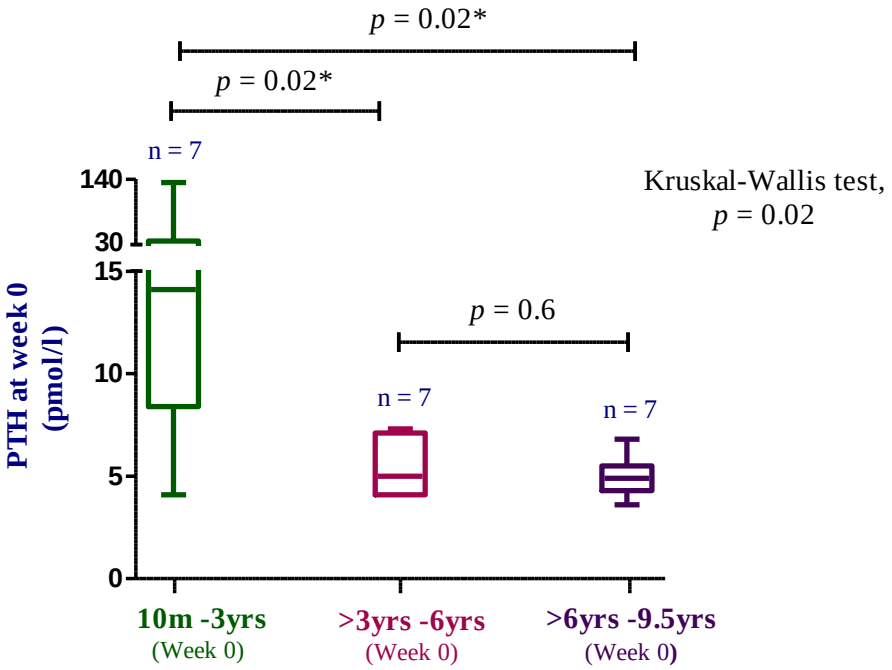


Figure 5-5: Comparison of basal serum PTH levels (medians) between study participants, as grouped by their ages.

The children were grouped into three age categories; from 10 months to 3 years, from >3 years to 6 years and from >6 years to 9.5 years. Significant differences in the median basal serum PTH levels between the youngest and each of older groups was observed, $p = 0.02$. No significant difference was observed between the age groups >3 years to 6 years and >6 years to 9 years, $p = 0.6$. Basal serum PTH levels also differed significantly between the three groups, $p = 0.02$. The boxes indicate quartile values; the whiskers indicate the upper and lower values. The numeric variables for two subgroups were compared using the Mann-Whitney U test; comparisons among the three subgroups were analysed using the Kruskal-Wallis test (non-parametric).

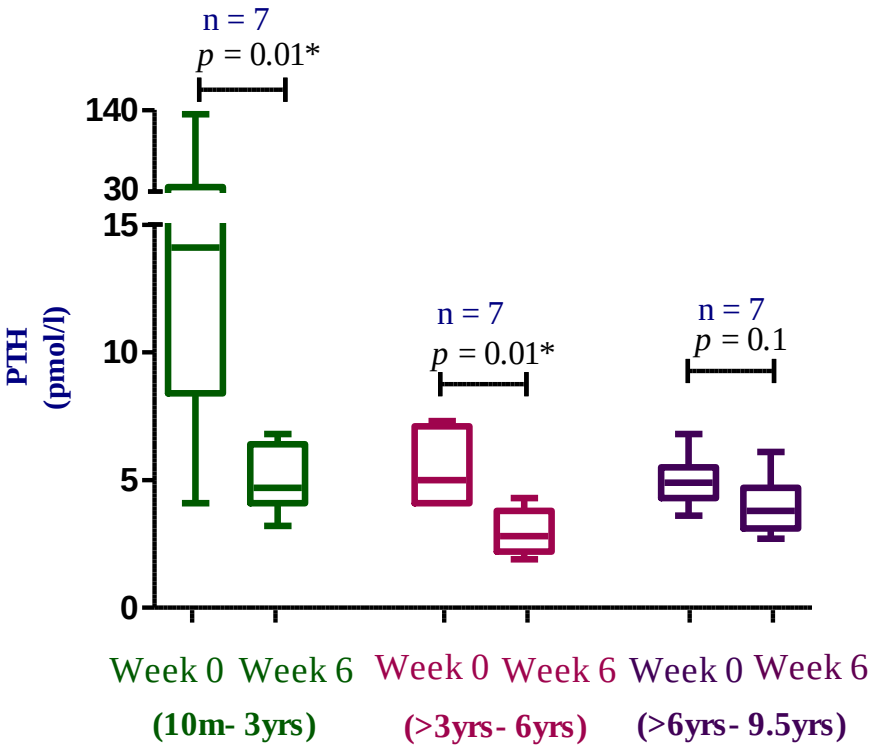


Figure 5-6: Effect of age on serum PTH concentrations before and after 6 weeks of receiving 5000 IU vitamin D3 treatment.

The median of serum PTH levels decreased significantly in response to vitamin D3 supplementation in both children age between 10 months to 3 years and children age > 3 years to 6 years. No significance reduction in the median of serum PTH levels in the oldest group, age > 6 years to 9.5 years. The boxes indicate quartile values; the whiskers indicate the upper and lower values. Wilcoxon signed-rank test was used to assess the change from baseline to week six within subgroups.

Table 5-3: Individual data for percentage change in serum 25(OH)D and PTH in study participants grouped according to age.

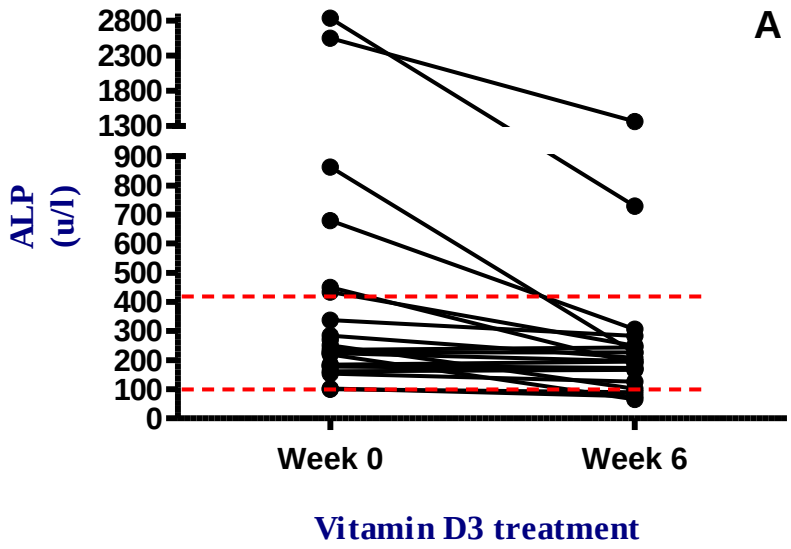
Patient ID	Age (Months)	Sex	% Change 25(OH)D*	% Change PTH*
	10 months- 3 years			
6	36	Girl	93.1	-51.1
12	33	Girl	730.4	-21.9
27	16	Boy	37	*
28	16	Girl	408.6	-82.3
29	19	Boy	307.6	-96.4
32	10	Boy	683.3	-51.75
33	18	Girl	505.2	-54.6
36	22	Girl	185.7	-76.1
Median	**18.5m (1.5year)**	-	**358%**	**-51.7%**
Patient ID	**Age (Months)**	**Sex**	**% Change 25(OH)D***	**% Change PTH***
	age > 3 years- 6 years			
1	43	Boy	203	*
8	46	Boy	394.1	-7.3
15	51	Girl	284.8	-61.9
17	71	Girl	200	-41
22	63	Girl	210.7	-46.3
26	63	Girl	73.5	-49
34	66	Boy	269.6	-40
37	72	Girl	106.4	-56.8
Median	**63m (5.2years)**	-	**206%**	**-46.3%**
Patient ID	**Age (Months)**	**Sex**	**% Change 25(OH)D***	**% Change PTH***
	> 6 years- 9.5 years			
3	102	Boy	268.4	-16.2
5	113	Girl	163.6	-20.3
7	115	Girl	130.3	-14.5
9	78	Boy	971.4	-36.7
10	79	Girl	371.4	-10.2
11	115	Boy	857.1	-37.2
30	96	Boy	89.7	8.3
Median	**102m (8.5years)**	-	**268.4%**	**-16.2%**

% change = [(v2-v1)/v1]*100.

5.4.4.2 Markers of bone remodelling (ALP, CTX and OCN)

By the end of the six-week study period, 21 paired ALP samples were available for analysis with a significant change in the median of ALP levels from baseline to week 6, p=0.001. As shown in Figure 5-7, of the 21 children, 15 (71.5%) children had basal serum ALP within normal reference range. Six (28.5%) children showed basal serum ALP levels exceeding the normal reference range, which returned to the normal range in 4 children and remained high in the remaining 2 children after vitamin D3 supplementation.

Within the subgroups analysis, the reduction in serum ALP was significant in only the younger age group (10 months-3 years) with p-value of 0.01. Non-significant reduction in the median of ALP in other groups, 3 to 6 years and > 6 to 9.5 years was observed with P-values of 0.07 and 0.1 respectively (see Figure 5-8). Table 5-4 summarises the percentage change in serum ALP concentration from baseline to 6 weeks in study participants, grouped according to the age.



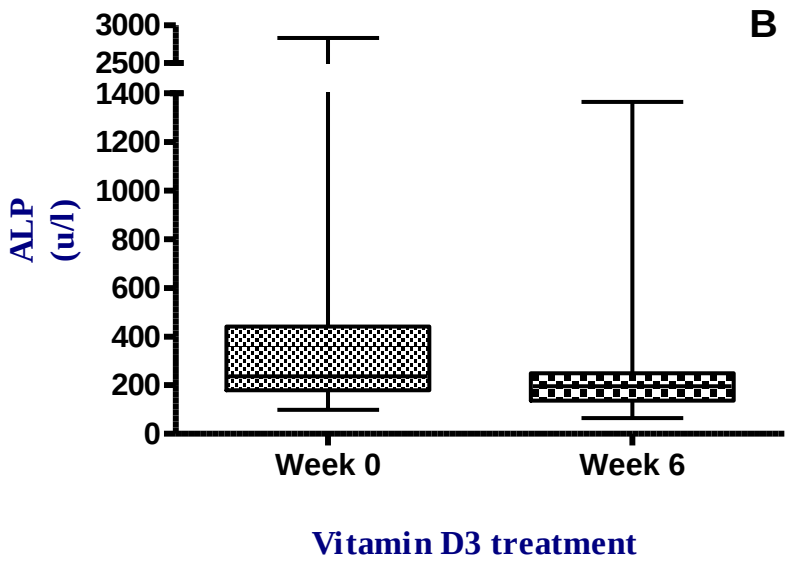


Figure 5-7: A; Individual changes in serum alkaline phosphatase (ALP) levels for 21 children before and after 6 weeks of receiving 5000 IU vitamin D3 treatment.

A line connecting week 0 with week 6 values represented each subject's ALP level. Area between two red lines; normal reference ranges for ALP. B: Box plot showing distribution of ALP levels among the study participants before and after receiving vitamin D3 supplementation. The boxes indicate quartile values; the whiskers indicate upper and lower values. A significant increase in the median of serum ALP levels was also observed ($p < 0.001$).

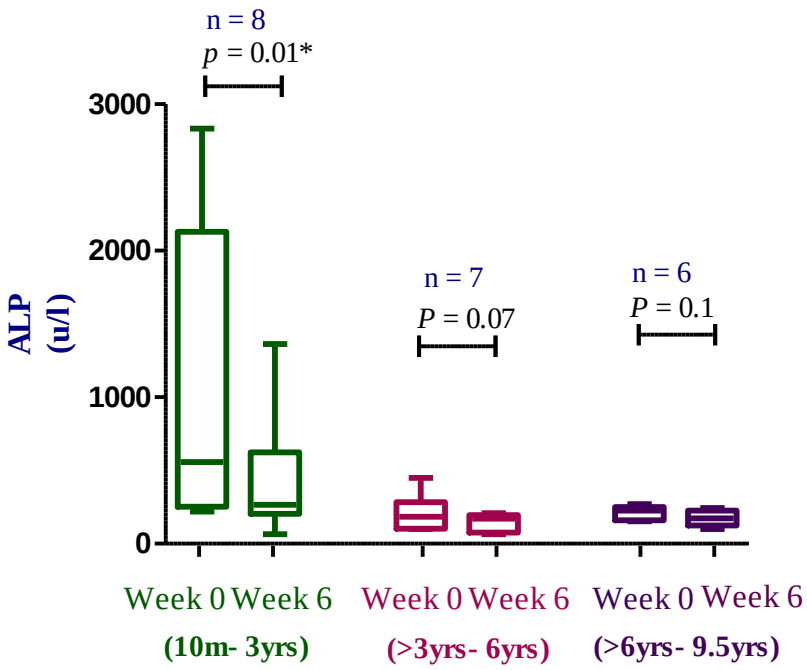


Figure 5-8: Effect of age on serum ALP concentrations before and after 6 weeks receiving 5000 IU vitamin D3 treatment.

The median of serum ALP levels decreased significantly in response to vitamin D3 supplementation in children aged between 10 months to 3 years. No significance reduction in serum ALP levels in the older groups, children age > 3 years to 6 years and age > 6 years to 9.5 years, *p*= 0.07 and 0.1 respectively. The boxes indicate quartile values; the whiskers indicate the upper and lower values. Wilcoxon signed-rank test was used to assess the change from baseline to 6 week within the subgroups.

Overall, vitamin D3 supplementation had no effect on median concentrations of OCN and CTX. Serum CTX was available for 19 children at the two time points with no significant change from baseline to 6 weeks, p=0.2 (see figure 5-9). Out of those 19 children, 7 (37%) children showed low basal CTX levels, 8 (42%) children showed basal CTX levels above the normal reference range whereas 4 (21%) children had basal CTX levels within the recommended level for corresponding age. After 6 weeks of vitamin D3 supplementation, 12 (63%) children had serum CTX within normal reference range, 3 (16%) children showed CTX levels above the normal reference range and 4 (21%) children showed low serum CTX levels. Interestingly, the post treatment 25(OH)D levels for the 4 children showing low serum CTX levels at 6 weeks was 56, 60, 64 and 168 nmol/l whereas post treatment 25(OH)D levels for the 3 children showing high serum CTX levels at 6 weeks was 40, 70 and 106 nmol/l (see Figure 5-10).

The subgroup analysis revealed that the younger age group (10 months-3 years) had basal serum CTX levels exceeding the normal reference range for corresponding age in 6/8 (75%) children whereas 2/8 (25%) children showed basal serum CTX falling within normal range. In contrast, basal serum CTX levels in the middle age group (3- 6 years) was below the recommended level for corresponding age group in 5/7 (71.5%) children and within the normal reference range in 2/7 (28.5%) children. In the older age group (> 6- 9.5 years), the four children showed variability in their basal CTX levels with 2 (50%) children higher and 2 (50%) children lower than normal reference range for their corresponding age. There were no significant differences in serum CTX level between the subgroups (p = 0.1) (see Figure 5-11).

No significant change was observed in the median for serum OCN levels from baseline to week 6, p=0.5. Serum OCN was available for 7 children at the two time points. Of those 7 children, 5 showed low basal OCN levels whereas 2 children had basal OCN levels within normal reference range for corresponding age. After supplementation, the OCN levels returned to normal reference value in 3 children and remained below reference value in the other 2 children, although their 25(OH)D and PTH levels went to the normal reference range. Due to lack of samples for OCN we were unable to look at changes based on subgroups analysis (see Figure 5-12).

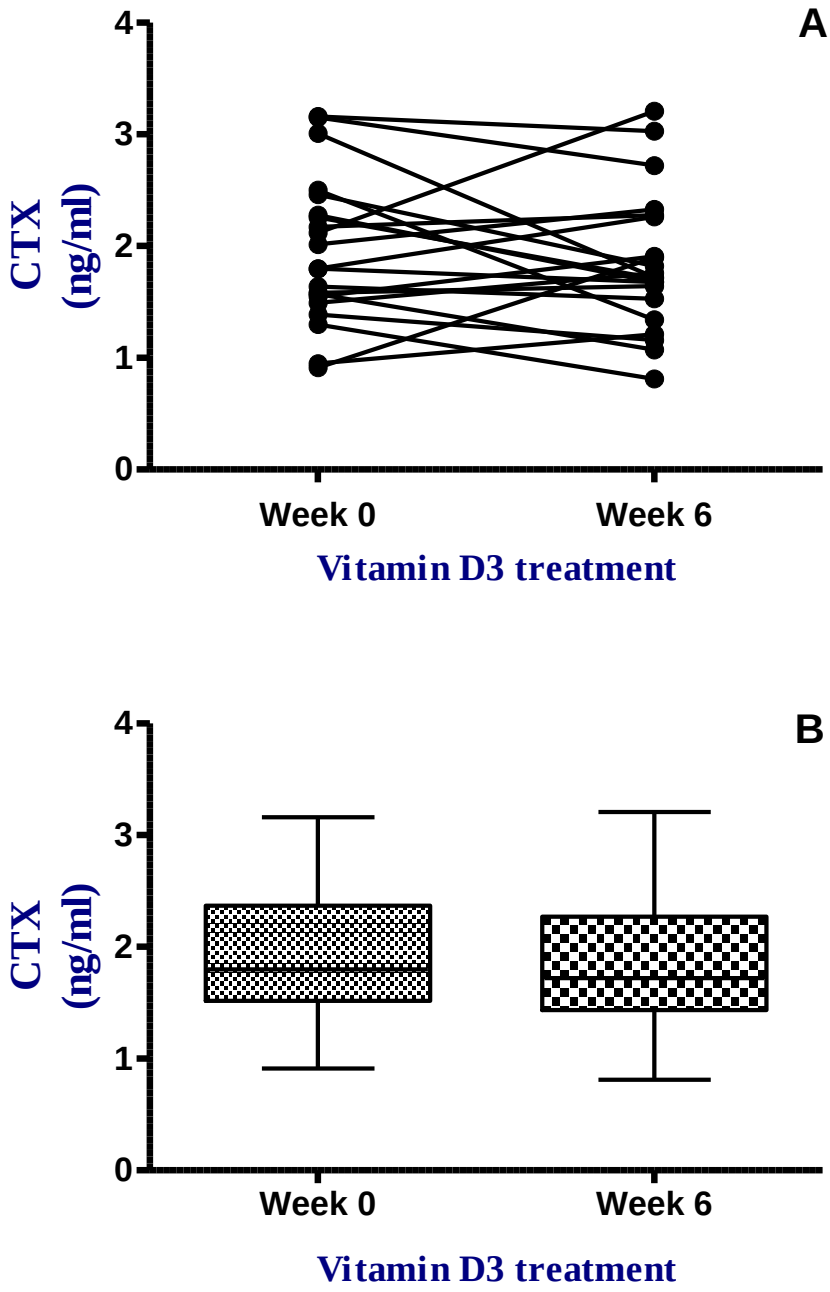
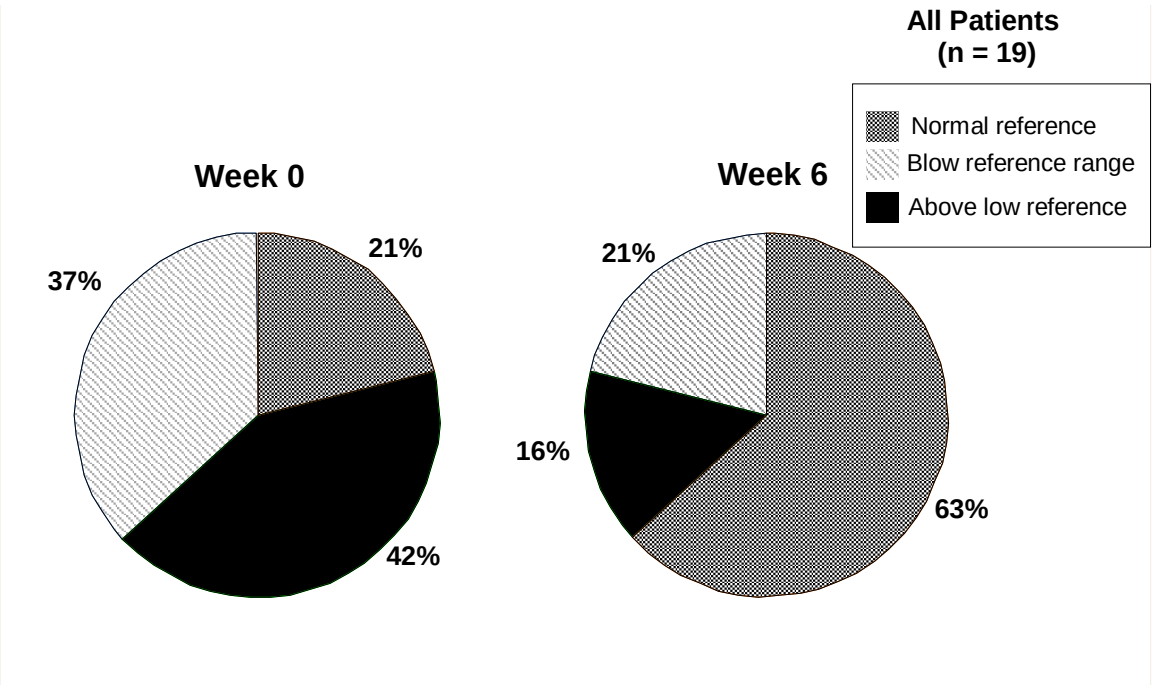


Figure 5-9 A; Individual changes in the serum cross linked C-telopeptide of type I collagen (CTX) for 19 children before and after 6 weeks of receiving 5000 IU vitamin D3 treatment.

Each subjects' CTX level is represented by a line connecting week 0 with week 6 values. B; Box plot showing distribution of CTX levels among the study participants before and after receiving vitamin D3 supplementation. The boxes indicate quartile values; the whiskers indicate the upper and lower values. No significant change in the median of serum CTX levels was observed, p =0.2.

Figure 5-10: Pie chart for the distribution of patients' CTX levels before and after six weeks of receiving vitamin D3 supplements.



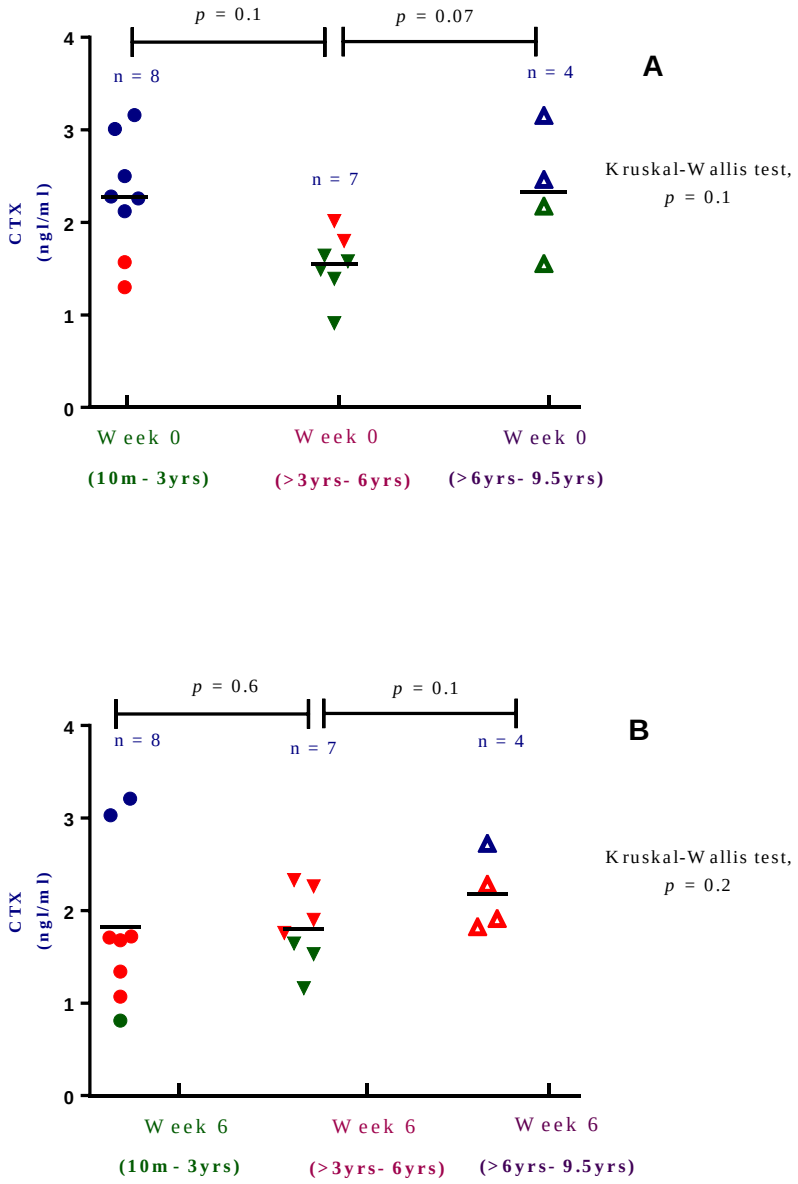


Figure 5-11: Comparison of basal (A) and 6 weeks (B) serum CTX levels (medians) between study participants according to age.

Children were grouped into three age categories; from 10 months to 3 years, from > 3 years to 6 years and from > 6 years to 9.5 years. No significant differences in the median of basal and week 6 serum CTX levels between subgroups or within groups. The numeric variables for the two subgroups were compared using the Mann-Whitney test; comparisons among the three subgroups were analysed using a one-way ANOVA or the Kruskal-Wallis test (non-parametric). Red colours; Children with normal CTX level, Blue; High serum CTX levels and Green: Low CTX levels for each corresponding age.

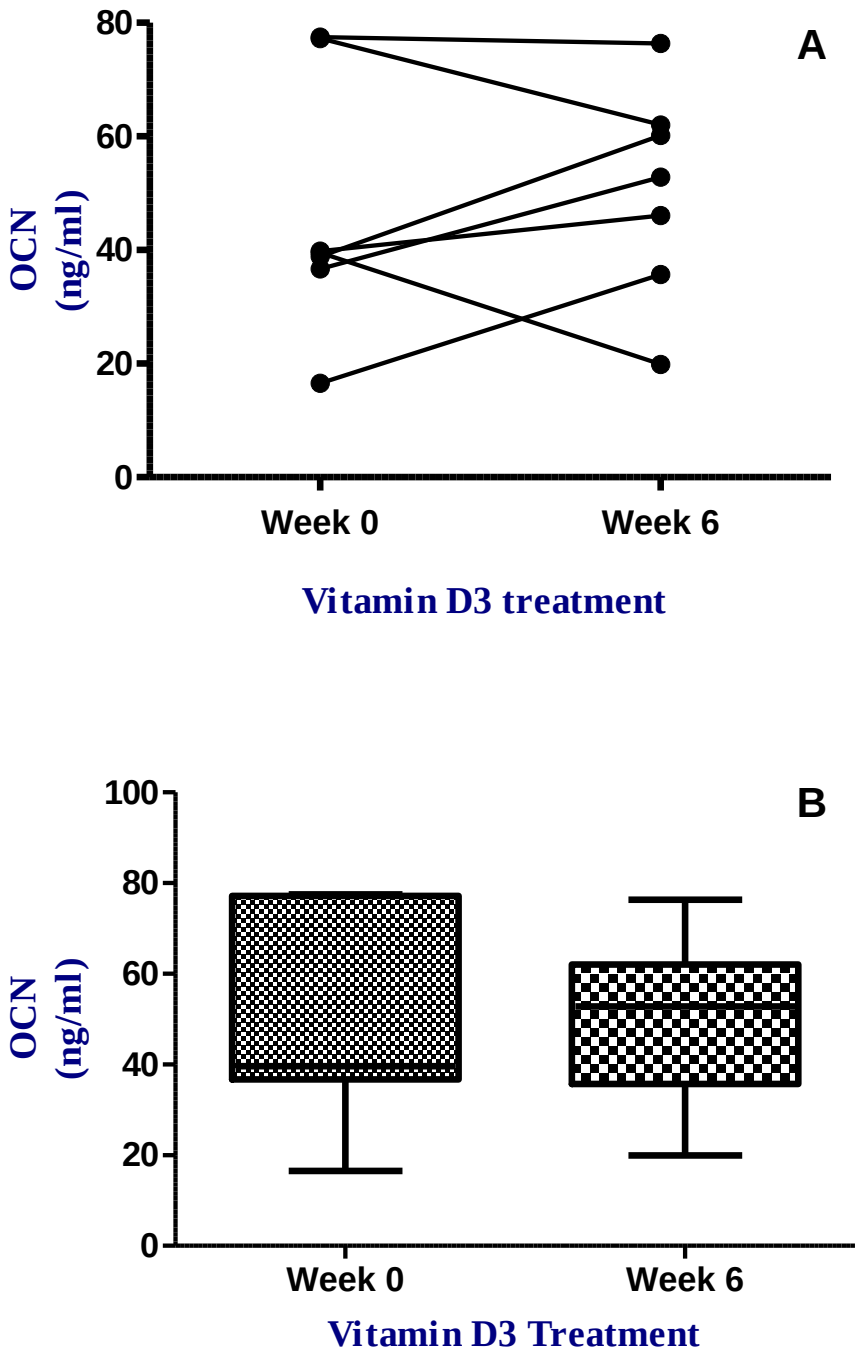


Figure 5-12: A; Individual changes in serum Osteocalcin (OCN) for 7 children before and after 6 weeks of receiving 5000 IU vitamin D3 treatment.

Each subject's OCN level is represented by a line connecting 0 week with 6 week values.B: Box plot showing distribution of OCN levels among the study participants before and after receiving vitamin D3 supplementation. The boxes indicate quartile values; the whiskers indicate upper and lower values. The median of serum OCN levels increased but not statistically significant, p =0.5.

Table 5-4: Individual data for percentage change in serum ALP and CTX in study participants grouped according to their age group.

Patient ID	Age (Months)	Sex	% Change ALP	% Change CTX
	10 months- 3 years			
6	36	Girl	-70.3	-37.8
12	33	Girl	-12.5	-31.9
27	16	Boy	-42.3	-42.8
28	16	Girl	-54.9	-46.3
29	19	Boy	-74.2	-4.3
32	10	Boy	-73.9	-26.3
33	18	Girl	-16.2	-24.3
36	22	Girl	-46.5	51.5
Median	**18.5m (1.5 year)**	-	**-54.9%**	**-29.1%**
Patient ID	**Age (Months)**	**Sex**	**% Change ALP**	**% Change CTX**
	age > 3 years- 6 years			
1	43	Boy	-9.9	15.4
8	46	Boy	-58.2	-6.9
17	71	Girl	-26.1	-16.6
22	63	Girl	5.4	107.6
26	63	Girl	-4.4	17.9
34	66	Boy	-26.7	25.8
37	72	Girl	-4.4	3.7
Median	**63m (5.2 years)**	-	**-9.9%**	**15.5**
Patient ID	**Age (Months)**	**Sex**	**% Change ALP**	**% Change CTX**
	> 6 years- 9.5 years			
3	102	Boy	-17.7	-13.8
5	113	Girl	-38.5	*
7	115	Girl	3.04	4.9
9	78	Boy	3.3	*
11	115	Boy	-59.7	22.9
30	96	Boy	0.44	-25.7
Median	**107m (9 years)**	-	**-8.6%**	**-4.4**

5.4.4.3 Calcium and phosphate

In general, vitamin D3 supplementation had no significant effect on the median of calcium and phosphate levels. Figure 5-13 shows individual changes in serum calcium for the 19 children for whom calcium data was available at the two time points. Most of the 19 children (with the exception of one child) had basal calcium level within the normal reference range, however, following 6 weeks of vitamin D3 treatment there was an increase in the median calcium levels with *p*-value of 0.05, which was close to statistically significance. Post treatment calcium levels remained within the normal reference range for all study participants. The subgroup analysis showed a non-significant increase in calcium levels in children age 10 months- 3 years and > 3- 6 years, p =0.1 and 0.2 respectively. Additionally, treatment with vitamin D3 had no effect on the calcium levels for children in the older group (6- 9.5 years) (see Figure 5-14).

Figure 5-15 shows individual changes in serum phosphate levels for the 19 children whose was available to the researcher. All 19 children had basal phosphate levels within the normal reference range. After 6 weeks, no significant effect from vitamin D3 treatment was observed, p=0.6. Post treatment serum phosphate levels remained within the normal reference range for nearly all study participants except for one child who showed 6 week phosphate level exceeding the normal reference range. Interestingly, pre-treatment bone profile for this child was; 25(OH)D;18 nmol/l, PTH; 6.8 pmol/l, calcium; 2.38 mmol/l, phosphate; 1.07 mmol/l, ALP; 864 u/l and CTX; 2.28 ng/ml. The post-treatment bone profile for this child was; 25(OH)D; 141 nmol/l, PTH; 14.1 pmol/l, calcium; 2.45 mmol/l, phosphate; 1.89 mmol/l, ALP; 225 u/l and CTX; 1.68 ng/ml.

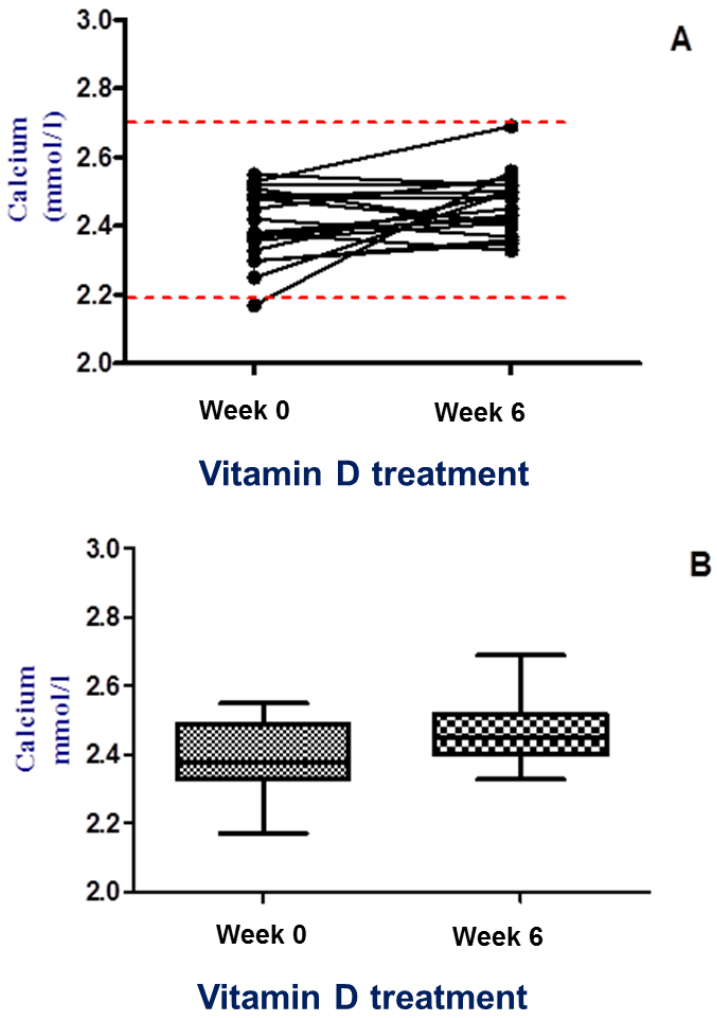


Figure 5-13: A; Individual changes in serum calcium levels for 19 children before and after 6 weeks of receiving 5000 IU vitamin D3 treatment.

Each subjects' calcium level is represented by a line connecting week 0 with week 6 values. Area between two red lines; normal reference ranges for calcium. B; Box plot showing distribution of calcium levels among the study participants before and after receiving vitamin D3 supplementation. The boxes indicate quartile values; the whiskers indicate the upper and lower values. No significant increase in the median of serum calcium levels was observed, p=0.05.

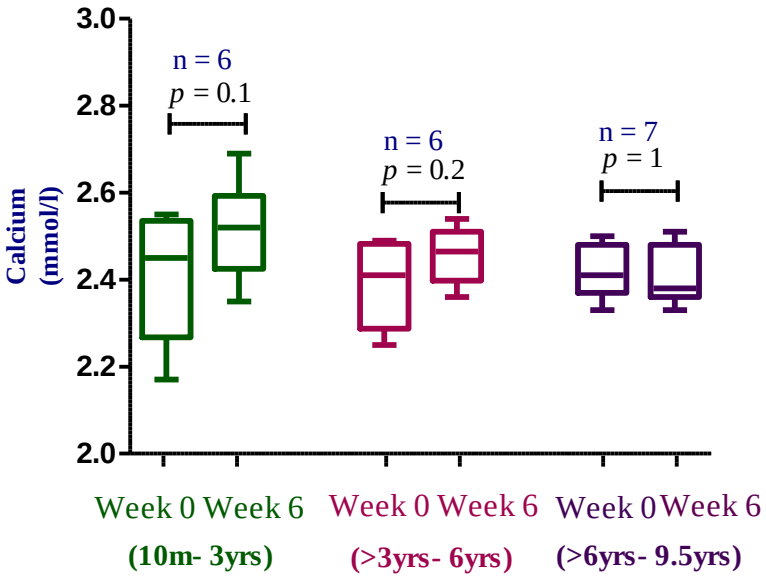


Figure 5-14: Effect of age on serum calcium concentrations before and after receiving 5000 IU vitamin D3 treatment.

Non-significant increase in the median of serum calcium levels in response to vitamin D3 supplementation in children age between 10 months to 3 years and age > 3 years to 6 years, *p*=0.1 and 0.2 respectively. Non-significant change in the median of calcium levels for children age > 6 years to 9.5 years, *p*=1. The boxes indicate quartile values; the whiskers indicate upper and lower values. The Wilcoxon signed-rank test was used to assess the change from baseline to week 6 within subgroups.

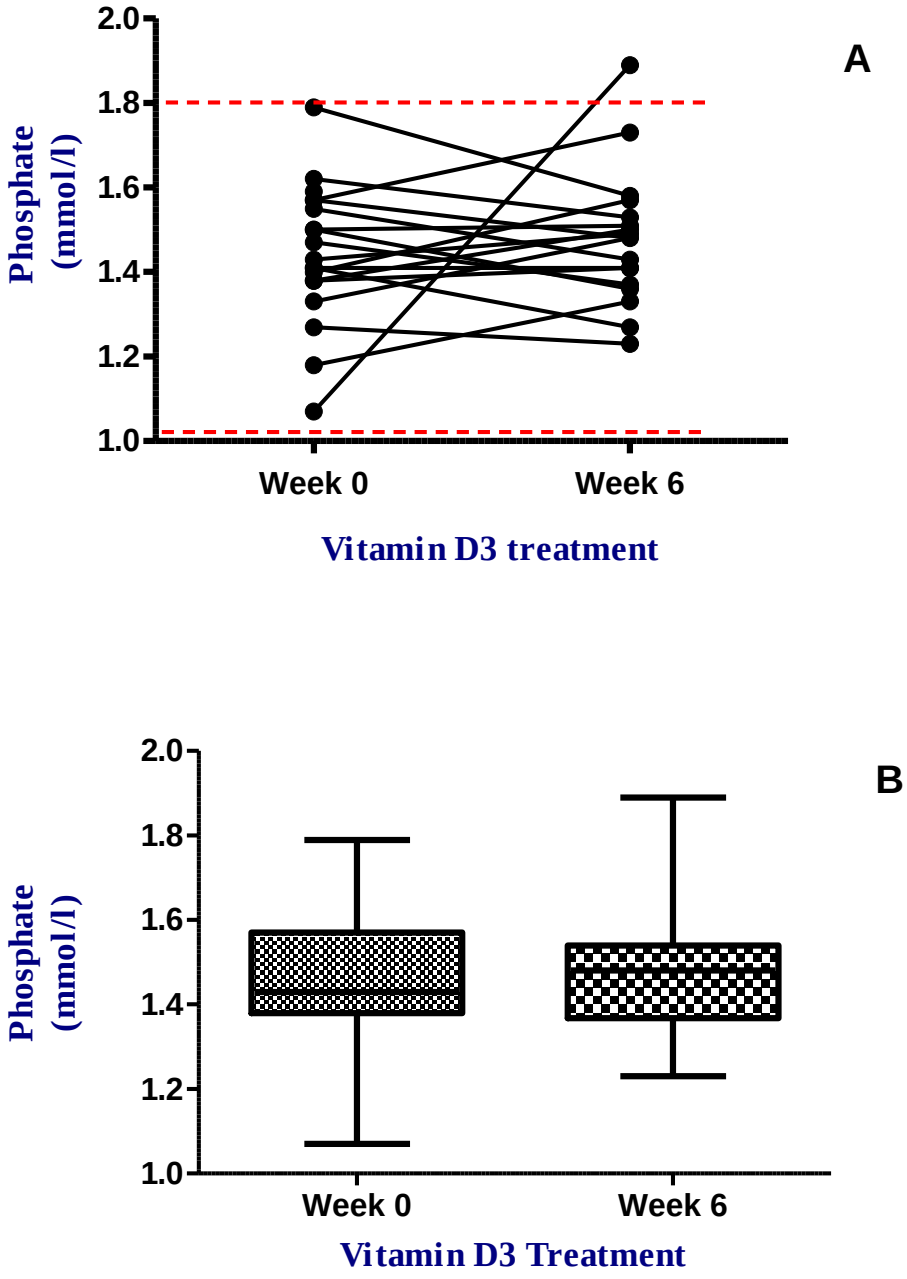


Figure 5-15: A; Individual changes in serum phosphate levels for 19 children before and after 6 weeks of receiving 5000 IU vitamin D3 treatment.

Each subjects' phosphate level is represented by a line connecting week 0 with week 6 values. Area between two red lines; normal reference ranges for phosphate. B; Box plot showing distribution of phosphate levels among the study participants before and after receiving vitamin D3 supplementation. The boxes indicate quartile values; the whiskers indicate upper and lower values. No significant increase in the median of serum phosphate was observed, p =0.6.

5.5 Correlation between vitamin D and bone remodelling markers

5.5.1 Relationships between 25(OH)D, parathyroid hormone (PTH) and calcium at the baseline and week six of the study

No significant correlation was observed between serum 25(OH)D and PTH or calcium at the baseline and week six of the study.

5.5.2 Relationships between 25 (OH) D, PTH and bone biomarkers, ALP, CTX and OCN at baseline and week six and between the changes over 6 weeks

There was no association between baseline and week six serum 25(OH)D with any of the bone remodelling markers. The multivariate analysis further confirmed this (see below). There was a significant positive association between PTH and ALP at baseline, r=0.4; *p*=0.02 which become statistically non-significant at week six, *p*=0.08 (Table 5-5). There was a trend towards a positive association between PTH and CTX, r= 0.4; *p*=0.08 at study baseline. Changes in serum 25 (OH) D were not associated with changes in any of the bone remodelling markers. However, change in serum PTH was correlated significantly with changes in serum ALP, r= 0.5; *p*=0.01 (see Figure 5-16) (Table 5-6).

Table 5-5: Univariate correlation coefficients.

	Correlation at baseline r	*p* value	Correlation at week six r	*p* value
Serum 25(OH)D and PTH	-0.3	0.08	0.2	0.2
Serum 25(OH)D and calcium	0.07	0.7	0.05	0.8
Serum PTH and calcium	-0.1	0.4	0.04	0.8

Changes in the correlation between 25(OH)D, PTH and calcium from baseline to week six of the study period.

Table 5-6: Univariate correlation between 25(OH)D, PTH, bone remodelling markers at baseline and at week six.

A Base line	**25(OH)D**		**PTH**	
Simple regression	r	***p*-value**	**r**	***p*-value**
Serum ALP	-0.2	0.2	0.4	0.02
Serum CTX	-0.2	0.2	0.4	0.08
Serum OCN	0.1	0.7	-0.3	0.4
B Week six	**25(OH)D**		**PTH**	
Simple regression	**r**	***p*-value**	**r**	***p*-value**
Serum ALP	0.1	0.5	0.4	0.08
Serum CTX	-0.1	0.6	0.04	0.8
Serum OCN	-0.3	0.4	-0.6	0.3
C Change over 6 weeks	**25(OH)D**		**PTH**	
Simple regression	**r**	***p*-value**	**r**	***p*-value**
∆ Serum ALP	0.05	0.8	0.5	0.01
∆ Serum CTX	0.007	0.9	0.09	0.7
∆ Serum OCN	-0.1	0.7	0.08	0.8

Univariate correlation between change in 25(OH)D, change in PTH and change in markers of bone remodelling over 6 weeks. ∆: 6 week value-baseline value.

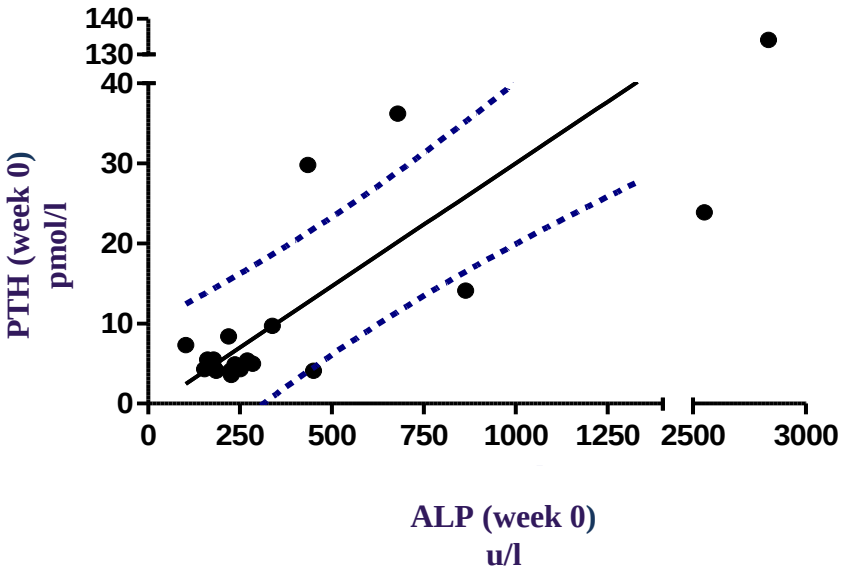


Figure 5-16: The relationship between PTH and ALP at baseline (week 0).

The black line indicates the line of best fit, and the blue lines indicate the 95% confidence limits. A significant association was observed, *p*=0.02.

5.5.3 Mixed Effects Model (MEM)

To conduct further analysis, a Mixed Effects Model (MEM) analysis (a hierarchical model with random intercept and slope) was employed. This analysis only took into account data for which values were available at both study time points. No independent effect between vitamin D with any of the bone remodelling parameters, although there was a trend towards a significant independent association between vitamin D with both PTH (p=0.08) and CTX (p=0.06). The parathyroid hormone independently associated with ALP and CTX with p values of 0.001 and 0.02, respectively. There was a trend for PTH to be independently associated with vitamin D, p=0.09. Age was independently associated with PTH with p values of 0.001. Time is independently associated with CTX and vitamin D with p values of 0.02 and 0.0001, respectively. Interestingly, there was a trend for a significant independent association between sex and CTX. The results from MEM are summarised in Table 5-7.

Table 5-7: Mixed model analysis for vitamin D, PTH and bone remodelling.

Variables	Estimate	SE	95% CI (Lower, Upper)	*P*-Value
Log Vitamin D				
Intercept	1.4	0.29	(0.8, 2.07)	0.0001
Time	0.4	0.06	(0.3, 0.6)	**0.0001**
Age	-0.002	0.001	(-0.004, 0.0001)	0.7
Sex	-0.08	0.07	(-0.2, 0.06)	0.2
Race	-0.05	0.05	(-0.1, 0.05)	0.2
Calcium	0.01	0.05	(-0.09, 0.1)	0.4
Log PTH	-0.2	0.12	(-0.4, 0.03)	0.09
Log PTH				
Intercept	1.79	0.3	(1.1, 2.4)	**0.0001**
Time	-0.1	0.1	(-0.3, 0.1)	0.3
Age	-0.004	0.001	(-0.006, -0.001)	**0.001**
Sex	-0.04	0.07	(-0.2, 0.1)	0.6
Calcium	0.03	0.05	(-0.07, 0.1)	0.5
Log vitamin D	-0.05	0.06	(-0.1, 0.07)	0.08
Log ALP				
Intercept	2.5	0.39	(1.7, 3.4)	0.0001
Time	0.03	0.09	(-0.1, 0.2)	0.7
Age	-0.002	0.001	(-0.005, 0.0006)	0.1
Sex	-0.09	0.09	(-0.2, 0.1)	0.3
Race	0.001	0.06	(-0.1, 0.1)	0.9
Calcium	-0.04	0.06	(-0.1, 0.09)	0.5
Log vitamin D	-0.11	0.1	(-0.4, 0.1)	0.4
Log PTH	0.4	0.1	(0.2, 0.7)	**0.001**
CTX				
Intercept	1.3	0.9	(-0.6, 3.3)	0.1
Time	0.6	0.2	(0.09, 1.1)	**0.02**
Age	0.002	0.003	(-0.003, 0.009)	0.3
Sex	-0.3	0.1	(-0.7, 0.03)	0.07
Race	0.2	0.1	(-0.03, 0.5)	0.08
Calcium	0.1	0.1	(-0.1, 0.4)	0.4
Log vitamin D	-0.7	0.3	(-1.5, 0.06)	0.06
Log PTH	0.7	0.3	(0.08, 1.3)	**0.02**

5.6 Discussion

A considerable number of studies have examined vitamin D status and the effect of vitamin D supplementation on markers of bone remodelling in the adolescent and adult population [461, 462]. Cross-sectional data studying the association of serum 25(OH)D with bone remodelling markers in children and young adults shows disagreement. For example, a study in Swiss children and adolescents found no association between bone remodelling markers and serum 25(OH)D [453], whereas others in Chinese and Irish adolescents demonstrated a significant negative association between serum 25(OH)D and bone remodelling markers [454, 455]. In a further interventional study, after 12 weeks of either 400, 1000, 2000, or 4000 IU of vitamin D3 supplementation daily to healthy pubertal multi-ethnic adolescents, neither baseline nor 12 week 25(OH)D levels were associated with bone remodelling markers [456]. Supplementation with either 200 IU or 400 IU of vitamin D3 over a period of 12 months to 221 vitamin D sufficient girls aged 11-12 years did not appear to have any significant effect on markers for bone remodelling. However, there was an effect on bone mineral density for those with the FF VDR genotype, indicating an influence from genotype [457]. These results reflect those observed in older subjects, aged between 18 and 27 years old, supplemented daily with 600 IU vitamin D3 and 1500 mg calcium for a period of eight weeks [463]. Four weeks of supplementation for healthy children aged between 6.2 and 13.7 years with 600 IU vitamin D3 does not appear to have any significant effect on the rate of bone remodelling [464]. However, these observations differ from those for younger vitamin D deficient children, aged 8.5-10.5 months. When this group received daily supplements containing 3000-4000 IU of vitamin D3, there was a significant effect on serum ALP levels as early as two weeks post treatment, and levels returned to normal within eight weeks of treatment [465]. Additionally, there was a transient but significant increase in osteocalcin during the first two weeks of treatment, which decreased gradually thereafter [465].

The majority of the available literature concerning vitamin D and bone health in children focuses on high-risk groups, i.e. those with rickets due to the very low content of vitamin D in breast milk, subjects with special dietary requirements, or those with particular ethnic origins [465-468]. However the effect of improving serum 25(OH)D status on markers of bone remodelling during infancy and early childhood continuous to be unclear, with a limited number of available studies [469, 470]. Such studies are of particular significance in Scotland, where people live at high latitude with prolonged winters, during which daylight hours are shorter.

In our study, short-term vitamin D3 supplementation significantly did reduce the overall median level of PTH. This reduction was most significant in children aged 10 months– 3 years and 3- 6 years. The younger group showed higher basal PTH levels and lower basal serum calcium. After supplementation, serum PTH concentrations decreased for all participants, regardless of their basal serum 25(OH)D levels.

The suppression of PTH with vitamin D3 supplementation was expected, and is consistent with previous observations, which have reported a linear reduction in serum levels of PTH at any basal 25(OH)D concentration below 30–40 ng/ml (75–100 nmol/l). At 25(OH)D concentration between 75–100 nmol/l, PTH level is suggested to plateau and remain stable [471, 472]. However, PTH concentrations were not elevated at the start of our study in many participants particularly in the age group older >6- 9.5 years. In addition to the serum 25(OH)D both serum calcium and age were found to be significant contributors in determining PTH level [473]. Therefore, the lack of elevation of PTH despite the lower vitamin D status in older children may be attributed to normal serum calcium levels, which are one of the main trigger of PTH secretion.

At the baseline, only two children in this study had low serum calcium levels with high PTH and very high ALP (Patients IDs: 29 and 36). Both children are < 24 months in age and both are still being exclusively breastfed at the time of study. Post treatment serum 25(OH)D levels for those two children reached the normal reference range in one child and remained below 50 nmol/l in another child. Additionally, in both children, there was an improvement in serum ALP but this still exceeded the upper normal limit at the end of the treatment course. Both children received a Sandocal tablet (400 mg/1 tablet twice a day). Calcium levels returned to the normal range in one child and was not available in the other child. Similarly, one previous study has reported that normal serum ALP levels and complete radiological healing were not observed before 12 weeks of combined vitamin D and calcium treatment [474]. Therefore, the response to vitamin D supplementation may depend on pre-treatment levels, treatment duration and vitamin D dose.

Rickets due to low dietary calcium intake has been reported in many developing countries including Northern Nigeria and parts of India. In the UK low dietary calcium intakes was linked with inappropriate infant feeding, such as using a soy based milk not formulated for infant. The biochemical features that have been used to differentiate dietary calcium deficiency rickets from vitamin D deficiency rickets are raised serum 1,25(OH)2D level, which nearly doubles in response to a single oral dose of vitamin D (due to high vitamin D requirements) and 25(OH)D concentrations being greater than those generally associated with vitamin D deficiency [474, 475].

Several studies report that serum 25(OH)D was inversely associated with PTH. This correlation was not observed in this study [472, 476]. The results from correlation study showed an inverse but non-significant relationship between serum PTH and both 25(OH)D and calcium. The lack of significance in the correlating study may be attributed to the weak responses to vitamin D3 supplementation in a considerable number of study participants (n=7), in addition to low basal PTH levels with minimal non-significant change after supplementation [477, 478].

Some reports also suggest that not everyone who is vitamin D deficient has an increased PTH level [479, 480]. Interactions between the endocrine and paracrine vitamin D network are not yet fully recognised, and this may also explain the apparent lack of a relationship between PTH and 25(OH)D levels. The main production site of vitamin D in the kidney, the active form of vitamin D is also produced locally in many organs/tissues. Therefore, the possibility of higher or lower serum PTH levels reflecting vitamin D status at tissue-level may explain the apparent dissociation between vitamin D and PTH. For example, in one recent study, the prevalence of secondary hyperparathyroidism was 32.5% in vitamin D insufficient/deficient critically ill patients, and both secondary hyperparathyroidism and vitamin D deficiency were associated with greater disease severity although there was no observable correlation between serum 25(OH)D and PTH ($r = -0.11$, $p = 0.3$) [481].

Before supplementation, the median serum level for ALP exceeded the normal reference range, returning to the normal range following supplementation. When we conducted a subgroup analysis, the median ALP concentration in the younger group (10 months– 3 years) was significantly higher than in the older groups, possibly indicating increased bone remodelling. However, following supplementation, serum ALP decreased significantly in almost all children, regardless of their basal ALP levels. A similar pattern of ALP

reduction in relation to basal vitamin D status for both children and adults is reported widely in the literature [465, 482]. Increased levels of serum ALP may be an indication of an abnormally late phase of mineralization, and the reduction in ALP, after treatment may be a sign of recovery of skeletal abnormality. However, due to the nature of our study which cited data at only two time points and involved one X-ray, we were unable to monitor changes in clinical/radiological abnormalities in relation to these biomarkers. Monitoring of biomarkers dynamic may be particularly important for children who showed very high bone biomarkers levels accompanied with profound clinical/radiological rickets at baseline which were not fully recovered after supplementation. Very high ALP levels were evident in just 2 children in our study with basal ALP levels of 2834 and 2551 u/l that decreased to 729 and 1364 u/l, respectively, following vitamin D3 supplementation (Patients IDs: 29 and 36). As these bone biomarkers remained above the normal reference values, even after supplementation, this might indicate the need for a longer duration of vitamin D3 treatment for individuals with profound rickets.

Osteocalcin is the main non collagen protein contained in the organic matrix of bone synthesised by the osteoblast. Few studies have investigated the effects of vitamin D supplementation on serum OCN and CTX. The majority of available studies involve adults and the elderly with abnormal bone remodelling such as post-menopausal women at risk of developing osteoporosis. In these studies an alteration in bone remodelling markers in response to vitamin D supplementation may be expected [483, 484]. For example, 6 months daily supplementation for both pre and post-menopausal women with 4000 IU vitamin D3 or placebo daily had different effects on rates of bone remodelling. In postmenopausal women, both CTX and OCN levels increased significantly (P=0.001, P=0.004 respectively), indicating an augmented rate of bone remodelling. However, in premenopausal women there was no significant response to vitamin D3 supplementation in either CTX or OCN [483]. Data linking serum 25(OH)D and bone remodelling markers in children and adolescents is inconclusive. To our knowledge, the majority of accessible studies that have explored the effect of vitamin D3 supplementation on OCN and CTX have been conducted in either older children or adolescents, with no significant changes observed [161, 457, 464] [456, 468].

A limited numbers of studies have examined the effect of vitamin D3 supplementation on OCN and CTX in infants or those in younger age groups [465]. In our study, vitamin D3 supplementation was found to have no effect on overall and subgroup median OCN and CTX levels, and most of the children who were still vitamin D deficient/insufficient, CTX

remained above and/ or below the normal reference values. Low OCN concentrations present in many children (5 children or 74%) might reflect suboptimal osteoblastic activity. The non-significant effect of vitamin D3 supplementation on both CTX and OCN in our study population could be attributed to multiple factors such as small sample size and study duration which may not be sufficient to observe a significance change. Additionally, vitamin D itself has been proposed to play a more vital role in bone mineralisation rather than bone remodelling [452]. For example, both vitamin D deficient rats and VDR knockout mice showed no abnormal intrauterine skeletal development, indicating that vitamin D and the VDR might not play an essential role in skeletal formation [485, 486]. The wide variation in end point serum 25(OH)D among our study participants could also influence the vitamin D threshold suggested to maintain bone metabolism [476, 487].

The results from the univariate correlation and multiple regression analyses performed here were consistent with previous observations, showing no significant association between vitamin D and bone remodelling markers. However, our results showed PTH is independently correlated with bone remodelling markers, ALP, CTX and OCN. Therefore, secondary hyperparathyroidism, due to vitamin D deficiency, may impose a more significant action on bone remodelling rather than vitamin D alone. Furthermore, there was a trend for vitamin D to be an independently associated with CTX, $p = 0.06$. The results from the mixed linear model also indicated age, sex and race, as independent factors to consider in future studies. The results from the correlation analysis further supported the evidence available for the significant independent role of PTH in the bone remodelling process, and in the regulation of serum calcium and phosphate levels. The principal action suggested for PTH in bone is through its main receptors, PTH/PTHrP type 1 receptor (PTH1R), leading to the activation of multiple signalling pathways, which are key regulators of bone metabolism, such as Wnt signalling [488].

Althogh defining the ideal level of vitamin D to optimize musculoskeletal health and fructure prevention remains under debate. However, achieving a level of 25(OH)D between 75-150 nmol/l is both safe and effective for multiple health outcomes. In this study there was a variability in the response to 5000 IU daily oral vitamin D3 supplementation. For example, 12 (52%) children reached a final serum 25(OH)D level of 75-150 nmol/l and three (13%) children exceeded the normal reference range with a final 25(OH)D level of > 150 nmol/l. Conversely, serum 25(OH)D in eight (35%) children remained at < 75 nmol/l. Therefore, this study could not confirm whether a dose of six weeks 5000 IU vitamin D3 supplementation is effective to increase serum 25(OH)D level

of > 75nmol/l in a considerable number of study participants. Poor compliance may be a factor for some children, while misuse of the medication may be the cause for the very high level of vitamin D. An alternative option suggested by other studies to address the problem of poor compliance is to use stoss therapy or a high single dose of 100,000-600,000 IU of oral (capsule, tablet or gel) or intramuscular vitamin D followed by a low maintenance dose [489].

The main strength of this study is the prospective and the interventional nature of the work, in addition to its unique population. However, there are several limitations that should be considered. Firstly, due to ethical issues specifically related to study design we were not able to construct a control group without vitamin D3 supplementation. Absence of a control group is a critical limitation of this part of the project. This study did not contribute to several potential confounding variables such as feeding practices, sun exposure and physical activity which may affect the individual responses to vitamin D3 treatment. Further, observed changes in the bone turnover markers possibly reflected high bone turnover during the infancy and early childhood, effect of vitamin D3 supplementation, or most likely, a combination of both. Finally, compliance might be affected by a combination of two possible confounding factors: a liquid form of vitamin D supplement and parental ambivalence regarding treatment.

In conclusion, supplementation of children with 5000 IU of vitamin D3 for six weeks significantly increased serum 25(OH)D levels and affected the level of bone formation marker, ALP. The effect of vitamin D3 supplementation on bone remodelling biomarkers may depend on the age in addition to on pre-treatment level and treatment duration. The results from this study, as well as previous studies have suggested ALP as a reliable marker for monitoring patients with nutritional vitamin D deficiency than CTX and OCN in addition to an indicate that PTH might have an independent role to play in bone remodelling process. Finally, an additional randomized control trial are needed to confirm the effectiveness vitamin D dose and to validate the clinical significance of our results and longer durations of high-dose vitamin D treatment, involving multiple doses at measurable intervals are advised for future studies.

Chapter 6

Effect of Vitamin D3 Supplementation on Glucose Homeostasis

6.1 Abstract

Introduction

Observational studies point to an association between lower circulating 25(OH)D status and an increased risk of diabetes mellitus. Interventional studies are inconclusive, and the effect of improving vitamin D status on glucose homeostasis in vitamin D deficient, otherwise healthy children is still unclear.

Objective

To investigate the effect of vitamin D3 supplementation on glycaemic status in vitamin D deficient children.

Method

Twenty-three pre-pubertal children aged 10 months to 9.5 years (10 boys) were diagnosed with vitamin D deficiency [25(OH)D level >50 nmol/l] and insufficiency [25(OH)D level >73 nmol/l] and recruited during 24 months of the study period. Treatment consisted of 5000 IU vitamin D3 (cholecalciferol) given orally once a day for six weeks. At baseline and completion of treatment the following were measured: 25 hydroxyvitamin D (25(OH)D), parathyroid hormone (PTH), glycated haemoglobin (HbA1c), sex hormone binding globulin (SHBG), fasting insulin, fasting glucose, homeostasis model assessment index - estimated insulin resistance (HOMA IR) and lipid profile.

Results

In 17 patients for whom glucose, insulin and HOMA IR data were available, median (range) serum 25(OH)D increased significantly from 26.5 (14, 49) nmol/l at the baseline to 96 (37, 191) nmol/l at week six ($p < 0.0001$). Serum PTH concentrations decreased significantly from a median of 6.3 (3.6, 134) pmol/l at the baseline to 4 (1.9, 6.8) pmol/l at week six ($p < 0.0001$). HOMA IR decreased significantly from a median of 2.18 (0.56,

20.29) at the baseline to 1.59 (0.37, 12.5) at week six (p = 0.04). Fasting serum insulin decreased from a median of 11.1 (2.46, 99.2) µiu/ml at the baseline to 8.1 (1.8, 66.6) µiu/ml (p = 0.05).

Conclusion

Short term improvement of vitamin D deficient status was associated with improvement in insulin resistance. Further interventional studies are still needed.

6.2 Introduction

As described earlier in the introduction of this thesis, there has been increased research on the effects of vitamin D on glucose homeostasis and conditions preceding type 2 diabetes, such as insulin resistance and impaired β cell function [490]. Epidemiological studies have linked low vitamin D status with impaired glucose tolerance and an increased risk of diabetes mellitus [256, 266, 491]. However, the majority of studies which involved the younger population have failed to prove relevant vitamin D effects on glucose homeostasis and insulin resistance.

Impaired insulin secretion and decreased glucose tolerance have been reported in VDR mutant mice compared with those with functional receptors, suggesting a direct role of vitamin D in the regulation of insulin secretion [229]. Evidence from non-genomic studies have also pointed to an indirect role of vitamin D in insulin secretion through regulation of intracellular calcium concentrations, known as the main stimulator for glucose-induced insulin secretion [490]. In peripheral tissues such as skeletal muscle, the active form of vitamin D is suggested to improve free fatty acid–induced insulin resistance accompanied by an increase in insulin responsive-ness for glucose transport in cultured myocytes (i.e., improved glucose uptake) [236, 237].

It has been suggested that combined assessment of both vitamin D status and PTH is needed for optimal evaluation of the impact of vitamin D on glucose homeostasis. There is evidence showing that PTH is positively correlated with the prevalence of type 2 diabetes. For example, PTH treatment of cultured adipocytes has been shown to induce a reduction in insulin-stimulated glucose uptake and to down regulate the insulin secretion signalling pathway [492]. Additionally, primary hyperparathyroidism has been suggested as a risk factor for insulin insensitivity and β cell dysfunction [493]. However, epidemiological studies which examined the effect of PTH on abnormal glucose homeostasis still show conflicting findings [494, 495].

To my knowledge, most, if not all, available interventional studies assessing the effect of vitamin D supplementation on glucose homeostasis involve higher-risk older children and/or adolescents (either obese or pre-diabetes). There is little information available about the effect of improving vitamin D status in vitamin D deficient healthy young children or infants or when raising serum 25(OH)D from < 50 nmol/l to > 75 nmol/l. In one interventional study, which aimed to treat obese children and adolescents (basal

25(OH)D < 56 nmol/l) with 20000 IU vitamin D2 daily for 28 days, both insulin sensitivity and glucose tolerance, but not insulin secretion, were improved [161].

This chapter aims to investigate the short term effects of vitamin D3 supplementation on insulin resistance in vitamin D deficient healthy children in addition to glycaemic status and lipid profile. This chapter also looked at the association between markers of glucose homeostasis and markers of bone remodelling.

6.3 Subjects and methods

6.3.1 Subjects

Chapter 4 of the thesis described in detail subject characteristics and inclusion/exclusion.

6.3.2 Study methods

Study protocol was detailed in Chapter 4 of this thesis. Briefly, the study design involved six weeks of 5000 IU vitamin D3 (cholecalciferol) given orally once a day to children referred to the endocrine department at RHSC for vitamin D deficiency.

6.3.3 Blood collection

Blood samples were collected in Ward 1C at RHSC, in the morning after a six-hour overnight fast. At baseline and completion of treatment, serum 25 hydroxyvitamin D (25(OH)D), parathyroid hormone (PTH), alkaline phosphatase (ALP), serum collagen type 1 cross-linked C-telopeptide (CTX), glycated haemoglobin (HbA1c), sex hormone binding globulin (SHBG), fasting serum insulin, fasting blood glucose, and homeostasis model assessment index estimated insulin resistance (HOMA IR) were measured. The original HOMA IR from Matthew et al. was used to assess the outcomes of the intervention [218]. This model employs a simple linear equation based on the combination of fasting serum glucose (FSG) and fasting serum insulin (FSI) to exhibit a measure for insulin resistance: HOMA IR = (FSI[mu/l] × FSG[mmol/l])/22.5. This method is simple, cheap and has been compared with other well validated methods of measuring insulin resistance and β cell function such as glycemic clamps [496].

6.3.4 Biochemical assays

All blood sampling was performed at the time of routine clinical visits; samples were immediately centrifuged at 2600-2800 rev/minute for 10 minutes, and the serum was subsequently stored at -80°C until the assays were performed. Serum 25(OH)D levels were assayed using the Abbott Architect Chemiluminescent Microparticle Immunoassay (CMIA). Parathyroid hormone measurements were assayed using intact PTH CMIA (Abbott Architect). Insulin was measured by enzyme immunoassay for the quantitative measurement of insulin in human serum (DRG Instruments, GmbH DRG International, Inc., Germany) with an intra-assay CV of 1 to 1.22%. Cholesterol measurements were

assayed using the Abbott ARCHITECT enzymatic cholesterol esterase method. Triglyceride measurements were assayed using the Abbott ARCHITECT glycerol phosphate oxidase method. The ELISA for the insulin was performed by Mr McMillan in the Department of Child Health at RHSC, Glasgow. Dr. McNeilly performed the assays for serum 25(OH)D and lipids profile in the Biochemistry Department at Southern General Hospital, Glasgow. The remaining samples were sent directly to the biochemistry laboratory at RHSC to test for fasting blood glucose, serum HbA1c and serum SHBG. HbA1c was measured using high performance liquid chromatography (HPLC). SHBG and fasting glucose were measured using an immunoassay analyser and glucose reagent kit respectively (ARCHITECT ci8200 System, Abbott Laboratories).

6.3.5 Statistical analysis

All statistical analyses were performed using GraphPad Prism 5 (San Diego, California, USA) or Mini Tab 17 (Minitab, Coventry, UK) statistical software and the results are presented as median and ranges, unless otherwise specified. The significance of the changes in the measurements between baseline and week six for all study participants were assessed using the Wilcoxon signed-rank test. Mann-Whitney U test were performed to analyse the two independent subgroups. A Spearman correlation analysis was also performed to reveal the relationship between vitamin D, bone remodelling and glucose homeostasis parameters. Using SPSS 22 Statistical Software (USA), a mixed model was employed where appropriate to explore the confounding effects of independent variables. For Mixed Effects Model analysis, the transformed values for 25(OH)D, PTH, glucose, insulin, HOMA, SHBG and HbA1c were used to satisfy the assumption of normal distribution. A significance level of $p < 0.05$ was used to evaluate all the study results.

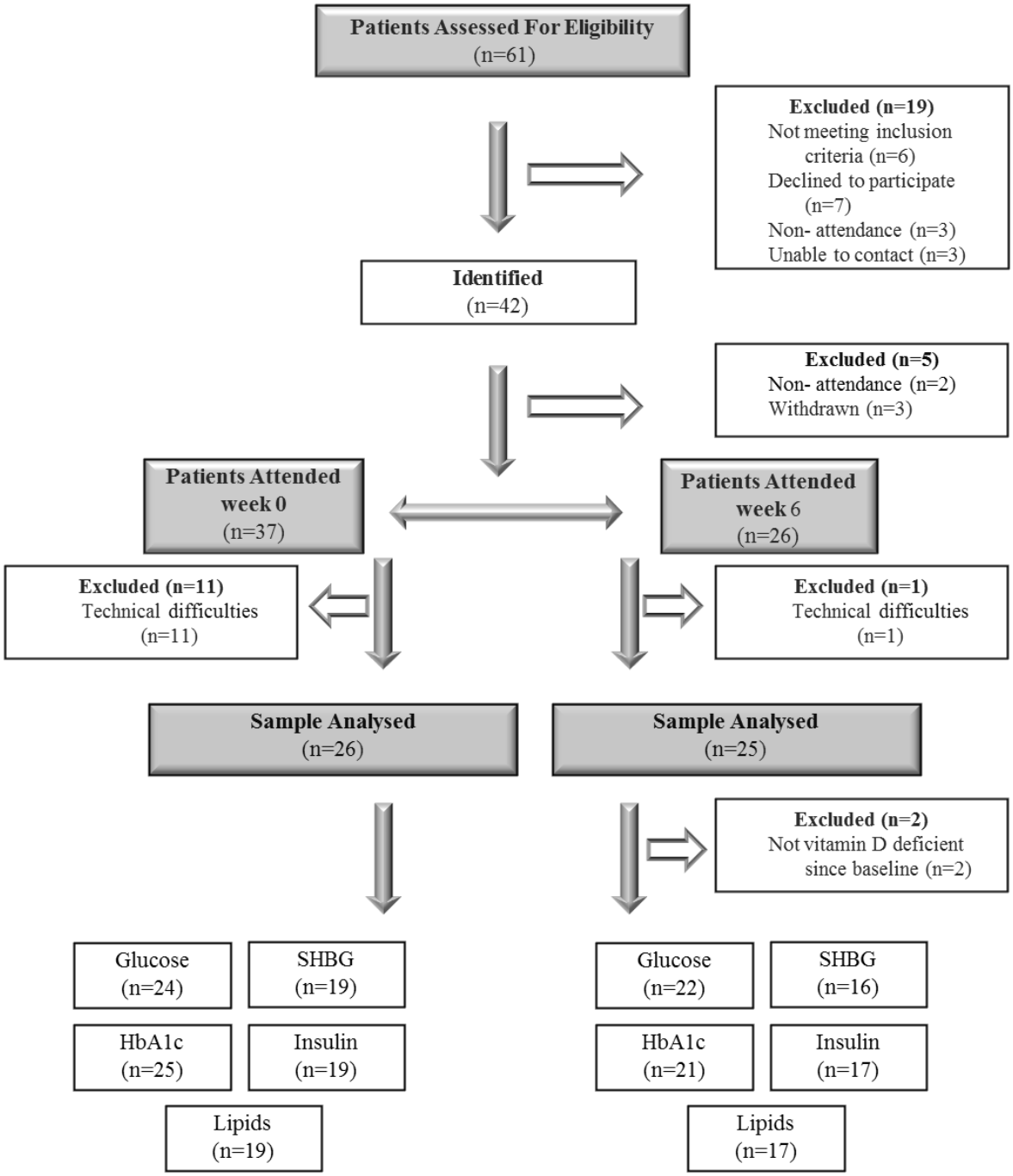
Only children with data available at both the baseline and week six of the study period were included in the MEM statistical analysis. Children for whom there was missing data recorded at either point in the study period were included in the univariate association analysis only.

6.4 Results

6.4.1 Recruitment summary

A total of 61 children were identified and screened during the 24 months of the study period between 2011 and 2014. Forty-two children met the main project inclusion criteria and agreed to participate. Thirty-seven children attended the first study visit, of whom 12 children were excluded due to difficulty with venous access, lack of patients/parents cooperation or insufficient blood sample volume. Twenty-six children's samples were analysed at the baseline and 25 samples at week six of the study. There was difficulty in obtaining full investigations for every child, particularly in the younger age group. This was mainly due to technical difficulties such as venous access, in addition to lack of cooperation from both parents and children. Figure 6-1 shows a flow diagram of study recruitment.

Figure 6-1: Flow diagram of study recruitment. Sixty-one children were identified through the endocrine clinic



*Thirty-eight children were excluded throughout the study period due to a variety of reasons as shown in the diagram.

6.4.2 Demographic characteristics

Twenty-three pre-pubertal children, 10 boys with a median age of 55 months (10 to 115 months) and 13 girls with a median age of 63 months (16 to 115 months) enrolled at the two time points of the study. Height (Ht) and body mass index (BMI) were converted into SDS using the 1990 British childhood standards [448, 449]. Standard Deviation Score (SDS) (BMI SDS) were 0.2 (-0.68, 2.29) and 0.67- (-1.45, 2.42) for boys and girls respectively. Weight Standard Deviation Score (Wt SDS) were 0.05 (-1.02, 2.88) and 0.39 (-4.5, 2.65) for boys and girls respectively. The main reason for referral was bone pain, with 14 children in total (61%) being referred for this reason. Out of those 14 children, 13 children were referred from the orthopaedic clinic and one child was referred directly from the general practitioner (GP). Radiological screening was performed in 20 study participants and we were unable to trace the reports in two children who were referred mainly due to bone pain. One other child did not have an X-ray due to parental preference. Other causes for referral included bowed leg, seizure, dietary concern, fracture and family history. Table 6-1 summarises the details of the 23 children enrolled in the study.

Childhood obesity has been suggested to be associated with impaired glucose tolerance and insulin resistance [497]. Therefore, increased BMI among study participants was identified and represented in a separate table. Three obese children (13%) were identified and their detailed information is provided in 6-2.

Table 6-1: Characteristics of recruited children.

	N	
Age (years)	**(23)**	**%**
0-2	5	22
2-6	9	39
6-10	9	39
Gender	**(23)**	**%**
Male	10	43.5
Female	13	56.5
Race	**(23)**	**%**
South Asian	10	43.5
Middle Eastern	4	17.5
Sub-Saharan African	3	13
Caucasian	6	26
Anthropometrics		**Median (range)**
BMI SDS	18/23	-0.22 (-1.45, 2.42)
Wt SDS	23/23	-0.25 (-4.5, 2.88)
Vitamin D supplementation	**(8)**	**%**
Prior high dose vitamin D3 supplementation	2	25
Prior multivitamin supplementation	4	50
Current multivitamin supplementation	2	25
Season of enrolment	**(23)**	**%**
Autumn	7	30.5
Spring	6	26
Summer	6	26
Winter	4	17.5
Reason for referral	**(23)**	**%**
Bone pain	14	61
Bowed legs	5	22
Other	4	17

Table 6-2: Baseline laboratory and demographic characteristics of the three obese children enrolled in the study.

Patient ID	34	30	7
BMI SDS	**2.3**	**2.1**	**2.4**
Wt SDS	**2.8**	**2.2**	**2.7**
Age (years)	7	8	9.5
Gender	Male	Male	Female
Race	Middle Eastern	Middle Eastern	South Asian
Referral	General paediatrician	Orthopaedics	General practitioner
Reason for referral	Bone pain	Bone pain	Bone pain
Season of referral	Summer	Summer	Autumn
Vitamin D supplementation	Multivitamin	No	No
25(OH)D, nmol/l	**33 (↓↓)**	**49 (↓↓)**	**33 (↓↓)**
PTH	5	3.6	5.5
Glucose (mmol/l)	5.7	4.4	4.1
Insulin (µiu/ml)	11.3	11.1	**99.2 (↑↑)**
HOMA IR	**2.86 (↑↑)**	2.18	**20.2 (↑↑)**
HbA1c (mmol/mol)	38	31	39
SHBG	**31 (↓↓)**	-	**17 (↓↓)**
Cholesterol (mmol/l)	4.1	4	**4.4 (↑↑)**
Triglycerides (nmol/l)	**2.2 (↑↑)**	0.7	1.5
HDL (mmol/l)	**1.1 (↓↓)**	2.4	**0.9 (↓↓)**
Cholesterol/ HDL	3.7	1.7	**4.9 (↑↑)**
Non-HDL cholesterol level (mmol/l)	3.5	3	2.3

Numbers in bold represent values outside the reference range (arrows indicate either above or below the reference range).

6.4.3 Effects of treatment

For the 23 children included in this study, the median (range) of serum 25(OH)D levels increased significantly from 27 (14, 65) nmol/l at the baseline to 106 (37, 202) nmol/l at week six ($p < 0.0001$). The median (range) of PTH levels in 21 children decreased significantly from 5.5 (3.6, 134.1) pmol/l at the baseline to 3.8 (1.9, 6.8) pmol/l at week six ($p < 0.0001$).

Compared with week 0, there was a significant reduction in HOMA IR ($p = 0.04$) and a trend towards a reduction in serum insulin was noticed at week six ($p = 0.05$). No significant changes in blood glucose, HbA1c and SHBG parameters, and any of the lipid profile measures were noticed after six weeks of supplementation (Table 6-3).

The median of the changes between week six and baseline (week six – baseline) for glucose, insulin, HOMA IR and SHBG parameters showed a reduction after six weeks of vitamin D3 treatment whereas HbA1c showed an increase. No change was observed in the lipid profile except for the cholesterol/HDL ratio which showed an increase following supplementation. Table 6-4 summarises the outcome variables expressed as difference /changes and percentage change between the baseline and the week-six visits.

For the 17 children whose insulin and HOMA IR data were available at the two time points, serum 25(OH)D increased significantly from 26.5 (14, 49) nmol/l at the baseline to 96 (37, 191) nmol/l at week six ($p < 0.0001$) (see Figure 6-2). Out of those 17 children, PTH data at week six was not obtained for two children due to insufficient samples, and serum PTH concentrations decreased significantly in the remaining 15 children from 6.3 (3.6, 134) pmol/l to 4 (1.9, 6.8) pmol/l at week six ($p < 0.0001$) (see Figure 6-3). Additionally, children showed variability in their responses to vitamin D3 treatment. For example, out of the 17 children with available HOMA IR at the two time points, 6 (35%) children had 25(OH)D of < 75 nmol/l at the end point, with a median (range) percentage change of PTH of -50.1(-76, -16)%. Eleven (65%) children reached a final 25(OH)D of > 75 nmol/l, with a median (range) percentage change of PTH of -45 (-96, 8.3)%.

High prevalence of abnormal glucose homeostasis in obese/overweight children, and significant associations between 25(OH)D level and measurements of glucose homeostasis were reported by several studies [263, 498]. Therefore the participants were grouped

according to their BMI SDS, to compare the study parameters between obese and normal weight children. Obese children showed a significant reduction in their HOMA IR from baseline to week six (p=0.007). However, the result showed no significant difference in HOMA IR between normal weight and obese children at week six, p=0.1. Additionally, a significant increase in serum 25(OH)D level and a significant decrease in serum PTH levels was observed in the normal weight group (p = 0.001 and < 0.0001 for 25(OH)D and PTH respectively). However, there was no difference observed between normal weight and obese children when we compared the change in both 25(OH)D and PTH from baseline to week six (Table 6-5 and Table 6-6).

At the baseline, a high cholesterol/HDL ratio was reported in three children. The first child was a 9.5 year old obese girl (Patient ID: 7) with a basal cholesterol/HDL ratio of 4.9. The other two children were 10 and 16 months normal-weight boys (Patient IDs: 27 and 32), with basal serum cholesterol/HDL ratio of 4.4 and 6.2. At week six, vitamin D3 treatment was successful in improving serum 25(OH)D in Patients 7 and 32, and their cholesterol/HDL also returned to normal range. The other child (Patient ID: 27), had a final serum 25(OH)D of 37 nmol/l, and showed an increase in cholesterol/HDL ratio from 4.4 at baseline to 5.1 at week six (Table 6-7). Abnormal triglyceride levels were reported in two children: 2.4 nmol/l in a normal-weight child (Patient ID: 32), and 2.2 nmol/l in an obese child (Patient ID: 34). After supplementation, both children showed an increase in final serum 25(OH)D to the normal levels. However, only Patient 34 showed a reduction in triglyceride level to the normal reference range, while serum triglyceride for the other child reduced slightly to 2.3 nmol/l (Table 6-7).

Finally, basal serum cholesterol was above the normal reference range in two children, Patient ID: 7 and Patient ID: 27, with basal serum cholesterol level of 4.4 and 4.8 mmol/l respectively. At week six, vitamin D3 treatment was successful in raising serum 25(OH)D in Patient ID 7, and serum cholesterol for this patient returned to normal range (4.1 mmol/l). The other child (Patient ID: 27) had final serum 25(OH)D of 37 nmol/l, and showed an increase in cholesterol level from 4.8 nmol/l at baseline to 5.1 nmol/l at week six (Table 6-7).

Table 6-3: Comparison of the biochemical and lipid profile measurements for the 23 participants enrolled in the study over two points of the study period.

	N	Baseline median (min, max)	N	Week 6 median (min, max)	Baseline vs week 6 *p*-value	Normal value
25(OH)D (nmol/l)	23	27 (14, 65)	23	106 (37, 202)	**<0.0001**	70-150
PTH (pmol/l)	21	5.5 (3.6, 134.1)	21	3.8(1.9, 6.8)	**<0.0001**	0.9-5.5
Metabolic	**N**	**Baseline median (min, max)**	**N**	**Week 6 median (min, max)**	**Baseline vs week 6 *p*-value**	**Normal value**
Glucose (mmol/l)	22	4.5 (2.9, 5.7)	22	4.4 (3.2, 5)	0.3	3.9-7
Insulin (µiu/ml)	17	11.1 (2.46, 99.2)	17	8.1 (1.8, 66.6)	0.05	*
HOMA IR	17	2.18 (0.56, 20.29)	17	1.59 (0.37, 12.5)	**0.04**	*
HbA1c (mmol/mol)	21	33 (29, 39)	21	34 (25, 42)	0.1	20-42
SHBG (nmol/l)	16	141 (17, 207)	16	115 (18, 223)	0.2	60-209
Lipids	**N**	**Baseline median (min, max)**	**N**	**Week 6 median (min, max)**	**Baseline vs week 6 *p*-value**	**Normal value**
Cholesterol (mmol/l)	17	4 (3.3, 4.8)	17	3.9 (2.9, 5.2)	0.8	≥4.3
Triglycerides (nmol/l)	17	0.9 (0.5, 2.4)	17	0.9 (0.4, 2.3)	0.6	≥1.7
HDL (mmol/l)	17	1.2 (0.6, 2)	17	1.2 (0.6, 1.9)	0.4	≤1.5
Cholesterol/ HDL	17	3.2 (2, 6.2)	17	3.5 (2.2, 6)	0.2	≥4
Non-HDL cholesterol (mmol/l)	17	2.6 (3.7, -1.9)	17	2.8 (4.1, 1.7)	0.5	>3.5

25(OH)D: 25 hydroxy vitamin D. PTH: Parathyroid hormone. HOMA IR: Homeostasis model assessment index - estimated insulin resistance. SHBG: Sex hormone binding globulin. HbA1c: Glycated haemoglobin. HDL: High density lipoprotein. Normal reference values for insulin and HOMA IR were compared with age- and sex-specific paediatric reference interval as indicated in references [499, 500].

Table 6-4: Summary statistics of differences/changes (week 6– baseline) and percentage changes (% = ([(v2-v1)/v1]*100) values after six weeks of vitamin D3 supplementation.

Metabolic	N	Change (min, max)	% change (min, max)
Glucose	22	-0.1(-1, 1.5) mmol/l	-2.2 (-23.8, 51.7)
Insulin	17	-0.7(-32.5, 8.97) µiu/ml	-9.3 (-64.5, 141.3)
HOMA IR	17	-0.21(-7.80, 1.4)	-13.6 (-71.4, 142)
HbA1c	21	1 (-4, 4) mmol/mol	2.63 (-13.8, 12.9)
SHBG	16	-3 (-87, 93) nmol/l	-3.5 (-53, 78.7)
Lipids	**N**	**Change (min, max)**	**% change (min, max)**
Cholesterol	17	0 (-0.4, 1) mmol/l	0 (-12, 23.8)
Triglycerides	17	0 (-1.1, 0.5) nmol/l	0 (-55, 50)
HDL	17	-0.1 (-0.3, 0.3) mmol/l	-5 (-30, 25)
Cholesterol/HDL	17	2.6 (-35.6, 53.7)	8 (-25, 46)

Results are shown as median (range).

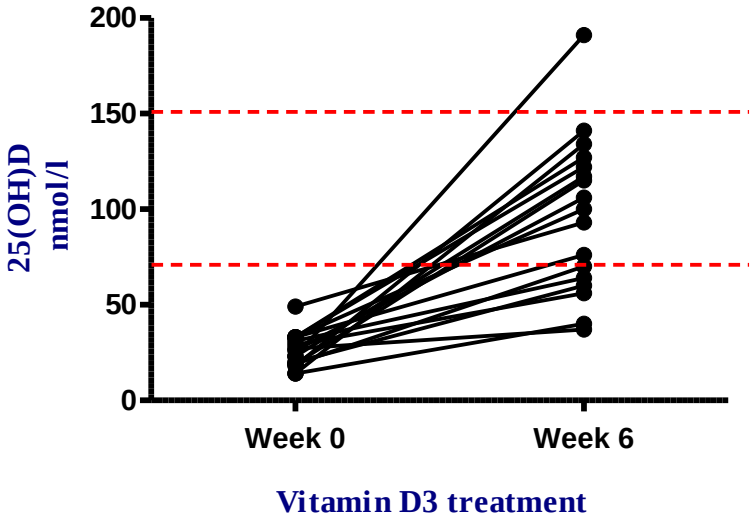


Figure 6-2: Individual changes in the 25(OH)D concentrations for 17 children with available insulin and HOMA IR data before and after six weeks of receiving 5000 IU cholecalciferol treatment.

Significant increase in the median of vitamin D levels was observed ($p < 0.0001$). Each subject's vitamin D level is represented by a line connecting week 0 with week 6 values. Area between two red lines; normal reference ranges for vitamin D.

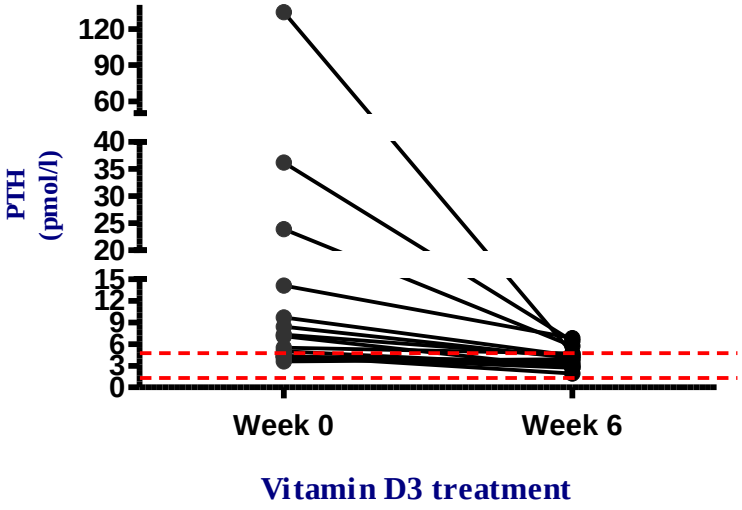


Figure 6-3: Individual changes in the PTH concentrations for 15 children with available insulin and HOMA IR data before and after six weeks of receiving 5000 IU cholecalciferol treatment.

Significant decrease in the median of PTH levels was observed ($p < 0.0001$). Each subject's PTH level is represented by a line connecting week 0 with week 6 values. Area between two red lines; normal reference ranges for PTH.

Table 6-5: Changes from baseline to six weeks of primary outcome measures for the study participants divided according to BMI SDS.

	N	Normal weight	*p*-value (Baseline vs week six)	N	Obese	*p*-value (Baseline vs week six)	*p*-value (Difference between groups at week six)
25(OH)D (nmol/l)							
Baseline	20	23 (14, 33)		3	33 (33, 49)	0.1	0.9
Week six	20	103 (37,191)	**0.001**	3	93 (76, 122)		
Change: End-Baseline		73.5 (10,168)			44 (43, 89)		
% change		277 (37, 857)			130 (89, 269)		
PTH (pmol/l)							
Baseline	18	6.18 (4,134)		3	5 (3.6, 5.5)	0.4	0.9
Week six	18	3.9 (1.9, 6.8)	**<0.0001**	3	3.9 (3, 4.7)		
Change: End-Baseline		-2.7 (-129, -0.3)			-0.8 (-2, -0.3)		
% change		-46 (-96, -7.3)			-14.5 (-40, -8)		
Metabolic							
Glucose (mmol/l)							
Baseline	19	4.4 (2.9, 5.1)		3	4.5 (4.4, 5.7)	0.4	0.3
Week six	19	4.4 (3.2, 5)	0.5	3	4.6 (4.1, 5)		
Change: End-Baseline		-0.1 (-1,1.5)			-0.2 (-0.7, 0.3)		
% change		-2 (-23,51.7)			-4.3 (-12, 6.8)		
Insulin (µiu/ml)							
Baseline	14	7.9 (2.4, 20)		3	11 (6, 99)	0.1	0.4
Week six	14	7.8 (1.8,11)	0.1	3	10 (5.9, 66)		
Change: End-Baseline		-0.9 (-12, 6.4)			-5.4 (-32.5, -1.3)		
% change		-14 (-64,141)			-32 (-47, -12)		
HOMA IR							
Baseline	14	1.5 (0.56, 3.89)		3	2.86 (2.28, 20)	**0.007**	0.1
Week six	14	1.4 (0.37, 2.4)	0.1	3	2.1 (1.31, 12.5)		
Change: End-Baseline		-0.23(-2.7,1.5)			-1.55 (-7.7, -0.2)		
% change		-16.6 (-71,142)			-38 (-54,-6.41)		
HbA1c (mmol/mol)							
Baseline	18	32.5 (29, 38)		3	38 (31, 39)	0.1	0.5
Week six	18	33.5 (25, 39)	0.1	3	7 (31, 42)		
Change: End-Baseline		1 (-4, 4)			0 (-1, 3)		
% change		2.7 (-13.7,12.9)			0 (-2.6, 7.7)		
SHBG (nmol/l)							
Baseline	15	141 (17, 207)		-		NA	-
Week six	15	115 (76, 223)	0.1		NA		
Change: End-Baseline		-3 (-87, 93)					
% change		-3.5 (-53,78.7)					

Table 6-6: Changes from baseline to six weeks of secondary outcome measures for the study participants divided according to BMI SDS.

	N	Normal weight	*p*-value (Baseline vs week six)	N	Obese	*p*-value (Baseline vs week six)	*p*-value (Difference between groups at week six)
Lipids							
Cholesterol (mmol/l)							
Baseline	14	3.8 (3.3, 4.8)		3	4.1 (4, 4.4)		
Week six	14	3.8 (2.9, 5.2)	0.5	3	4 (3.7, 4.2)	0.3	0.9
Change: End-Baseline		0.05 (-0.4,1)			-0.3 (-0.4, 0)		
% change		1.2 (-12, 23.8)			-6.8 (-9.7, 0)		
Triglycerides (nmol/l)							
Baseline	14	0.85 (0.5, 2.4)		3	1.5 (0.7, 2.2)		
Week six	14	0.9 (0.4, 2.3)	0.8	3	1.1 (0.8,1.1)	0.4	0.4
Change: End-Baseline		0 (-0.5, 0.5)			-0.4 (-1.1, 0.1)		
% change		0 (-55, 50)			-26.6 (-50,14)		
HDL (mmol/l)							
Baseline	14	1.2 (0.6, 2)		3	1.1 (0.9,1.7)		
Week six	14	1.2 (0.6, 1.9)	0.6	3	1.1 (0.9,1.6)	0.3	0.5
Change: End-Baseline		-0.1 (-0.3, 0.3)			-0.1 (-0.2, 0)		
% change		-6 (-30, 25)			-5.8 (-18, 0)		
Cholesterol/ HDL							
Baseline	14	3.2 (2, 6.2)		3			
Week six	14	3.4 (2.2, 6)	0.2	3	3.7 (2.4, 4.9)	0.7	0.5
Change: End-Baseline		0.25 (0.8,1.9)			4.1 (2.5, 4.6)		
% change		8.7 (-25, 46)			-0.1 (-0.3, 0.4)		
					4.1 (-6.1,10.8)		
Non-HDL Cholesterol (mmol/l)							
Baseline	14	2.5 (3.7, 1.9)		3	3 (3.5, 2.3)		
Week six	14	2.75 (-4.1, 1.7)	0.3	3	2.8 (3.2, 2.4)	0.4	0.7
Change: End-Baseline		0.1 (0.8, 0.5)			0.2 (0.2, 0.3)		
% change		4.3 (19.2, 30.4)			6.6 (8.5, 4.3)		

Table 6-7: Abnormal lipid parameters reported in four patients. Numbers in bold represent values outside a reference range.

Patient ID	7	27	32	34
Age	7 years	16 months	10 months	7 years
Gender	Female	Male	Male	Male
Race	South Asian	Middle Eastern	Caucasian	Middle Eastern
Anthropometrics	2.4 **(BMI SDS)**	-0.5 **(WT SDS)**	> -2 **(WT SDS)**	2.3 **(BMI SDS)**
Main presentation	Bone Pain	Fracture/Clear rickets on X-ray	Wrist swelling/Clear rickets on X-ray	Bone Pain
Patient's medical history	Clear	Iron deficiency anaemia/Poor feeding	B12 deficiency anaemia/Poor feeding	Clear
Family history	Hypertension/High cholesterol (Father)	Clear	Clear	Asthma (Father) Diabetes (Mother)
25(OH)D (nmol/l)				
Baseline	33	27	18	33
Week six	76	37	141	122
Cholesterol (mmol/l)				
Baseline	**4.4**	**4.8**	3.3	4.1
Week six	4.1	**5.1**	2.9	3.7
Triglycerides (nmol/l)				
Baseline	1.5	1	**2.4**	**2.2**
Week six	1.1	1.5	**2.3**	1.1
HDL (mmol/l)				
Baseline	0.9	1.1	0.6	1.1
Week six	0.9	1	0.6	0.9
Cholesterol/ HDL				
Baseline	**4.9**	**4.4**	**6.2**	3.7
Week six	**4.6**	**5.1**	**5.5**	**4.1**
Non-HDL cholesterol (mmol/l)				
Baseline	**3.5**	**3.7**	3.1	3
Week six	3.2	4.1	2.7	2.8

6.4.4 Individual changes in markers of glucose homeostasis

6.4.4.1 Insulin

Seventeen paired samples were available for insulin analysis at the two time points of the study period. There was no significant change in the median of insulin levels after six weeks of vitamin D3 supplementation ($p = 0.05$). Abnormal baseline insulin levels were identified in four children (Patient IDs: 7, 12, 15 and 28). After supplementation, insulin levels returned to the normal reference range in three children (Patient IDs: 12, 15 and 28), and remained above the normal reference range in one child (Patient ID: 7) (see Figure 6-4 and Table 6-8).

When the 17 children was divided into two groups based on their final 25(OH)D concentrations, children with final 25(OH)D levels of ≥ 75 nmol/l had a significant reduction in the median of serum insulin ($p = 0.02$), whereas those who were still vitamin D deficient after six weeks of vitamin D3 supplementation showed no significant change ($p = 0.8$) (see Figure 6-5). There was no significant difference in the percentage change of insulin levels between the two groups ($p = 0.05$) (see Figure 6-6). Vitamin D3 treatment was successful to reach serum 25(OH)D concentrations of ≥ 75 nmol/l in 11 children. The median of the insulin levels for those children who achieved serum 25(OH)D concentrations of ≥ 75 nmol/l at the end of the study was significantly decreased from 13.2 (2.46, 99.2) µiu/ml at the baseline to 9.3 (1.8, 66.67) µiu/ml at week six ($p = 0.02$) (Table 6-8).

The percentage changes in serum insulin were compared with percentage changes in serum 25(OH)D (see Figure 6-7). With the exception of two children (Patient IDs: 29 and 33), participants who reached a serum 25(OH)D of ≥ 75 nmol/l at week six showed a reduction in serum insulin. One of those two children (Patient ID: 29) showed a 141% increase in serum insulin after supplementation compared with the baseline. This was an 18-month-old male who had co-existing vitamin B12 deficiency anaemia. Additionally, markers of bone remodelling (ALP and CTX) for this child showed abnormal levels even after supplementation. The other child (Patient ID: 33) was a 19-month-old male who had a 15% increase in serum insulin after supplementation compared with the baseline, and had no significant medical background.

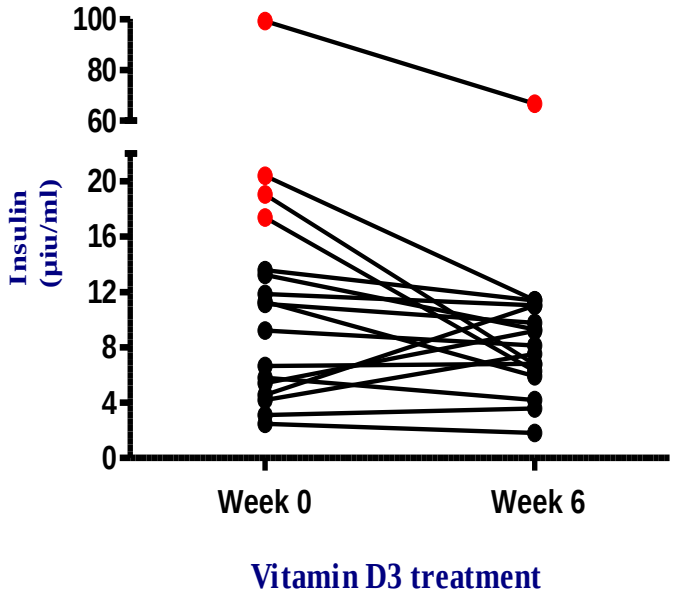


Figure 6-4: Individual changes in serum insulin for 17 children before and after six weeks of receiving 5000 IU cholecalciferol treatment.

No significant change in the median of serum insulin levels was observed (p = 0.05). Each subject's insulin level is represented by a line connecting week 0 with week 6 values. Red dots represent values exceeding normal reference range for serum insulin. Abnormally high insulin was observed in four children at baseline and in one child at week six.

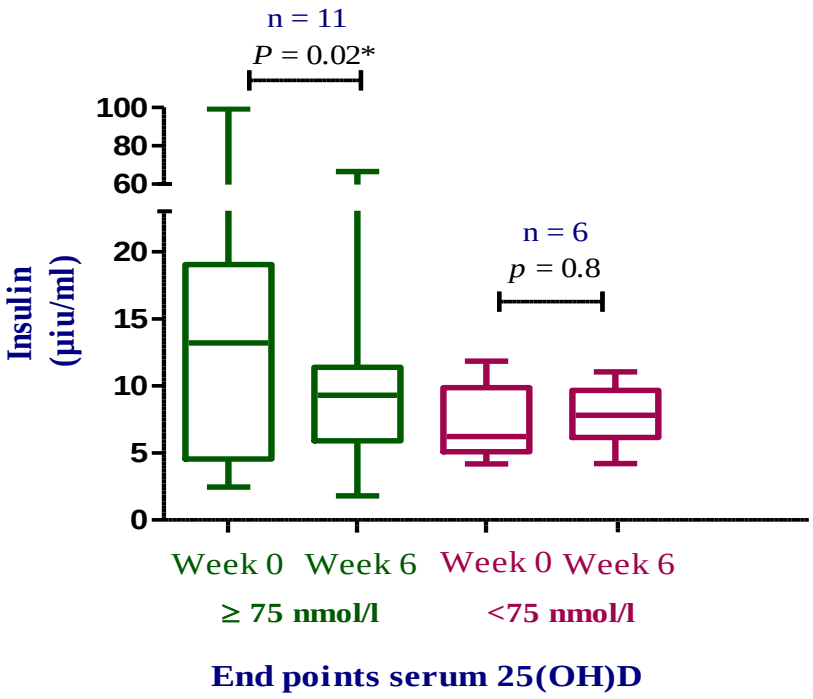


Figure 6-5: Changes in serum insulin from baseline to week six in 17 children grouped by serum 25(OH)D concentration reached by the end point.

Significant reduction in serum insulin was observed for the children with final 25(OH)D concentration of ≥ 75 nmol/l ($p = 0.02$) and no significant change in serum insulin was observed in those who were still vitamin D deficient after six weeks of supplementation ($p = 0.8$).

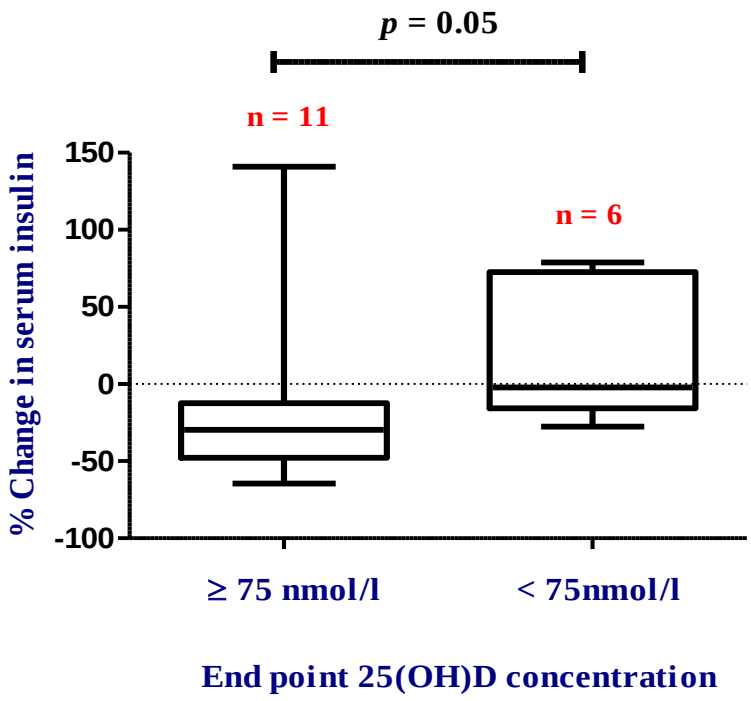


Figure 6-6: The percentage changes in serum insulin in 17 children grouped by serum 25(OH)D concentration at the end point.

Children who reached a final 25(OH)D concentration of ≥ 75 nmol/l showed a trend towards a significant decrease in % change of serum insulin compared with those who had a final 25(OH)D of < 75nmol/l (p = 0.05).

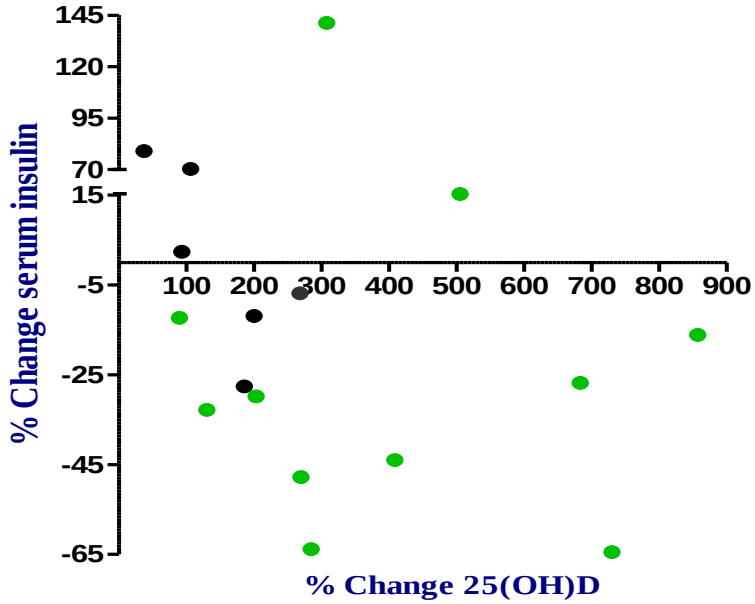


Figure 6-7: The percent changes in serum insulin in 17 children in relation to the percent changes in serum 25(OH)D concentration.

Green dots represent children who reached a final 25(OH)D level of ≥ 75 nmol/l while black dots represent children who reached a final 25(OH)D < 75nmol/l. With an exception of two children (patients IDs: 29 and 33). Children with a sufficient final 25(OH)D concentration showed a trend toward a significant decrease in % serum insulin compared with those who were still vitamin D deficient/insufficient at week six.

6.4.4.2 Glucose

Twenty-two paired samples were available for glucose analysis at the two time points of the study period. They showed no significant change in the median of blood glucose levels after six weeks of vitamin D3 supplementation ($p = 0.3$) (see Figure 6-8). Low basal blood glucose (defined by a serum glucose) of < 3.9 mmol/l was observed in one child (Patient ID: 22), whereas another child (Patient ID: 8) showed a low week-six blood glucose level (Table 6-8).

When the 22 children with available glucose data at the two time points were divided into two groups based on their final 25(OH)D concentrations (25(OH)D ≥ 75 nmol/l (n = 14) and < 75 nmol/l (n = 8)), no significant change in the medians of blood glucose levels from baseline to week six in either groups was observed ($p = 0.5$ and $p = 1$ respectively). Out of the 22 children with available glucose data, vitamin D3 treatment was successful to reach serum 25(OH)D concentrations of ≥ 75 nmol/l in 14 children. The median glucose levels for those 14 children who achieved serum 25(OH)D concentration of ≥ 75 nmol/l at the study end was 4.6 (2.9, 5.7) mmol/l at the baseline and non-significantly decreased to 4.55 (3.2, 5) mmol/l at week six ($p = 0.2$). Additionally, there was no significant difference in the percentage change of blood glucose between children who reached a final 25(OH)D concentration of ≥ 75 nmol/l compared with those who had a final 25(OH)D concentration of < 70 nmol/l (see Figure 6-9).

In order to examine the change in blood glucose in relation to the change in vitamin D levels individually for each child, the percentage change of blood glucose was compared with percentage change of serum 25(OH)D. Out of 14 children who were vitamin D deficient at the baseline and reached final 25(OH)D concentrations of ≥ 75 level nmol/l at week six, 9 children had a reduction in the % of glucose, four children showed an elevation in % of glucose while glucose level remained unchanged in one child (see Figure 6-10).

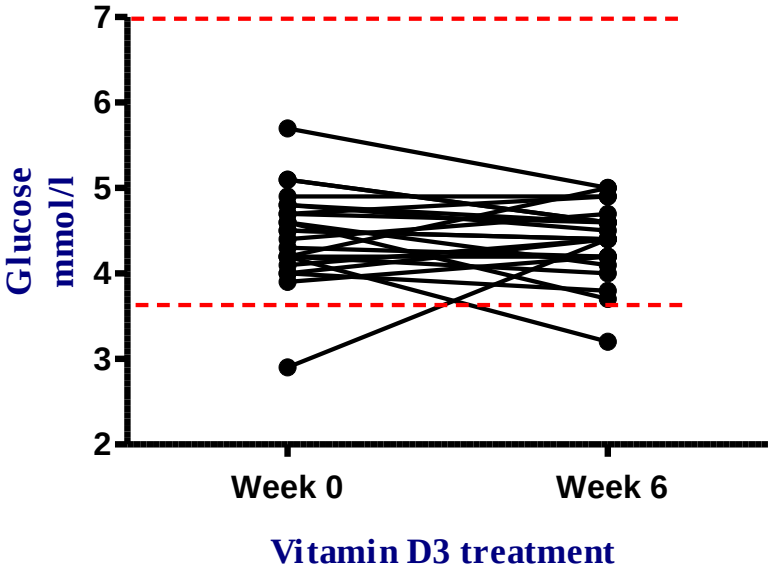


Figure 6-8: Individual changes in blood glucose for 22 children before and after six weeks of receiving 5000 IU cholecalciferol treatment.

No significant change in the median fasting glucose level was observed (p = 0.3). Each subject's glucose level is represented by a line connecting week 0 with week 6 values. Area between two red lines; normal reference ranges for blood glucose. Low blood glucose level was observed in two children.

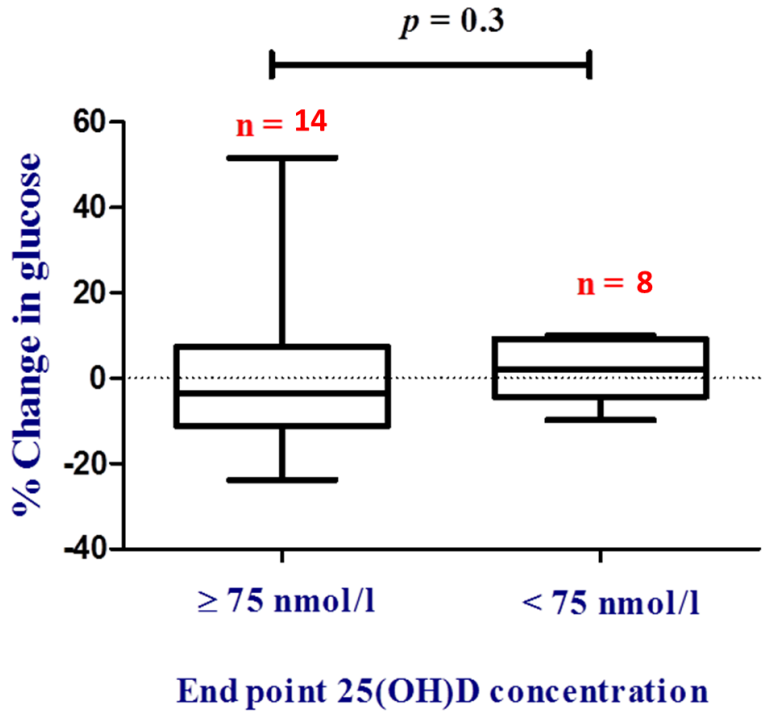


Figure 6-9: The percentage change in blood glucose in 22 children grouped by the end point vitamin D concentration.

No significant difference in % change of fasting glucose was found between the two groups (p = 0.3).

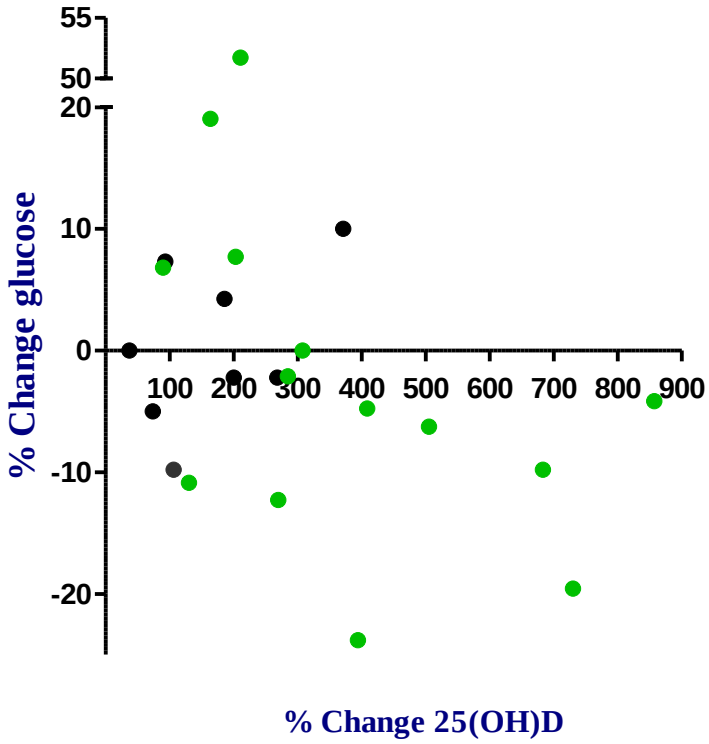


Figure 6-10: The percentage changes in blood glucose in 22 children in relation to the percentage changes in serum 25(OH)D concentration.

Green dots represent children who reached a final 25(OH)D level of ≥ 75 nmol/l, while black dots represent children who reached a final 25(OH)D level of < 75nmol/l.

6.4.4.3 Homeostasis model assessment index - estimated insulin resistance (HOMA IR)

Seventeen paired samples were available for HOMA IR analysis at the two time points of the study period. They had a significant reduction in the median of HOMA IR levels after six weeks of vitamin D3 supplementation ($p = 0.04$) (see figure 6-11). Abnormal baseline HOMA IR was observed in four children (Patient IDs: 7, 12, 15 and 28). Following supplementation, HOMA IR declined to the normal reference range in three children (Patient IDs: 12, 15 and 28) and remained above the normal reference range in one child (Patient ID: 7) (Table 6-8).

When the 17 children were divided into two groups, based on their final 25(OH)D levels, a significant reduction in HOMA IR was observed in children with final 25(OH)D concentrations of ≥ 75 nmol/l ($p = 0.02$), whereas no significant change in HOMA IR was observed in those who were still vitamin D deficient after six weeks of vitamin D3 supplementation ($p = 0.8$) (see Figure 6-12). There was no significant difference in the percentage change of HOMA IR between the two groups ($p = 0.05$) (see Figure 6-13). Vitamin D3 treatment was successful in 11 children. The HOMA IR for those children who achieved 25(OH)D concentrations of ≥ 75 nmol/l at the study end was significantly decreased from 2.23 (0.56, 20.29) at the baseline to 1.34 (0.72, 12.5) at week six ($p = 0.02$) (Table 6-8). The percentage changes in HOMA IR were compared with the percentage changes in serum 25(OH)D. Only two of the children who reached serum 25(OH)D levels of ≥ 75 nmol/l at week six did not show a reduction in HOMA IR (Patient IDs: 29 and 33) (see Figure 6-14).

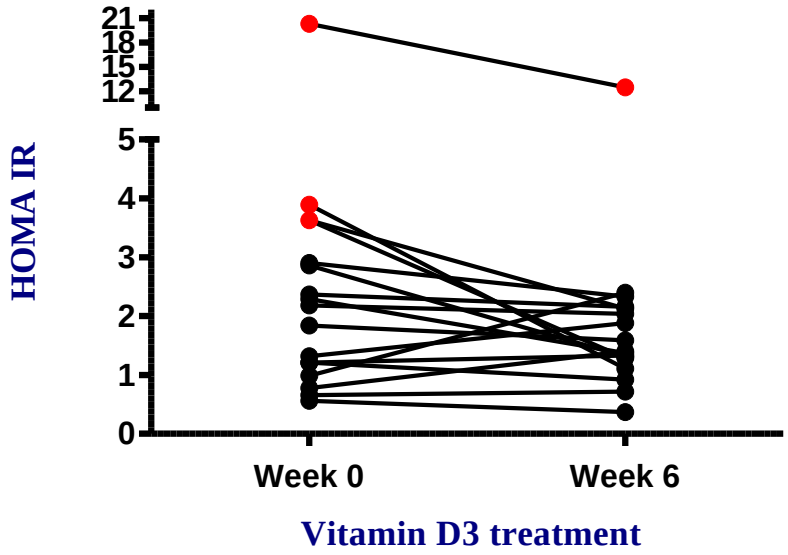


Figure 6-11: Individual changes in HOMA IR for 17 children before and after six weeks of receiving 5000 IU cholecalciferol treatment.

Significant reduction in the median of HOMA IR levels was observed (p = 0.04). Each subject's HOMA IR level is represented by a line connecting week 0 with week 6 values. Red dots represent values exceeding normal reference ranges for HOMA IR. Abnormal HOMA IR was observed in four children at baseline and in one child at week six.

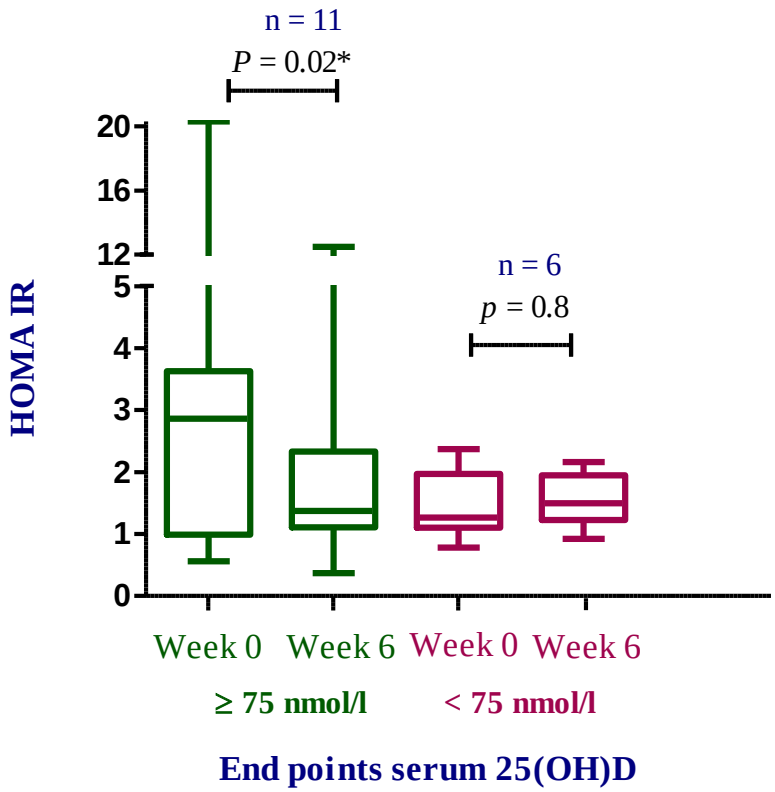


Figure 6-12: Changes in HOMA IR from baseline to week six in 17 children grouped by the end point serum 25(OH)D concentration.

A significant reduction in HOMA IR was observed for children with final 25(OH)D concentration of ≥ 75 nmol/l (p = 0.02) and no significant change was observed in those who were still vitamin D deficient after six weeks (p = 0.8).

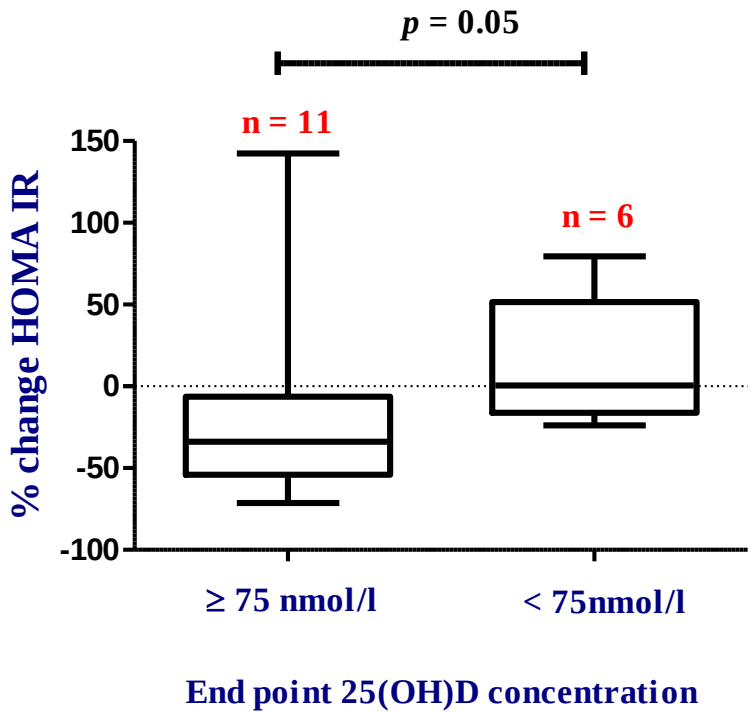


Figure 6-13: The percentage changes in HOMA IR in 17 children grouped by the end point serum 25(OH)D concentration.

Children who reached a final 25(OH)D concentration of ≥ 75 nmol/l showed a trend toward a significant reduction in % HOMA IR compared with those who had a final 25(OH)D level of < 75nmol/l (*p* = 0.05).

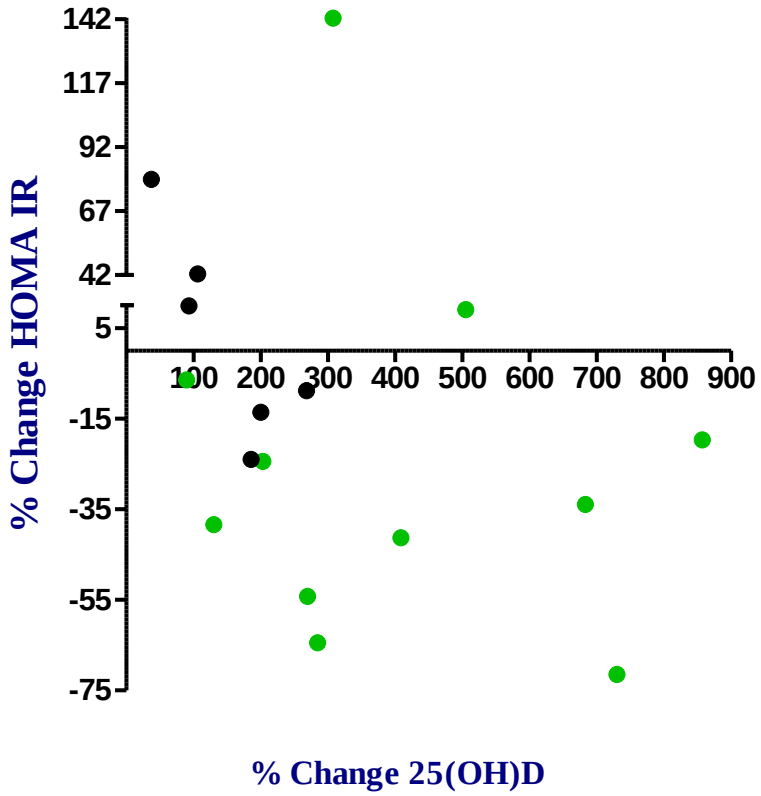


Figure 6-14: The percentage change in HOMA IR in 17 children in relation to the percentage changes in serum 25(OH)D concentration.

Green dots represent children who reached a final 25(OH)D level of ≥ 75 nmol/l, while black dots represent children who reached a final 25(OH)D level of < 75 nmol/l. With an exception of two children (Patient IDs: 29 and 33), children with sufficient final 25(OH)D concentration showed a trend towards a significant decrease in % HOMA IR compared with those who were still vitamin D deficient/insufficient at six weeks.

Table 6-8: Summary data of age (months), fasting blood glucose, fasting serum insulin and HOMA IR of the 17 children with available glucose, insulin and HOMA IR data at the two time points divided according to their final 25(OH)D level.

End points 25(OH)D ≥ 75 nmol/l										
No	**Age**	**Glucose Week 0**	**Glucose Week 6**	***p*-value**	**Insulin Week 0**	**Insulin Week 6**	***p*-value**	**HOMA IR Week 0**	**HOMA IR Week 6**	***p*-value**
1	43	3.9	4.2		13.2	9.3		2.29	1.37	
7	115	4.6	4.1		**99.2**	**66.6**		**20.29**	**12.5**	
11	115	4.1	4.4		13.5	11.3		2.9	2.33	
12	33	4.6	3.7		**19**	6.7		**3.89**	1.11	
15	51	4.7	4.6		**17.3**	6.2		**3.63**	1.29	
28	16	4.2	4	0.1	**20.3**	11.4	0.02	**3.63**	2.13	0.02
29	19	4.9	4.9		4.5	11		0.99	2.4	
30	96	4.4	4.7		11.1	9.7		2.18	2.04	
32	10	5.1	4.6		2.4	1.8		0.56	0.37	
33	18	4.8	4.5		3.1	3.5		0.66	0.72	
34	115	5.7	5		11.3	5.9		2.86	1.31	
Median		4.6	4.5		13.2	9.3		2.23	1.34	
End points 25(OH)D < 75 nmol/l										
No	**Age**	**Glucose Week 0**	**Glucose Week 6**	***p*-value**	**Insulin Week 0**	**Insulin Week 6**	***p*-value**	**HOMA IR Week 0**	**HOMA IR Week 6**	***p*-value**
3	102	4.5	4.4		11.8	11		2.37	2.16	
6	36	4.1	4.4		6.6	6.8		1.21	1.33	
17	16	4.5	4.4	1	9.2	8.1	0.8	1.84	1.59	0.8
27	16	4.2	4.2		4.1	7.5		0.78	1.4	
36	70	4.7	4.9		5.8	4.2		1.21	0.92	
37	21	5.1	4.6		5.4	9.2		1.32	1.88	
Median		4.5	4.4		6.2	7.3		1.26	1.49	

**p*-values refer to the change in the median of each parameter within each group. Numbers in bold represent values above the normal reference range.

6.4.4.4 Sex Hormone Binding Globulin (SHBG) and Glycated Haemoglobin (HbA1c)

Sixteen paired samples were available for SHBG analysis and 21 samples were available for HbA1c analysis at the two time points of the study period. There were no significant changes in the medians of these parameters following six weeks of vitamin D3 supplementation ($p = 0.2$ and $p = 0.1$ for SHBG and HbA1c, respectively) (see Figure 6-15 and Figure 6-16).

At the baseline, HbA1c levels were within the normal reference range for all participants. At week six, one child showed HbA1c at the upper limit of the normal range (Patient ID: 7) (see Figure 6-15). Out of the 21 children with available HbA1c data, vitamin D3 treatment was successful in increasing 25(OH)D concentrations to ≥ 75 nmol/l in 14 children, while seven children had final 25(OH)D concentrations of < 75nmol/l. The median percentage change in HbA1c levels from week 0 to week 6 was compared between those two groups of children, with no significant difference in % change of HbA1c between children who had final 25(OH)D concentrations of ≥75 nmol/l and those with final 25(OH)D concentrations of <75 nmol/l ($p = 0.7$) (see Figure 6-17).

At the baseline, SHBG levels were within the normal reference range except for one child (Patient ID: 7) who had a basal SHBG below the normal reference range, and still had low SHBG after six weeks. A high SHBG level was reported in one child at week six (Patient ID: 9) (see Figure 6-16). Of the 16 children who had available SHBG data, vitamin D3 treatment was successful in increasing 25(OH)D concentrations to ≥ 75 nmol/l in 11 children, while five children had a final 25(OH)D levels of < 75nmol/l. Median percentage change in SHBG levels from week 0 to week 6 was compared between those two groups of children, with no significant difference in % change of SHBG between children who had a final 25(OH)D concentrations of ≥ 75 nmol/l and those with a final 25(OH)D concentrations of < 75 nmol/l ($p = 0.4$) (see figure 6-18).

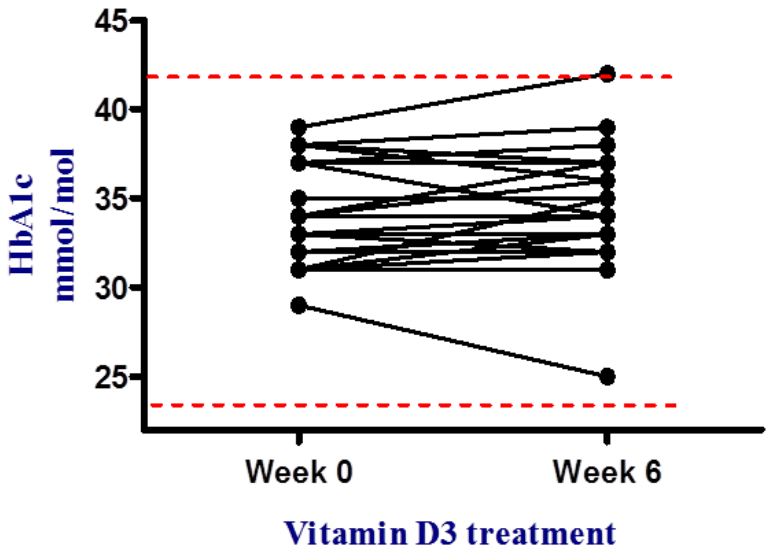


Figure 6-15: Individual changes in glycated haemoglobin (HbA1c) for 21 children before and after six weeks of receiving 5000 IU cholecalciferol treatment.

No significant change in the median of HbA1c levels was observed (p = 0.1). Each HbA1c's level is represented by a line connecting week 0 with week 6 values. Area between two red lines; normal reference ranges for HbA1c.

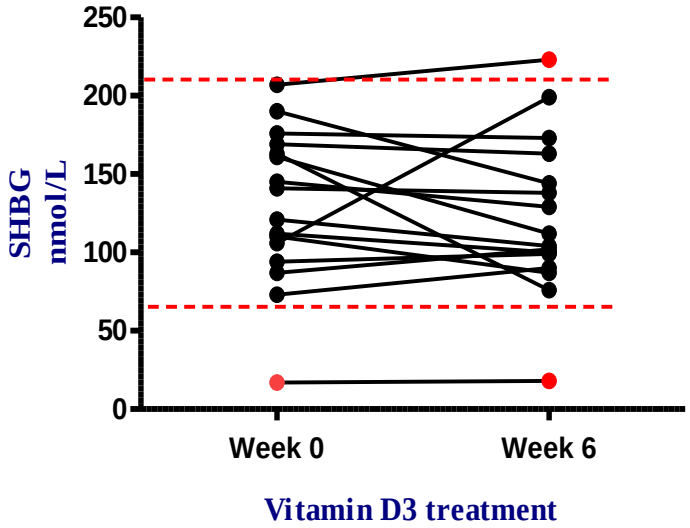


Figure 6-16: Individual changes in sex hormone binding globulin (SHBG) for 16 children before and after six weeks of receiving 5000 IU cholecalciferol treatment.

No significant change in the median of SHBG levels was observed (p = 0.2). Each subject's SHBG level is represented by a line connecting week 0 with week 6 values. Area between two red lines; normal reference ranges for SHBG. Low SHBG was observed in one child and high SHBG level was observed in one child (red dots).

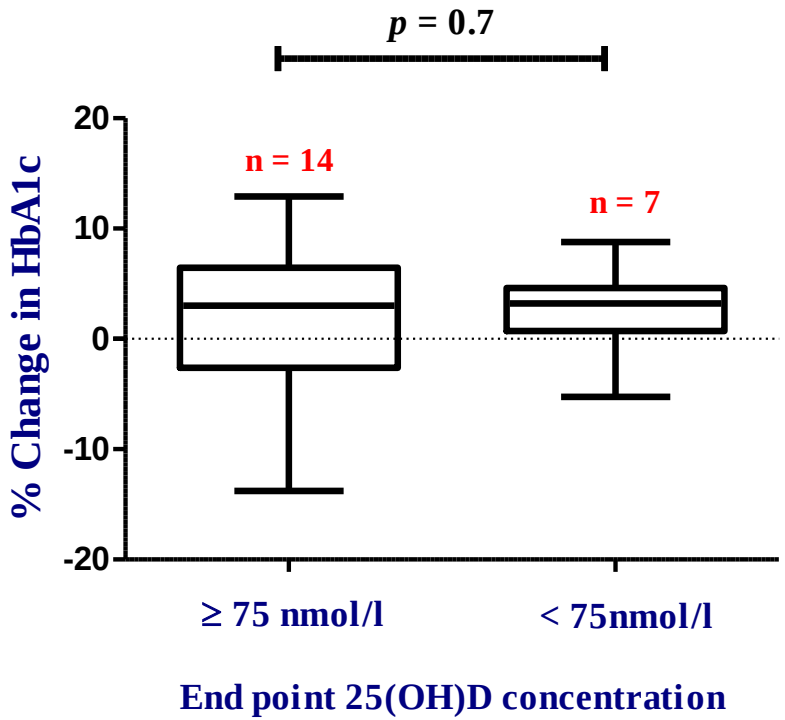


Figure 6-17: The percentage change in HbA1c in 21 children grouped by end point vitamin D concentration (children who had final 25(OH)D ≥ 75 nmol/l and those with final 25(OH)D < 75 nmol/l).

No significant difference in % change of HbA1c was found between the two groups (p = 0.7).

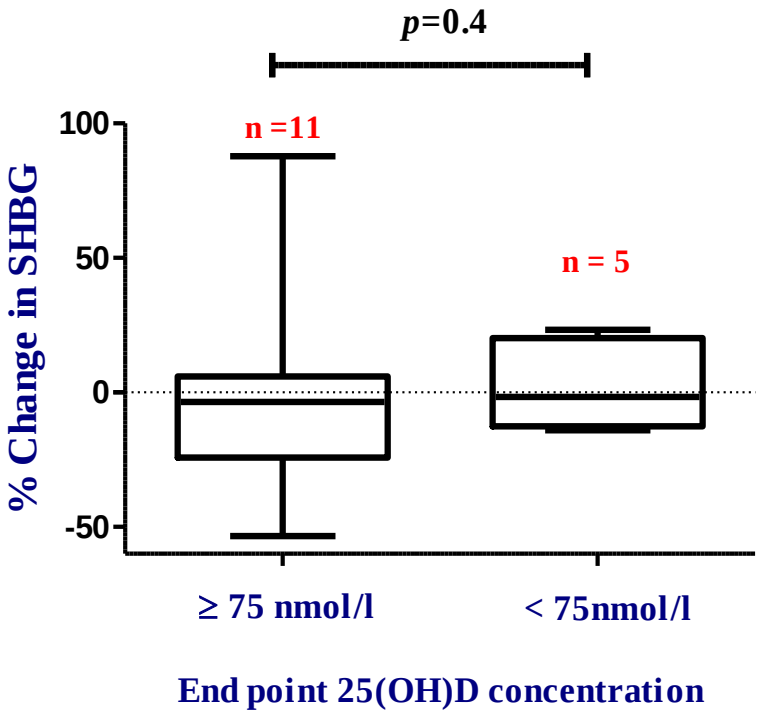


Figure 6-18: The percentage change in SHBG for 16 children grouped by end point of vitamin D concentration (children who had final 25(OH)D ≥ 75 nmol/l and those with final 25(OH)D < 75 nmol/l).

No significant difference in % change of SHBG was found between the two groups (p = 0.4).

6.4.5 Correlation between glucose homeostasis parameters: SHBG, HbA1c, glucose, insulin and HOMA IR

Fasting measurements of SHBG, HbA1c, glucose, insulin and HOMA IR levels were examined for correlations.

A significant negative association was observed between SHBG and HbA1c levels at baseline (r = -0.4; p = 0.04) and after six weeks of vitamin D3 treatment (r = -0.5; p = 0.03) (see Figure 6-19 and Figure 6-20). No significant association was observed between HOMA IR and HbA1c at baseline (r = 0.3; p = 0.1) and after six weeks of vitamin D3 treatment (r = 0.2; p = 0.2) (see Figure 6-21 and Figure 6-22). A non-significant negative association was observed between HOMA IR and SHBG at baseline (r = -0.4; p = 0.1), and after six weeks of vitamin D3 treatment (r = -0.3; p = 0.2) (see Figure 6-23 and Figure 6-24). There was no significant association observed between glucose and HbA1c levels at baseline (r = 0.003; p = 0.9), and after six weeks of vitamin D3 treatment (r = -0.1; p = 0.5). Similarly, no significant association was observed between glucose and SHBG at baseline (r = 0.4; p = 0.1) and after six weeks of vitamin D3 treatment (r = -0.01; p = 0.9) (Table 6-9). No significant association was observed between insulin and HbA1c levels at baseline (r = 0.3; p = 0.1), and after six weeks of vitamin D3 treatment (r = 0.3; p = 0.2). There was also no significant association observed between insulin level and SHBG at baseline (r = -0.4; p = 0.1) and after six weeks of vitamin D3 treatment (r = -0.4; p = 0.1) (Table 6-9).

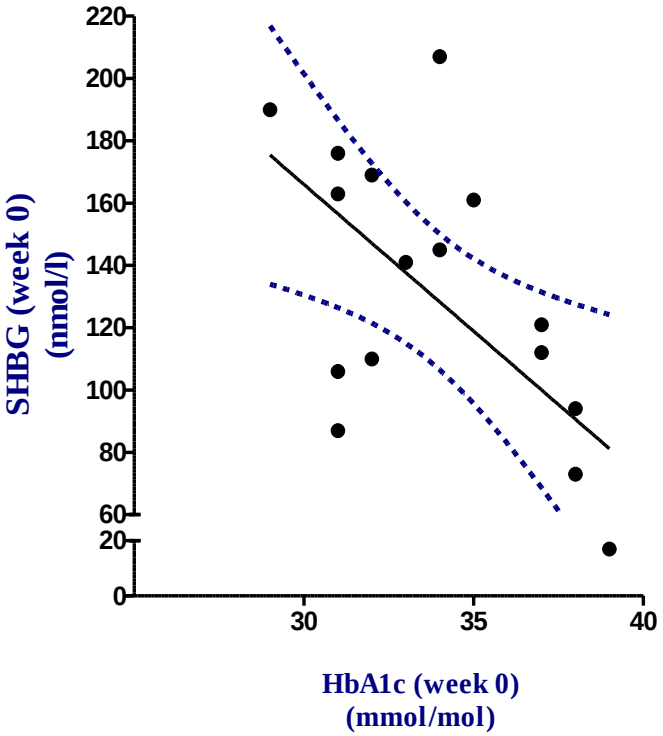


Figure 6-19: The relationship between SHBG and HbA1c in 16 vitamin D deficient children at the baseline.

The black line indicates the line of best fit, and the blue lines indicate the 95% confidence limits. A significant association was observed (r = -0.4; *p* = 0.04).

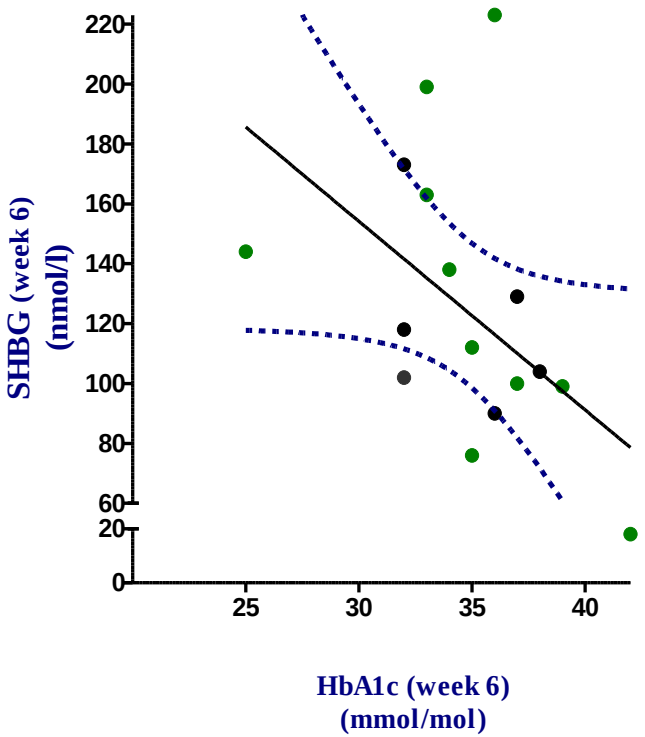


Figure 6-20: The relationship between SHBG and HbA1c in 16 children following six weeks of vitamin D3 supplementation.

Green dots represent children who reached a final 25(OH)D level of ≥ 75 nmol/l. Black dots represent children with a final 25(OH)D level of < 75nmol/l. The black line indicates the line of best fit, and the blue lines indicate the 95% confidence limits. A significant association was observed (r = -0.5; *p* = 0.03).

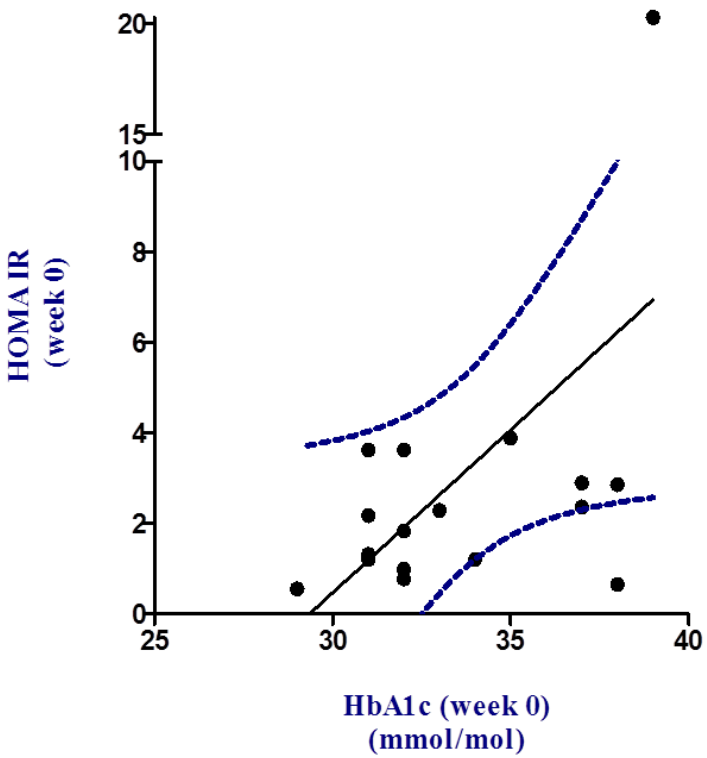


Figure 6-21: The relationship between HOMA IR and HbA1c in 17 vitamin D deficient children at the baseline.

The black line indicates the line of best fit, and the blue lines indicate the 95% confidence limits. No significant association was observed (r = 0.3; *p* = 0.1).

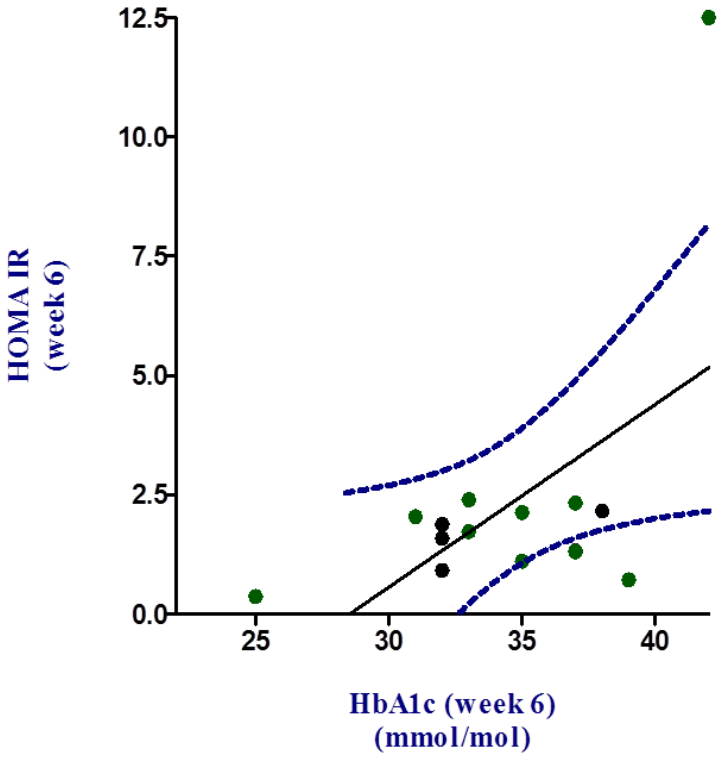


Figure 6-22: The relationship between HOMA IR and HbA1c in 15 children following six weeks of vitamin D3 supplementation.

Green dots represent children who reached a final 25(OH)D level of ≥ 75 nmol/l whereas black dots represent children who reached a final 25(OH)D level of < 75nmol/l. The black line indicates the line of best fit, and blue lines indicate the 95% confidence limits. No significant association was observed (r = 0.2; *p* = 0.2).

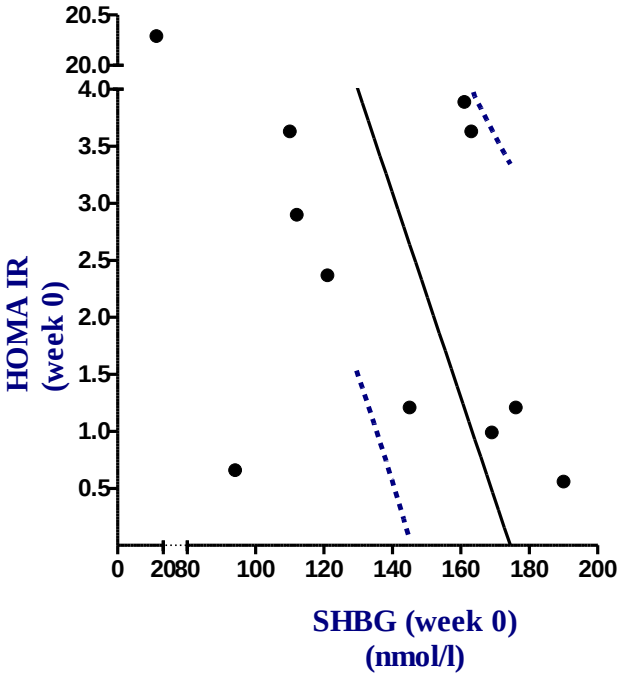


Figure 6-23: The relationship between HOMA IR and SHBG in 11 vitamin D deficient children at the baseline.

The black line indicates the line of best fit, and the blue lines indicate the 95% confidence limits. No significant association was observed (r = -0.4; *p* = 0.1).

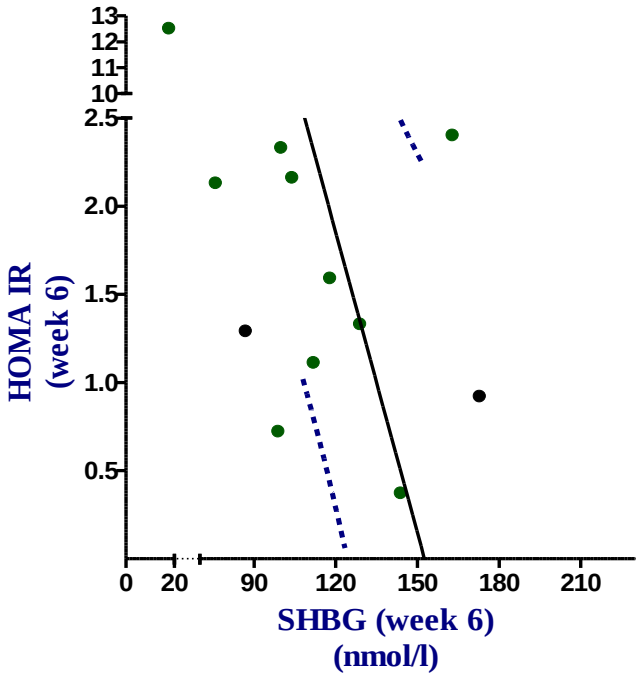


Figure 6-24: The relationship between HOMA IR and SHBG in 12 children following six weeks of vitamin D3 supplementation.

Green dots represent children who reached a final 25(OH)D level of ≥75 nmol/l whereas black dots represent children reached a final 25(OH)D level of < 75nmol/l. The black line indicates the line of best fit, and blue lines indicate the 95% confidence limits. No significant association was observed (r = -0.3; *p* = 0.2).

Table 6-9: Univariate correlation coefficients: Changes in correlation between different glucose homeostasis parameters from baseline to week six.

	At baseline							
	N	**Fasting glucose (mmol/l)**	**N**	**Fasting insulin (µiu/ml)**	**N**	**HOMA IR**	**N**	**SHBG (nmol/l)**
		r (*p*)		**r (*p*)**		**r (*p*)**		**r (*p*)**
SHBG (nmol/l)	15	0.1 (0.4)	11	-0.4 (0.1)	11	-0.4 (0.1)	-	-
HbA1c (mmol/mol)	22	0.003 (0.9)	17	0.3 (0.1)	17	0.3 (0.1)	16	-0.4 (0.04)
	At week six							
	N	**Fasting glucose (mmol/l)**	**N**	**Fasting insulin (µiu/ml)**	**N**	**HOMA IR**	**N**	**SHBG (nmol/l)**
		r (*p*)		**r (*p*)**		**r (*p*)**		**r (*p*)**
SHBG (nmol/l)	17	-0.01 (0.9)	11	-0.4 (0.1)	12	-0.3 (0.2)	-	-
HbA1c (mmol/mol)	21	-0.1 (0.5)	15	0.3 (0.2)	15	0.2 (0.2)	16	-0.5 (0.03)

6.4.6 Correlation of vitamin D with markers of glucose homeostasis

No significant correlation was observed between 25(OH)D and PTH in the 17 children whose 25(OH)D and PTH data were available at both week 0 and week 6 of the study period (r = -0.01; p = 0.9 and r = -0.1; p = 0.7 at baseline and week 6 respectively).

6.4.6.1 Changes in correlation between serum 25(OH)D, glucose homeostasis parameters and bone remodelling markers from baseline to week six.

Univariate correlation coefficients between study parameters are shown in Table 6-10. No significant correlations were found between serum 25(OH)D and any of the glucose homeostasis parameters throughout the study period. At the baseline, PTH was significantly and negatively correlated with HOMA IR (r = -0.5; p = 0.03). PTH was significantly and positively correlated with cholesterol/HDL ratio (r = 0.5; p = 0.04). Additionally, there was a significant positive correlation between SHBG and ALP (r = 0.5; p = 0.03). At week six, there was a significant positive correlation observed between serum PTH and triglyceride levels (r = 0.5; p = 0.03) (see Figure 6-26, Figure 6-27, Figure 6-28, Figure 6-29, Figure 6-30, Figure 6-31, Figure 6-32).

6.4.6.2 Mixed Effects Model (MEM)

The mixed effects model (MEM) were employed for further analysis. Out of 17 patients whose glucose, insulin and HOMA IR data were available, only parameters with available data at the two time points are included in this analysis. The results from the MEM are summarised in Table 6-11. The MEM showed that both HOMA IR and glucose are independently correlated with vitamin D after correction for other covariance such as BMI SDS, age and PTH. Additionally, HbA1c independently correlated with PTH and time.

Table 6-10: Univariate correlation coefficients.

At baseline														
	Glucose (mmol/l)		Insulin (µiu/ml)		HOMA IR		Chol/HDL		Triglycerides (mmol/l)		SHBG (nmol/l)		HbA1c (mmol/mol)	
	N	r (*p*)	N	r (*p*)	N	r (*p*)	n	r (*p*)	N	r (*p*)	N	r (*p*)	N	r (*p*)
25(OH)D	22	-0.3(0.08)	17	0.2(0.2)	17	0.3(0.2)	17	-0.007(0.9)	17	-0.1(0.4)	17	-0.4(0.09)	23	-0.1(0.3)
PTH	22	0.1(0.4)	15	-0.4(0.08)	17	**-0.5(0.03)**	17	**0.5(0.04)**	17	0.3(0.2)	17	0.2(0.2)	23	-0.1(0.4)
Calcium	18	-0.1(0.6)	14	0.2(0.3)	14	0.2(0.3)	15	-0.16(0.5)	15	-0.2(0.4)	15	-0.1(0.4)	20	-0.007(0.9)
ALP	20	0.3(0.1)	16	-0.4(0.07)	16	-0.4(0.08)	15	0.1(0.6)	15	0.09(0.7)	15	0.5**(0.03)**	21	-0.3(0.1)
CTX	19	0.2(0.2)	16	-0.2(0.4)	16	-0.2(0.3)	15	0.2(0.3)	15	0.2(0.4)	13	0.4(0.07)	19	-0.09(0.6)
At week six														
	Glucose (mmol/l)		Insulin (µiu/ml)		HOMA IR		Chol/HDL		Triglycerides (mmol/l)		SHBG (nmol/l)		HbA1c (mmol/mol)	
	N	r (*p*)	N	r (*p*)	N	r (*p*)	N	r (*p*)	N	r (*p*)	N	r (*p*)	N	r (*p*)
25(OH)D	22	-0.02(0.8)	17	-0.1 (0.6)	17	0.1-(0.5)	17	-0.2(0.2)	17	-0.2(0.2)	17	0.1(0.6)	21	0.05(0.8)
PTH	20	0.1(0.5)	15	-0.07(0.7)	15	-0.1(0.6)	15	0.4(0.07)	15	**0.5(0.03)**	17	-0.02(0.9)	20	-0.003(0.9)
Calcium	19	0.1(0.7)	13	-0.1(0.6)	13	-0.1(0.5)	15	-0.02(0.9)	15	0.2(0.4)	16	0.2(0.9)	19	-0.2(0.3)
ALP	19	0.3(0.1)	16	-0.3(0.1)	16	-0.1(0.5)	15	0.04(0.7)	15	0.1(0.5)	14	0.3(0.1)	20	-0.1(0.4)
CTX	19	0.4(0.05)	16	0.2(0.3)	16	0.3(0.2)	15	-0.2(0.3)	15	0.04(0.8)	14	0.4(0.1)	18	0.1(0.6)

Changes in correlation between serum 25(OH) D, glucose homeostasis parameters and bone remodelling markers from baseline to week six. Non-paired samples were included in this analysis. Significant results are represented in bold.

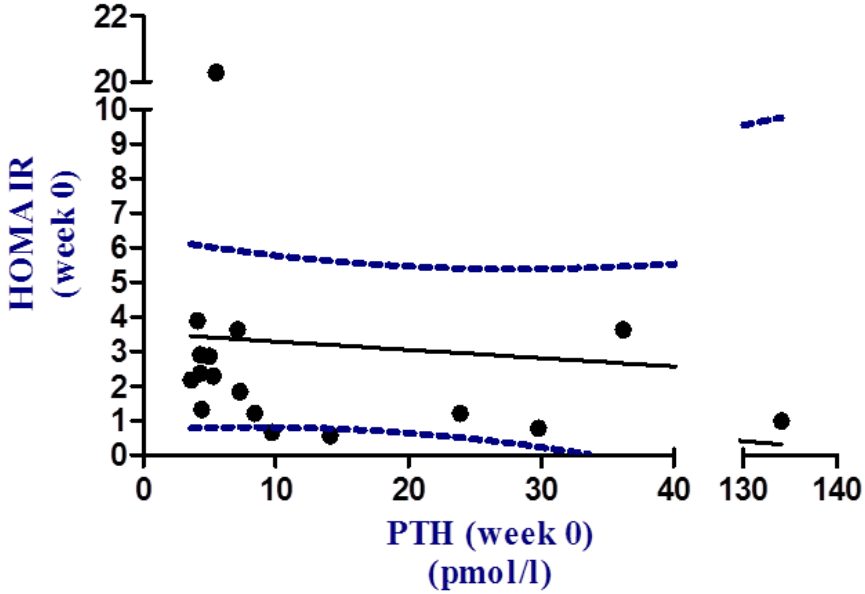


Figure 6-25: The relationship between HOMA IR and PTH in 17 vitamin D deficient children at the baseline.

The black line indicates the line of best fit, and the blue lines indicate the 95% confidence limits. A significant negative association was observed (r = -0.5; *p* = 0.03).

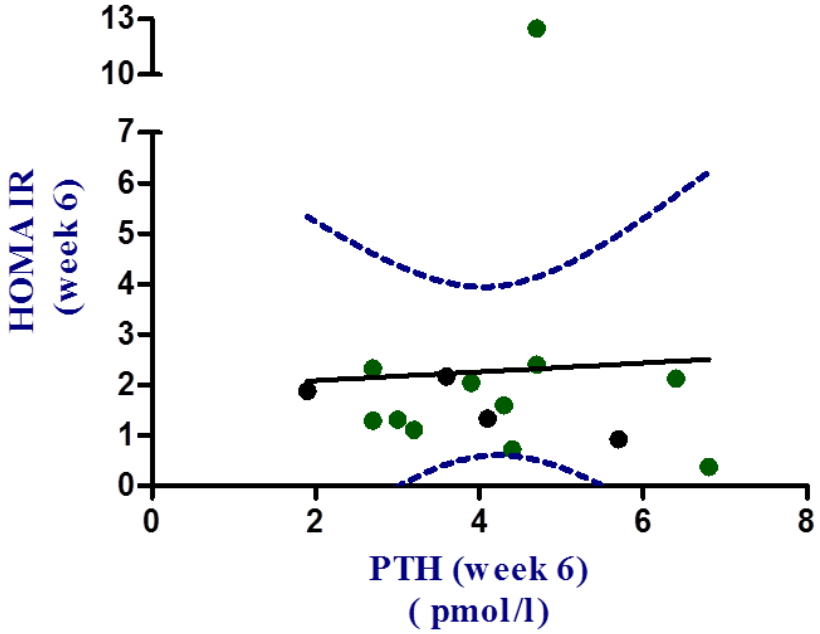


Figure 6-26: The relationship between HOMA IR and PTH in 15 children following six weeks of vitamin D3 supplementation.

The black line indicates the line of best fit, and the blue lines indicate the 95% confidence limits. Green dots represent children who reached a final 25(OH)D level of ≥ 75 nmol/l, whereas black dots represent children who reached a final 25(OH)D level of < 75nmol/l. No significant association was observed (r = -0.1; *p* = 0.6).

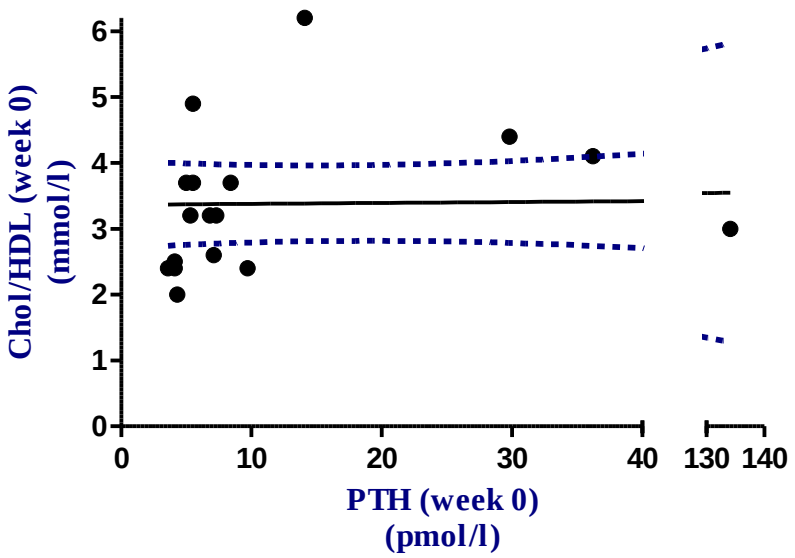


Figure 6-27: The relationship between cholesterol/HDL ratio and PTH in 17 vitamin D deficient children at the baseline.

The black line indicates the line of best fit, and the blue lines indicate the 95% confidence limits. A significant association was observed (r = 0.5; *p* = 0.04).

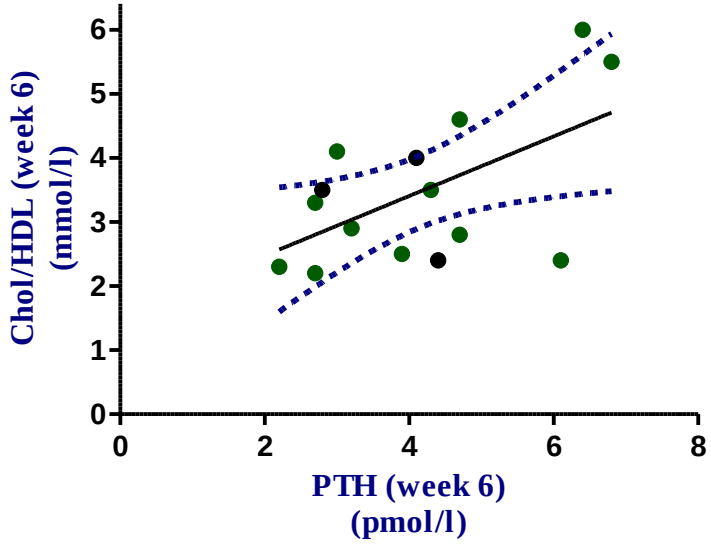


Figure 6-28: The relationship between cholesterol/HDL ratio and PTH in 15 children following six weeks of vitamin D3 supplementation.

Green dots represent children who reached a final 25(OH)D level of ≥75 nmol/l whereas black dots represent children who reached a final 25(OH)D level of < 75nmol/l. The black line indicates the line of best fit, and the blue lines indicate the 95% confidence limits. No significant association was observed (r = 0.4; *p* = 0.07).

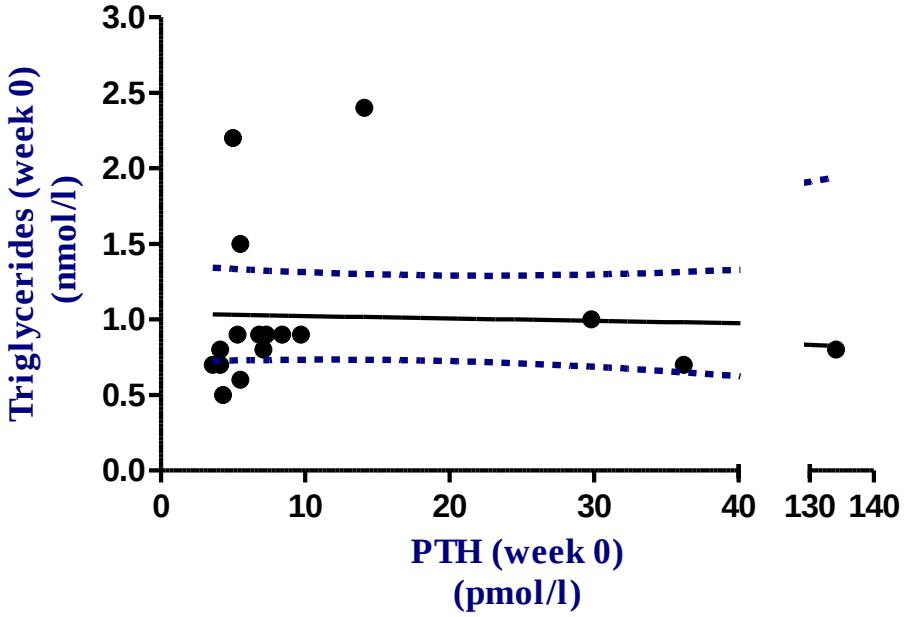


Figure 6-29: The relationship between Triglycerides and PTH in 17 vitamin D deficient children at the baseline.

The black line indicates the line of best fit, and the blue lines indicate the 95% confidence limits. No significant association was observed (r = 0.3; *p* = 0.2).

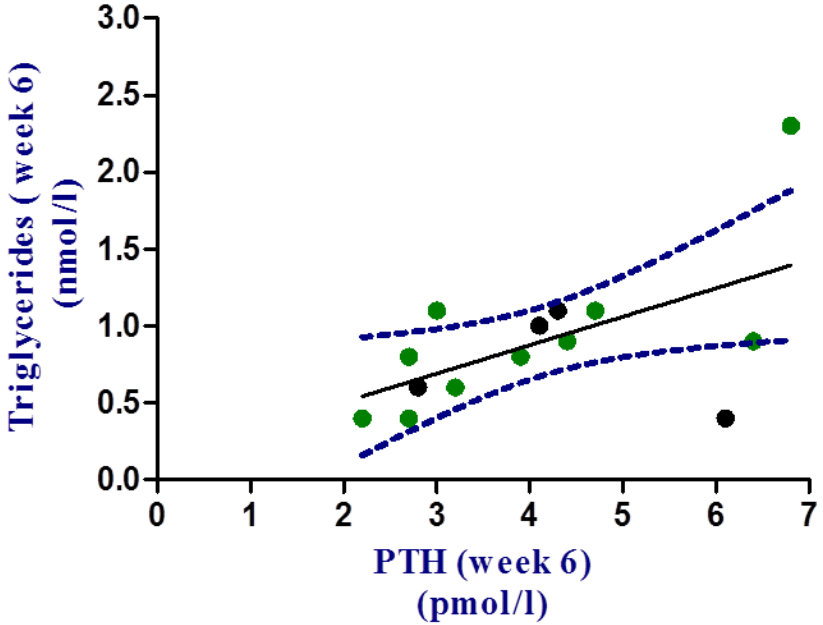


Figure 6-30: The relationship between triglycerides and PTH in 15 children following six weeks of vitamin D3 supplementation.

Green dots represent children who reached a final 25(OH)D level of ≥ 75 nmol/l whereas black dots represent children who reached a final 25(OH)D level of < 75nmol/l. The black line indicates the line of best fit, and the blue lines indicate the 95% confidence limits. A significant association was observed (r = 0.5; *p* = 0.03).

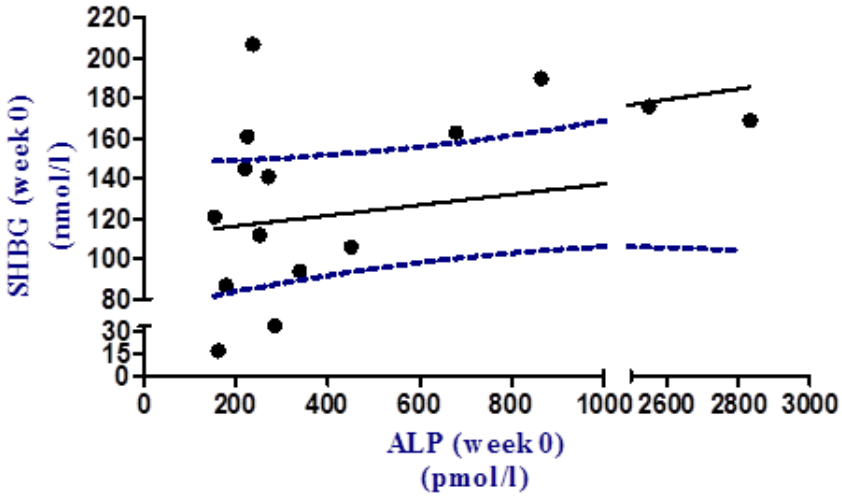


Figure 6-31: The relationship between SHBG and ALP in 15 vitamin D deficient children at the baseline.

The black line indicates the line of best fit, and the blue lines indicate the 95% confidence limits. A significant association was observed (r = 0.5; *p* = 0.03).

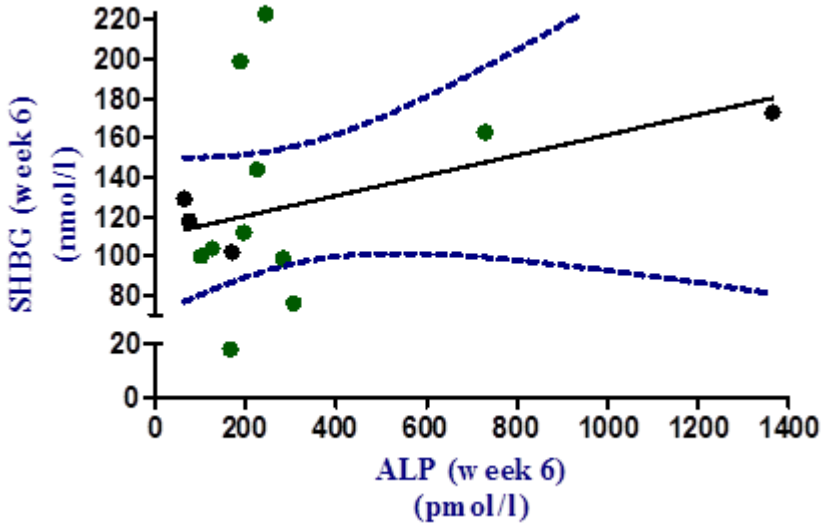


Figure 6-32: The relationship between SHBG and ALP in 14 vitamin D deficient children.

Green dots represent children who reached a final 25(OH)D level of ≥ 75 nmol/l whereas black dots represent children who reached a final 25(OH)D level of < 75nmol/l. The black line indicates the line of best fit, and the blue lines indicate the 95% confidence limits. No significant association was observed (r = 0.3; *p* = 0.1).

Table 6-11: Mixed model analysis to identify predictors of vitamin D, PTH and glucose homeostasis parameters.

Variables	Estimate	SE	*p*-value	95% CI (Lower, Upper)
Log vitamin D				
Intercept	4.46	3.67	0.2	(-3.01,11.93)
Time	0.56	0.05	0.0001	(0.44,0.67)
Log HOMA IR	1.01	2.82	0.7	(-4.72,6.75)
Log insulin	-1.13	2.80	0.6	(-6.82,4.54)
Log glucose	-4.11	3.08	0.1	(-10.35,2.12)
Log SHBG	-0.041	0.30	0.8	(-0.63,0.55)
Log HbA1c	-0.009	0.19	0.9	(-0.60,0.58)
Log PTH				
Intercept	-10.79	7.24	0.1	(-26.22,4.63
Time	-0.40	0.10	0.001	(-0.62,-0.18
Log HOMA IR	-9.87	5.55	0.09	(-21.63,1.88)
Log insulin	9.35	5.50	0.1	(-2.31,21.01)
Log glucose	9.20	5.89	0.1	(-3.15,21.56)
Log SHBG	-0.10	0.53	0.8	(-1.17,0.96)
Log HbA1c	-0.06	0.28	0.8	(-0.77, 0.63)
Log HOMA IR*				
Intercept	0.83	0.95	0.4	(-1.55,3.22)
Time	0.11	0.09	0.3	(-0.15,0.38)
BMI SDS	-0.02	0.30	0.9	(-0.75,0.71)
Age	0.002	0.03	0.9	(-0.06,0.06)
Log vitamin D	-0.51	0.18	0.02	(-0.95,-0.07)
Log PTH	0.03	0.16	0.8	(-0.38,0.44)
Log insulin				
Intercept	1.29	0.99	0.19	(-0.67, 3.25)
Time	0.09	0.10	0.4	(-0.30, 0.49)
BMI SDS	-0.02	0.31	0.9	(-0.64, 0.60)
Age	0.003	0.03	0.9	(-0.06,0.07)
Log vitamin D	-0.40	0.18	0.1	(-0.96, 0.14)
Log PTH	0.12	0.16	0.5	(-0.44,0.68)
Log glucose				
Intercept	0.78	0.1	0.0001	(0.4,1.147)
Time	0.04	0.01	0.02	(0.005,0.08)
BMI SDS	-0.001	0.06	0.9	(-0.12,0.12)
Age	-0.0004	0.005	0.9	(-0.01,0.01)
Log PTH	-0.04	0.03	0.3	(-0.12,0.04)
Log vitamin D	-0.09	0.03	0.005	(-0.16,-0.02)
Race	0.001	0.01	0.8	(-0.02,0.02)
Log SHBG				
Intercept	1.83	0.97	0.08	(-0.26, 3.93)
Time	0.06	0.08	0.5	(-0.31,0.44)
BMI SDS	-0.06	0.34	0.8	(-0.77,0.65)
Age	0.0003	0.02	0.9	(-0.05, 0.05)
Log vitamin D	-0.08	0.11	0.5	(-1.39,1.21)
Log PTH	0.31	0.16	0.1	(-0.28,0.90)
Log HbA1c				
Intercept	0.35	0.77	0.6	(-1.38,2.09)
Time	0.21	0.05	0.0001	(0.1,0.32)
BMI SDS	-0.01	0.33	0.9	(-0.79,0.77)
Age	0.01	0.01	0.5	(-0.02,0.051)
Log PTH	1.01	0.12	0.0001	(0.76,1.25)
Log vitamin D	-0.131	0.08	0.14	(-0.31,0.051)

Vitamin D independently correlated with HOMA IR and glucose. PTH independently correlated with HbA1c. * Race could not be added to HOMA IR, insulin and SHBG due to low sample size.

6.5 Discussion

Epidemiological studies suggest an inverse relationship between vitamin D status and glycaemic parameters, such as fasting glucose, fasting insulin and HbA1c [265, 266]. Several clinical intervention studies have supported the hypothesis that vitamin D replacement improves insulin sensitivity/resistance indices, and may had a positive impact on glycaemic control in patients with (or at high risk of developing) type 2 diabetes mellitus [279, 282]. Therefore, we hypothesized that these effects were likely to be more evident in vitamin D deficient individuals.

A dose of 5000 IU of vitamin D3 for 4-8 weeks is the suggested minimum dose that is effective to achieve optimal serum 25(OH)D concentrations of ≥ 75 nmol/l for musculoskeletal benefit in vitamin D deficient healthy adults [501]. Additionally, serum 25(OH)D levels between 75-100 nmol/l have been suggested as in the ideal range for multiple health outcomes [441]. A daily vitamin D3 dose of 4000 IU was successful in improving HOMA IR and decreasing plasma insulin at six months, but not at three months, in obese adolescents [282]. In the present study, daily treatment of vitamin D deficient children without diabetes mellitus with 5000 IU of vitamin D3 for six weeks had a significant effect on insulin resistance.

Although we found no effect from the correction of vitamin D status on fasting glucose, HbA1c, SHBG and lipid profile, results from the study reveal favourable effects from oral vitamin D3 supplementation on insulin resistance and fasting serum insulin levels. A significant reduction in insulin resistance *(p* = 0.04), and a trend towards a significant reduction in fasting serum insulin (*p* = 0.05) was observed for the 17 children for whom glucose, insulin and HOMA IR data was available at both baseline and week six of the study period. This improvement in insulin resistance and the reduction in serum insulin was more apparent in children who responded well to vitamin D3 supplementation and who had an end point serum 25(OH)D level of ≥ 75 nmol/l. In one child (Patient ID: 7), both HOMA IR and serum insulin decreased remarkably from baseline to week six, although still remaining outside the normal reference range. The improvement in insulin resistance at the end point serum 25(OH)D level of ≥ 75 nmol/l was consistent with previous observations in adults. In a cross sectional study by Chiu *et al* 2004, involving 126 glucose-tolerant healthy non obese adults, a positive association between serum 25(OH)D and insulin sensitivity indices was observed, and an improvement in insulin

sensitivity of 60% was demonstrated when serum 25 (OH)D increased from 24.96 to75 nmol/l [258].

Observational studies exploring the relationship between vitamin D status and markers of glucose homeostasis also show conflicting data. Several studies support an inverse relationship between 25(OH)D status and insulin resistance [265, 266]. On the other hand, other studies have failed to prove that relationship [264, 502]. For example, no association was found between 25(OH)D with measures of glucose homeostasis and β cell function in 175 obese youths (aged 9-20 years) who had either normal glucose tolerance, impaired glucose tolerance or had type 2 diabetes mellitus [502]. Another study involving both obese and normal weight girls aged between 12-18 years found that PTH, but not 25(OH)D, was correlated inversely with HOMA IR and positively with QUICKI only in obese girls [503].

The limited number of published interventional studies evaluating the effects of vitamin D replacement on insulin resistance in paediatric populations are inconsistent. The discrepancy among studies may be attributed to the differences in vitamin D dose, basal and end points serum 25(OH)D levels and a variable risk of bias. Additionally, the majority of the available clinical trial data is based on trials targeting obese and/or high risk children. Six months, but not three months, of daily supplementation of 4000 IU vitamin D in obese adolescents showed a significant improvement in both HOMA IR and QUICKI compared with a placebo [282]. Using the HOMA to measure insulin resistance, two recent randomised trials including obese adolescents have reported conflicting results. In the first trial, a 2000 IU daily oral vitamin D3 supplementation in 20 healthy adolescents for 12 weeks did not have any effect on the lipid profile and markers for insulin resistance [504]. On the other hand, weekly 300000 IU oral vitamin D3 supplementation for 12 weeks in 21 obese adolescents with metabolic syndrome improved the lipid profile and markers for insulin resistance [283]. When comparing the last study with our study, there are important differences in baseline vitamin D levels (mean baseline 25(OH)D level in the last study was 45 nmol/l compared with 28 nmol/l in our study) and study population. Additionally, the mean HOMA IR in the last study was 3.21 which decreased following oral vitamin D3 supplementation by 0.4 to 2.81 whereas in our study the mean HOMA IR was 3.09 which decreased following vitamin D treatment by 0.9 to 2.19.

In older children and adolescents, lower 25(OH)D status has been linked to a higher risk of metabolic syndrome, including increased triglyceride level and insulin resistance [283].

Few available studies have considered the association between vitamin D and serum lipids specific to a younger age group. One cross-sectional study, which included 1961 children aged between 1 and 5 years reported a significant association between serum 25(OH)D and non-HDL cholesterol, as well as non-fasting triglycerides and total cholesterol (even after adjustment for specific confounders) [505]. Both triglycerides and non-HDL cholesterol are known predictors, used to identify the risk of cardiovascular disease in healthy adults. The association between serum 25(OH)D and lipids still needs to be confirmed in an interventional study. If an association between vitamin D and lipid parameters in early childhood exists, this may provide a basis for early life stage interventions to decrease future cardiovascular risk.

In the present work, vitamin D3 treatment was not found to improve the median lipids profile. However, when examining individual data for a small number of children with an abnormal lipid profile, demonstrable improvement in lipids, particularly in obese children, occurred following vitamin D3 supplementation.

The link between lower vitamin D status and obesity is well-documented [86]. Obese children generally have low vitamin D status compared with normal weight children [85]. Clinical trials have linked obesity to lower vitamin D status, which in turn seems to have negative consequences on insulin resistance and glucose homeostasis [265, 266]. In the present study, three obese children were identified. The three children had basal serum 25(OH)D levels of < 50 nmol/l, and two showed an alarmingly abnormal metabolic profile (increased insulin resistance, low basal SHBG level and an abnormal lipid profile). Additionally, both of those children with abnormal metabolic profiles had a positive family history of either diabetes or hypertension with high cholesterol level. Following supplementation, the metabolic profile returned to normal level in one child while it improved (but remained abnormal) in the other. Therefore, the study participants were divided into two groups based on their BMI, to compare the effect of vitamin D supplementation between normal weight children and obese children. The results showed a significant reduction in HOMA IR in obese children following vitamin D supplementaion ($p = 0.007$).

The correlation study found a significant association between HOMA IR and PTH r(p): -0.5(0.03), which was also reported in another study [503]. Previous reports suggest PTH may have a role on glucose-mediated insulin secretion [506, 507]. The proposed role concerned the ability of PTH to impair insulin release mechanisms, involving ATP

depletion and increasing resting calcium concentrations [508]. In addition, PTH has been suggested to either directly increase insulin resistance, or to at least be linked to a deterioration in insulin resistance [509, 510]. However, the effect of lowering serum PTH by vitamin D supplementation on glucose homeostasis requires further evaluation.

There was no association between 25(OH)D and measures of glucose homeostasis was found in the universal correlation analysis. However, the results from MEM analysis revealed vitamin D as an independent predictor of HOMA IR and glucose levels, after correction for other variables such as time, BMI SDS, age, PTH, and race. The association between vitamin D and glycaemia markers has been reported previously in both adults and children [265, 511].

Although the results do not demonstrate any significant effect of vitamin D3 treatment on serum SHBG level, those from the universal correlation analysis showed a significant association between HbA1c and SHBG at the baseline, as well as at the end of the study. Additionally, MEM analysis suggested PTH as an independent predictor for HbA1c, which may further supports reports that correlate hyperparathyroidism with the prevalence of hyperglycaemia.

Finally, several children (n = 6) demonstrated low final serum 25(OH)D levels. Since patient compliance was only assessed verbally and may not reflect actual intake, the wide range in the end point of serum 25(OH)D may indicate poor compliance. A larger study may have disclosed a significant effect from vitamin D intervention on other glucose homeostasis parameters. Additionally, since vitamin D3 treatment lasted only six weeks, we could not assess any long-term effects on glucose and lipid parameters or the safety of the 5000 IU vitamin D3 dose.

The main strength of this study is the prospective and interventional nature of our work. In contrast to other available interventional studies, our study involved younger and healthy children. However, this part of the thesis has several limitations. Firstly, several children were still vitamin D deficient following vitamin D replacement, which may limit our ability to detect significant changes in some parameters. However, it could be hypothesised that the relationship between vitamin D status and these parameters is dependent on other factors such as age, production of sex hormone and diabetic risk and the absence of the significant association between 25(OH)D and these measurements is due to the nature of study participants: pre-pubertal non-obese children at lower risk of diabetes

mellitus. Secondly, our study involved only a small number of participants and a larger study may have disclosed a positive effect of vitamin D intervention on other glucose homeostasis parameters. An additional limitation is that there was no control group which may limits our ability to attribute the changes exclusively to the intervention and to eliminate the confounding effects of age, sex, race, BMI, nutritional status and family history. Finally, since vitamin D3 treatment lasted only six weeks, we cannot assess the long-term effects of vitamin D3 supplementation on glucose and lipid parameters.

In summary, six weeks of vitamin D3 supplementation has a favourable effect on key glucose homeostasis parameters, HOMA IR and fasting serum insulin in healthy children with low vitamin D level. Although our results may not have any immediate implication in term of clinical practice and public health, a larger sample size with a longer period of follow-up would possibly have obtained more favourable results and the correction of poor vitamin D status may be considered as an additional effective measure to decrease the insulin resistance and/or improve glycemic status.

Chapter 7

Effect of Vitamin D3 Supplementation on Immune Function

7.1 Abstract

Introduction

The immunomodulatory function of vitamin D is not fully understood. One key mechanism through which vitamin D influences human immune responses may be related to the regulation of specific cytokines and chemokines produced by antigen-presenting cells. Additionally, some reports suggest that vitamin D may play a central role in alterations to the balance of different T helper cell subsets, and may affect the tissue-specific lymphocyte homing capability. Finally, several immune cell-secreting cytokines have been suggested as mediators for the bone remodelling process, and as regulators of bone cell development and activity.

Objective

The effect of short-term vitamin D3 supplementation on main T helper 1 (Th1) and T helper 2 (Th2) cytokines, Th17 cytokine, T regulatory cytokine (Treg) and chemokines linking the balance between the Th1 and Th2 subset was assessed. Additionally, an association between these cytokines and bone remodelling markers before and after vitamin D3 supplementation was also investigated.

Method

Children deficient in vitamin D were treated with daily 5000 IU Cholecalciferol (vitamin D3) for six weeks. Baseline and end of treatment serum 25 hydroxyvitamin D (25(OH)D), parathyroid hormone (PTH), alkaline phosphatase (ALP) and serum collagen type 1 cross-linked C-telopeptide (CTX) were measured. Leukocyte subset analysis was performed for T and B cells and T regulatory cells and a Luminex assay was used to measure Th1 cytokines (IL-2, TNF-α, INF-γ), Th2 cytokines (IL-4, IL-5, IL-6), Th17 cytokine (IL-17),

Treg cytokine (IL-10) and the Th1/Th2 subset balance linked to chemokines/cytokines and/or differentiation (IL-8, IL-12, EOTAXIN, MIP-1β, IP-10, RANTES, MCP-1).

Results

A total of 14 children were enrolled in the study, with a median (range) age of five years (10 months, 9.5 years). Over a six-week period, 25(OH)D increased from 24.5 (14, 49) nmol/l at the baseline to 103 (37, 191) nmol/l at the week six ($p = 0.0001$). Serum PTH concentrations decreased from 7.2 (3.6, 134) pmol/l at the baseline to 4.3 (2.7, 6.8) pmol/l at week six ($p = 0.006$). No significant alteration in measured lymphocyte subsets in response to vitamin D3 treatment was detected, although there was a significant increase in the main Th2 secreted cytokine IL-4 ($p = 0.001$) and a tendency towards a significant increase in other Th2 secreted cytokines (IL-5, $p = 0.05$ and IL-6, $p = 0.05$) following vitamin D3 supplementation. The results from the Mixed EffectsModel (MME) study showed that IL-17 is independently correlated with PTH. Additionally, IL-6, IL-8, IL-12, IL-10, IL-17 and IFN-γ are independently correlated with bone remodelling markers.

Conclusion

Six weeks vitamin D3 supplementation in vitamin D deficient children is associated with an alteration in multiple cytokines. However, it is unclear whether serum 25(OH)D in these children or the response to vitamin D treatment are directly associated with a change in these cytokines. The results also showed a significant association between inflammation and bone remodelling markers in vitamin D deficient children.

7.2 Introduction

As described in the introduction, it has become increasingly clear that hosts' defences against harmful events require complex communication between the innate and adaptive sides of the immune system [287]. Signals from innate immune cells, stimulated by infectious agents and inflammatory stimuli, prompt naive CD4-positive T helper (CD4+Th) cells to proliferate and differentiate into one of several T cell effectors. These included T helper 1(Th1), T helper 2 (Th2), T helper 17 (Th17) and regulatory T (iTreg) cells, as determined by the specific effector cytokines produced by antigen-presenting cells (APCs) [294, 296].

CD4-positive T helper cells are also important manufacturers of a range of cytokines that determine the features of immune responses [294]. Type 1 helper cells produce pro-inflammatory cytokines such as IL-2, IFN-γ and tumour necrosis factor-alpha (TNF-α).Type 2 helper cells are generally considered to be anti-inflammatory and produce cytokines such as IL-4, IL-5, IL-13 and IL-6 [302, 512]. T regulatory cells suppress the activation of the immune system and therefore maintain self-tolerance [513]. T helper 17 cells produce IL-17 and IL-22 and have been suggested to play a key role in resistance to extracellular pathogens [514]. Other cytokines, such as IL-8, IL-10 and IL-12, can influence T-cell activation and/or differentiation. For example, IL-10 inhibits IL-2 production by activating human T cells and down-regulates Th1 secreted cytokines [515], and both IL-8 and IL-12 are essential for Th1 development. Additionally, IL-12 is suggested as a regulator of Th1/ Th17 balance [516-518].

Upon activation, CD4+Th cells are able to express a variety of chemokine receptors, depending upon the cellular context in which they are activated. T helper 1 cells are able to express chemokine receptors such as CCR5 (RANTES and MIP-1β), CXCR3 (IP-10) and CCR2 (MCP-1), which play a role in the differentiation of Th1 cells and/or in assisting their migration into different inflammatory tissue sites [519-521]. The activation of Th2 cells is associated with an expression of CCR3 (Eotaxin) receptors on Th2 cells, promoting their migration into target sites, such as sites of allergic reaction in the airways [364, 522].

Ideally, humans should produce a well-balanced Th1 and Th2 response suited to the immune challenge [513, 523]. A key role of cytokines is the maintenance of a proper balance between Th1 and Th2 cellular subsets. Any dysregulation of the Th1/Th2 balance may cause a disruption in immune system's responses to infections (immunodeficiency), or

excessive and incorrect immune responses to harmless environmental antigens (atopy) or self-antigens (autoimmunity) [513, 524]. Both Treg and Th17 can play a role in Th1 and Th2 activity and function. For example, IL-4 signaling through the IL-4 receptor in naive CD4+T cells maintains the Th2/Treg balance via activation of transcription factor fork head box P3 (FoxP3) and signal transduction and activator 6 (STAT6) [304]. Improper Th1/Th17 cell balance has been linked with the development of inflammatory bowel disease [305].

Experimental reports have shown that vitamin D may play a critical role in determining immune response through an alteration in the functional status of CD4+ Th cells subsets. It has been suggested that vitamin D may affect Th cell polarization by enhancing Th2 cell and/or inhibiting Th1 development. For example, it has been found that vitamin D3 enhances the production of Th2 cytokine IL-4, and inhibits the production of Th1 cytokines IL-2, IFN-γ and TNF-α [401, 525-527]. Additionally, vitamin D3 has been suggested as a direct inducer of T regulatory cell development via the regulation of FoxP3 expression and has been shown to inhibit IL-17 secretion by Th17 cells [528, 529].

Both the immune system and bone remodelling process are interrelated with a complex overlapping regulatory network [530, 531]. Osteoblasts regulate the haematopoietic stem cell niche, which gives rise to all blood and immune cells [532], whereas osteoclasts originate mainly from the same myeloid precursor cells that produce macrophages and myeloid dendritic cells [533]. Evidence from experimental studies has suggested that cytokines such as IL-2, IL-6, IL-8, IL-10, IL-12, INF-γ and TNF-α may act as regulators of bone cell metabolism and skeletal remodelling [530]. Bone remodelling factors such as vitamin D and PTH may also play a key role in the process of systemic inflammation and the modulation of cytokine secretion [197, 534, 535].

Based on available evidence, the impact of vitamin D supplementation on different T cell subsets remains unclear, particularly among both the healthy and the younger population. This study was designed to test the hypothesis that vitamin D can influence Th1/Th2 skewing by examining the effect of short-term vitamin D3 supplementation on main T helper 1 (Th1) and T helper 2 (Th2) cytokines, Th17 cytokine, T regulatory cytokine (Treg) and chemokines linking the balance between the Th1 and Th2 subsets. An additional objective was to determine whether these cytokines are associated with markers of bone remodelling before and after vitamin D3 supplementation.

This study also aimed to document the change in the peripheral blood lymphocyte subset following vitamin D supplementation by examining the relative percentages of B cells (CD19+), T cells (CD4+/CD8+/CD3+) and T regulatory cells (CD4+CD25+FoxP3+) using Multi-Parameter Flow Cytometry (FACS).

7.3 Subjects and methods

7.3.1 Patient methods

7.3.1.1 Ethics approval and funding

This study was given a favourable ethical opinion by the West of Scotland Research Ethics Service (REC3) (REC Reference: 11/AL/0291) and received additional funding from TENOVUS (sponsored research project no. S11/37).

7.3.1.2 Recruitment of Subjects

A total of 18 children aged between 10 months and 9.5 years, who had been referred for evaluation and management of suspected vitamin D deficiency, were investigated. These cases were recruited in the outpatient clinical endocrinology department of the Royal Hospital for Sick Children (RHSC). All patients had already consented to participate in the bone and glucose parts of this study.

7.3.1.3 Inclusion/Exclusion criteria

All vitamin D-deficient children aged 10 years or under were eligible for this study. In addition to the main exclusion criteria listed earlier in Chapter 4. Patients with a history of any chronic inflammatory condition or atopy were further excluded in this chapter. The study protocol is described in Chapter 4.

7.3.1.4 Blood collection

Blood samples were collected on Ward 1C at RHSC. Serum 25(OH)D and PTH levels were obtained as a part of routine clinical care, and an additional 3ml of peripheral blood was collected in EDTA vacuettes for the immune study. For 25(OH)D, samples were collected in lithium heparin vacuettes and immediately centrifuged at 2600-2800 rev/minute for 10 minutes. The serum was subsequently stored at -80C until the assay was performed. Samples for PTH were collected in lithium heparin vacuettes and sent directly on the same day to the biochemistry laboratory at RHSC. Blood samples for the immune analysis were collected into two EDTA vacuettes. The first blood sample was immediately centrifuged at 2600-2800 rev/minute for 10 minutes, and the plasma fraction stored in aliquots at -80^{o}C until the end of the recruitment period for the study in order to perform the Luminex Multiplexed Cytokine Assay. The second sample was transferred directly to

the research unit at the Institute of Infection, Immunity and Inflammation for the lymphocyte subset analysis.

7.3.2 Routine laboratory measurements

The assay for serum 25(OH)D levels was performed by Dr Jane McNeilly in the Biochemistry Department at the Southern General Hospital, Glasgow using an Abbott Architect Chemiluminescent Microparticle Immunoassay (CMIA). Parathyroid hormone (PTH) measurements were assayed by the biochemistry laboratory at RHSC using intact PTH CMIA (Abbott Architect).

7.3.3 Molecular biology: Luminex Multiplexed Cytokine Assay

This assay was performed at the laboratory of the Institute of Infection, Immunity and Inflammation at the University of Glasgow. All samples were assayed on the same plate at the same time in duplicates.

Luminex assay, an alternative to classical ELISA, enables the simultaneous detection and quantification of multiple cytokines in a single well of a 96-well plate. The advantages of Luminex over ELISA are the smaller sample volume requirement and a lower protein detection limit in addition to the production of more reliable results [536]. In this study, the cytokines and chemokines IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-17, EOTAXIN, MIP-1β, IP-10, TNF-α, IFN-γ, RANTES and MCP-1 were simultaneously examined in each sample from the study participants using a custom made Bio-Plex Pro Human Cytokine Group I, 15-plex assay. The immunoassay reagents were purchased from Bio-Rad laboratories (Hemel Hempstead, Hertfordshire, UK) and the method was performed according to the manufacturer's instructions.

This method involves the use of a capture antibody-antigen sandwich to detect the marker of interest, as described in Figure 7-1. Briefly, standards and reagents were prepared following the manufacturer's instructions. After thawing, 50 µl of each patient's sample, various concentrations of the assay standards and the control were added to a 96-well plate containing magnetic beads and pre-wet with assay buffer. The magnetic beads were pre-coated with analyte/biomarker-specific capture antibodies and had distinctive fluorescence wavelengths for each antibody. The plate was then incubated for 30 minutes before a wash step was applied. Subsequently, 25 µl of a biotinylated detection antibody specific to the

biomarker of interest was added to each well and incubated on a plate shaker for one hour at room temperature. Following the incubation, 50 µl of diluted reporter streptavidin-PE antibody was added, followed by incubation for a further 30 minutes, agitation at room temperature and washing. Finally, 125 µl assay buffer was added to each well and analysis was performed using a Bio-Plex 100 multiplex plate reader (Bio-Rad). A standard curve was obtained using the different concentrations of the assay standards. Concentrations of target proteins were calculated by matching each value to the standard curves. Data were analysed using Bio-Plex Manager software v6.1 (Biorad).

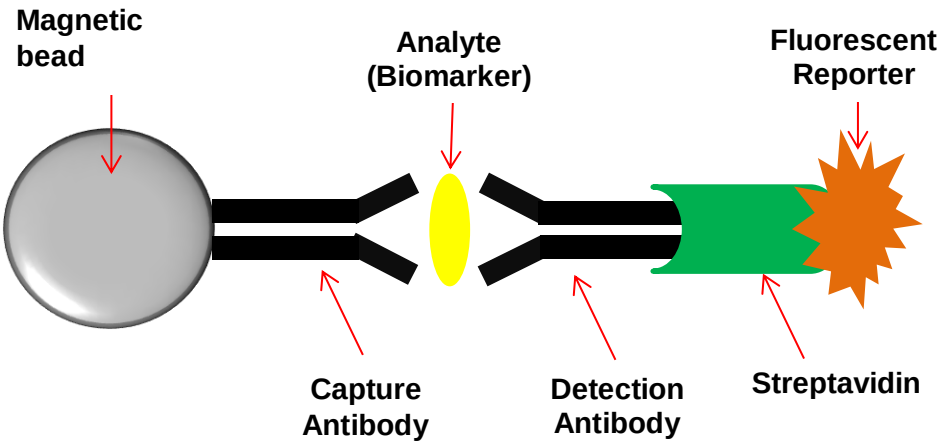


Figure 7-1: Luminex Multiplexed Cytokine Assays.

Magnetic bead pre-coated with analyte-specific capture antibody and biotinylated detection antibody specific to the biomarker of interest form an antibody-antigen sandwich. This is followed by the addition of phycoerythrin (PE)-conjugated streptavidin and read by a detection instrument.

7.3.4 Cell based assays

7.3.4.1 Cell purification

Lymphocytes (CD4+ T cells) were indirectly isolated from whole blood by layering over the Ficoll-Paque density medium (Sigma-Aldrich) using the RosetteSep™ CD4+ T cell enrichment cocktail (Stem Cell Technologies). This cocktail contains a combination of monoclonal antibodies directed against cell surface antigens on human leukocytes (CD16, CD19, CD36, CD56, CD66b) and glycophorin A on RBCs, which crosslink unwanted haematopoietic cells. The method was performed according to the manufacturer's instructions as described in Figure 7-2.

7.3.4.2 T reg Detection (Miltenyi Treg detection kit)

Following the purification steps, cells were washed and surface-stained with CD4-FITC, CD25-APC and CD127-BV421 (all BioLegend). Dead cells were labelled using fixable viability stain eFluor780 (eBioscience). Cells were fixed and permeabilized using the eBioscience fixation and permeabilization kit (as per the manufacturer's instructions), before incubation with Foxp3-PE (eBioScience) antibody. Finally, cells were resuspended in cold PEF buffer (flow cytometry buffer: PBS + 0.5% FCS + 2mM EDTA). Samples were acquired on the MACSQuant flow cytometer and analysed using the MACSQuantify software v2.4 (Miltenyi Biotec).

7.3.4.3 Lymphocyte analysis (T/B cells)

Lymphocyte analysis (T/B cells) was performed following an addition of 1ml of RBC lysis buffer (Miltenyi Biotec) to 100 µL of whole blood. After 10 minutes of incubation, centrifuging at 300g/5 minutes and removing the supernatant, cells were washed and suspended in 200 µL PEF, and labelled with 5 µL of CD19-PE (B cells; BioLegend), CD3-FITC (T cells; BioLegend), CD4-Vioblue (Miltenyi Biotec) and CD8-APC (Miltenyi Biotec) antibodies. Dead cells were labelled using 1 µL DRAQ7 (BioStatus). Samples were acquired on the MACSQuant flow cytometer and analysed using the MACSQuantify software v2.4 (Miltenyi Biotec).

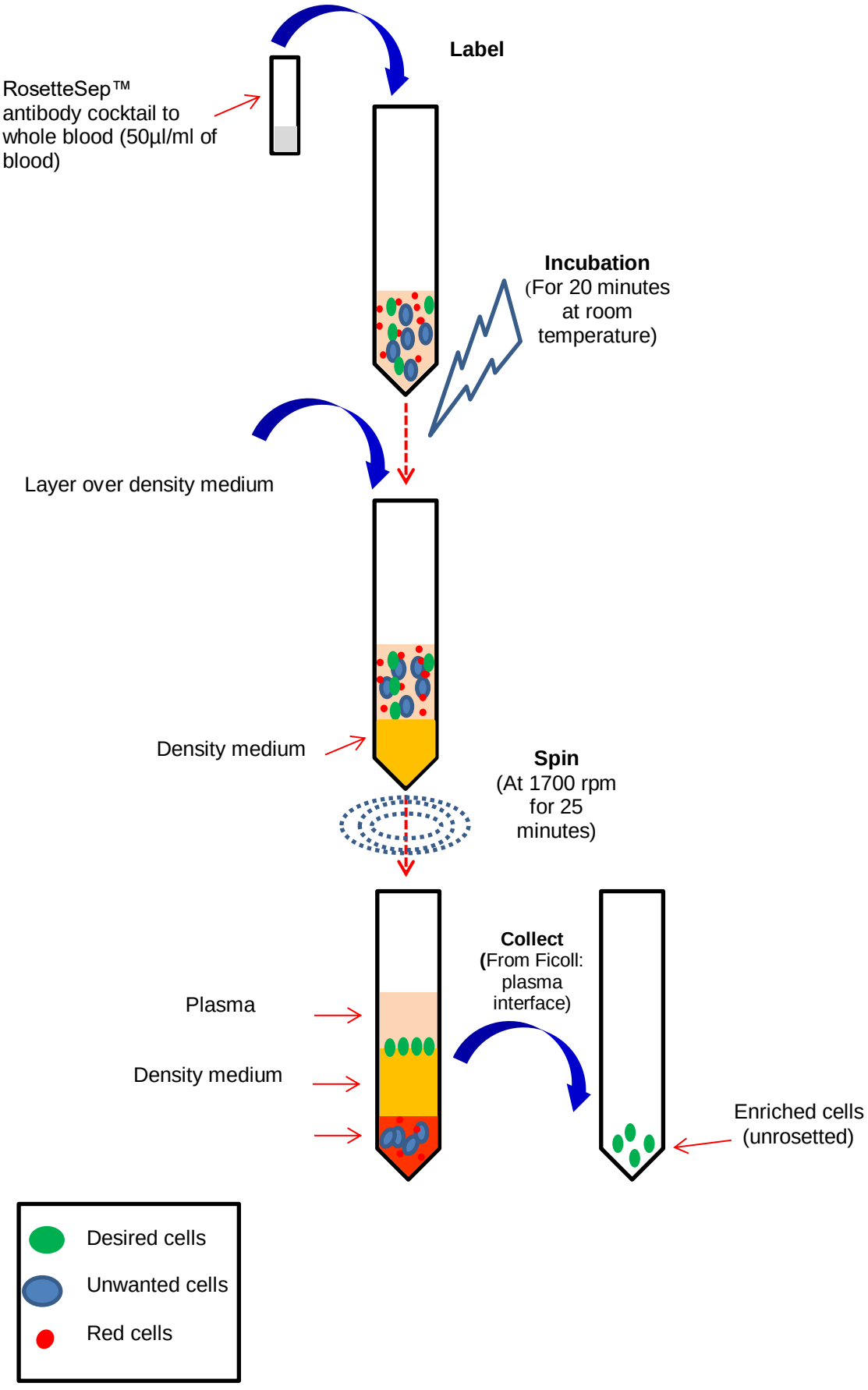


Figure 7-2: Schematic diagram showing the main steps used for isolation of CD4+ T cells using the Human CD4+T Cell Enrichment cocktail.

7.3.4.4 Flow Cytometry

Flow cytometry is a technology that enables researchers to simultaneously examine, count, and sort cells by detecting surface or intracellular markers of interest via measuring the fluorescence signals emitted from cells. The process starts with the preparation of a cell suspension and labelling with fluorescently conjugated antibodies. The suspension is then forced to pass through a complex tubing system in a liquid jet stream followed by excitation with a laser beam to emit light at different wavelengths (depending on the fluorophore used). Several detectors are carefully placed around the stream at a point where the fluid passes through the laser beam. One of these detectors is in line with the laser beam and is used to measure Forward Scatter (FSC). Another detector is placed perpendicular to the stream and is used to measure Side Scatter (SSC). Light scatter and fluorescence released from fluorochromes are detected by these detectors and used to provide a multiparametric analysis of the physical and chemical characteristics of single cells. For example, both FSC and SSC together can provide information about cell size and granularity, while markers of interest can be evaluated via fluorescence. In this chapter, flow cytometry was employed for the lymphocyte subset analysis [537].

7.3.5 Statistical analysis

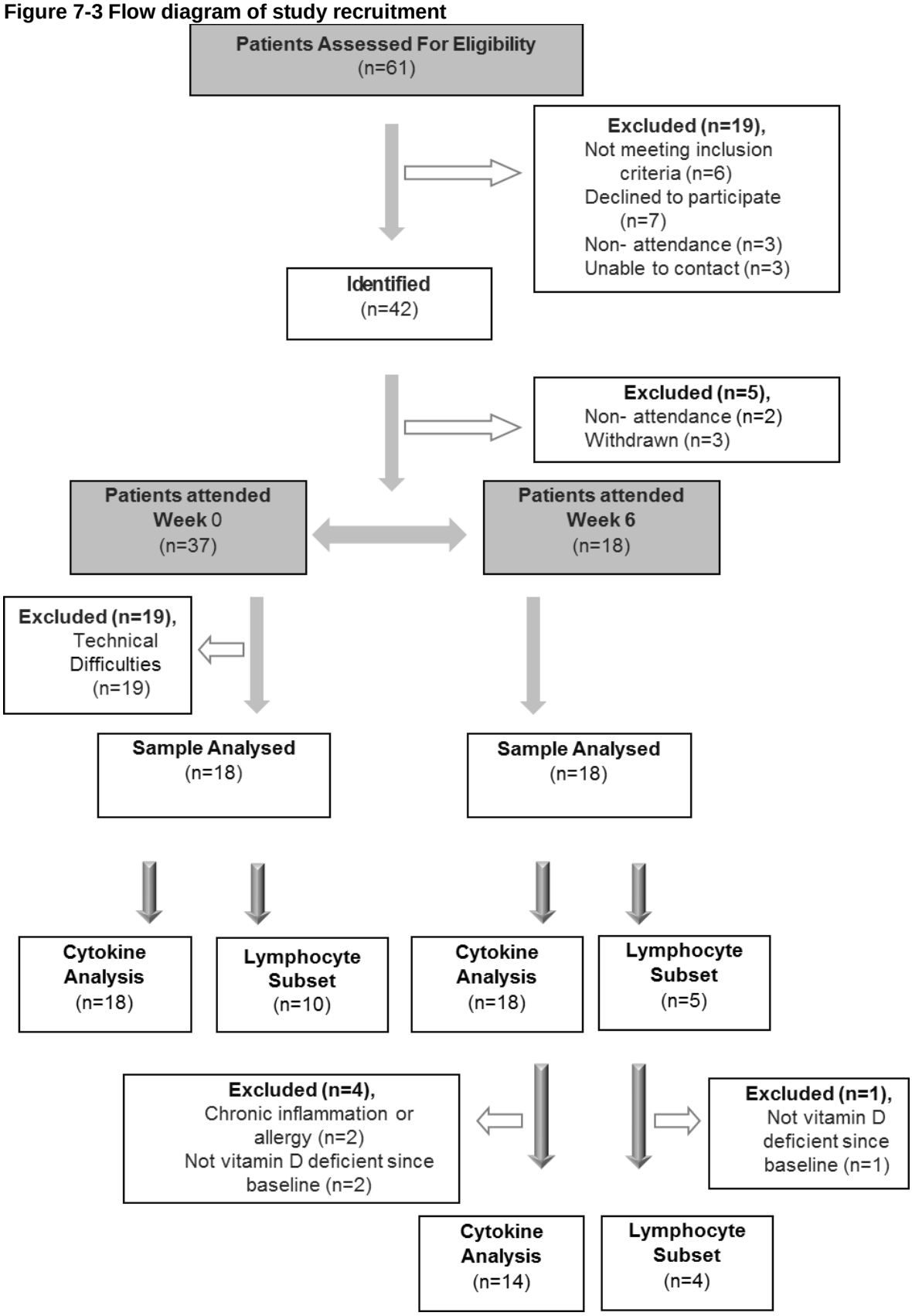
Statistical analyses were performed with GraphPad Prism 5 (San Diego California, USA) or Mini Tab 17 (Minitab, Coventry, UK) statistical software. Results are presented as median and ranges unless stated otherwise. The significance of changes in cytokine and lymphocyte measurements from the baseline to the week six was assessed using the Wilcoxon signed-rank test. Spearman correlation analysis was performed to reveal the association of 25(OH)D levels and markers of bone remodelling with laboratory parameters. Using IBM SPSS 22 statistical software, the Mixed Effectsmodel (MEM) was employed where appropriate to explore the confounding effects of independent cytokines. For MEM analysis, the laboratory measures were transformed with logarithmic transformation to obtain a more normal distribution (as data were skewed, a natural log transformation was applied). All study results were evaluated at the level of significance $p < 0.05$.

7.4 Results

7.4.1 Recruitment summary

A total of 61 children were identified and screened during the 24-month study period between 2011 and 2014, of whom 42 met the main project inclusion criteria and agreed to participate. In total, 37 children attended the first study visit, of whom 19 were excluded due to difficulty with venous access, lack of patient/parent cooperation or insufficient blood sample volume. Samples from 18 children were available for cytokine analysis and 10 for lymphocyte subset analysis at the baseline. At week six of the study, 18 samples were available for cytokine analysis and only five samples were available for lymphocyte subset analysis. The main reason for the drop-out at week six was the difficulty in obtaining a sufficient blood sample during venipuncture due to either difficult venous access or parental refusal. During the process of data analysis, four children were excluded as two of them showed vitamin D levels within the normal reference range at the baseline study visit. A further two patients were excluded due to the coexistence of chronic inflammatory/atopic conditions, chronic eczema and cow's milk allergy (see figure 7-3, flow diagram of study recruitment).

Figure 7-3 Flow diagram of study recruitment



Sixty-one children were identified through the endocrine clinic. Forty-seven children were excluded throughout the study period due to a variety of reasons as shown in the diagram.

7.4.2 Demographic characteristics

At the baseline, the median age (range) of the children was four years (10 months to 9.5 years). Height (Ht) and body mass index (BMI) were converted into SDS using 1990 British childhood standards [448, 449]. The main cause of referral was bone pain (six children; 43%). Other causes included bowed legs, seizures, dietary concern fractures and family history. Table 7-1 summaries the details of the 14 children enrolled in the study.

Table 7-1: Characteristics of recruited children.

Variables	N	%
Age (years)	**(14)**	
0-2	5	36
2-6	5	36
6-10	4	28
Gender	**(14)**	
Male	7	50
Female	7	50
Race	**(14)**	
South Asian	6	43
Middle Eastern	3	21.5
Sub-Saharan African	2	14
Caucasian	3	21.5
Anthropometrics		**Median (range)**
BMI SDS	9/14	0.5(-1.4, 2.4)
Wt SDS	14/14	0.09(-4.5, 2.6)
Vitamin D supplementation	**(14)**	**%**
Prior high dose vitamin D3 supplementation	1	7
Prior multivitamin supplementation	3	21.5
No supplementation	10	71.5
Season of enrolment	**(14)**	**%**
Autumn	6	43
Spring	3	21.5
Summer	3	21.5
Winter	2	14
Reason for referral	**(14)**	**%**
Bone pain	6	43
Bowed leg	4	28.5
Other	4	28.5

7.4.3 Individual changes in cytokines

As previously described, in order to examine the effect of short-term vitamin D3 supplementation on Th1/Th2 cytokine skewing, Th17 cytokine, Treg cytokine and chemokines linking the balance between the Th1 and Th2 subsets, the cytokine profiles from the serum of 14 children was studied at the baseline and following six weeks of vitamin D3 supplementation. Table7-8 and Table 7-9 show the baseline and week six cytokine profiles for the 14 children. Table 7-2 shows the median and median percent changes for each individual's cytokines.

The researcher is not aware of any scientific data specifying what level of change in serum cytokines is considered to be clinically meaningful. An increase in the cytokine serum level from the baseline value was therefore defined as a positive change, while a decrease was defined as a negative change, regardless of magnitude.In order to further compare the effect of the response to vitamin D3 supplementation on circulatory cytokines, children were grouped according to their final serum 25(OH)D, 25(OH)D > 75 and 25(OH)D < 75 nmol/l. Data were represented according to these figures. However, the representation was not found to affect the interpretation of the results.

For the Th1-secreting cytokines, the results showed a borderline trend towards an increase in IL-2 levels from 16.6 pg/ml at baseline to 40.5 pg/ml at week 6 ($p = 0.09$). However, patients' responses to high-dose vitamin D3 were not homogenous. While 10 patients (71.5%) had increased serum IL-2 levels after six weeks, three patients (21.5%) showed decreased IL-2 levels after the same period of high dose supplementation. Due to a technical error in the Luminex machine, the IL-2 value for one patient (7%) was unavailable at week 6 and was excluded from further analysis. No significant changes were noticed in other Th1 secreted cytokines IFN-γ and TNF-α (see figure 7-4).

A significant increase in serum IL-4 from 7.3 pg/ml at baseline to 10.8 pg/ml at week six ($p = 0.001$) was detected. Additionally, there were increases in the other Th2 secreting cytokines (IL-5 and IL-6) after supplementation (18.3 to 24.6 pg/ml; $p = 0.05$ and 19.2 to 30.5 pg/ml; $p = 0.05$), although this was not statistically significant. However, serum IL-4, IL-5 and IL-6 response to high dose vitamin D3 was non-homogenous. Of the 14 patients included in the analysis, one patient (7%) showed an 18% reduction in serum IL-4 value after six weeks of supplementation, while the other 13 patients showed an increase. A total of 11 patients (78.5%) had increased serum IL-5 and IL-6 levels after six weeks. Three

patients (21.5%) had lower IL-5 and IL-6 levels after the same period of high dose supplementation (see figure 7-5).

No significant change was noted in the other cytokines or chemokines related to other Th cell subtype activity and Th1/Th2 balance (see figure 7-6 and see figure 7-7).

A non-significant change in serum chemokines related to Th1 function and activity was noted: RANTES, IP-10, MCP-1 and MIP-1β levels as shown in figure 7-8 see figure 7-9. The serum level of EOTAXIN, a chemokine related to Th2 activity, also showed no significant change after supplementation (see figure 7-10).

Interestingly, one child (Patient ID: 33) had an inconsistent/different response to vitamin D3 supplementation compared with other children. This patient experienced a substantial drop in both pro- and anti-inflammatory cytokines, although there was an increase in IFN-γ and an almost steady IL-4 level. Although the immunological profile for this child had a significant impact on the study results, the child had no coexisting medical/ immunological condition.

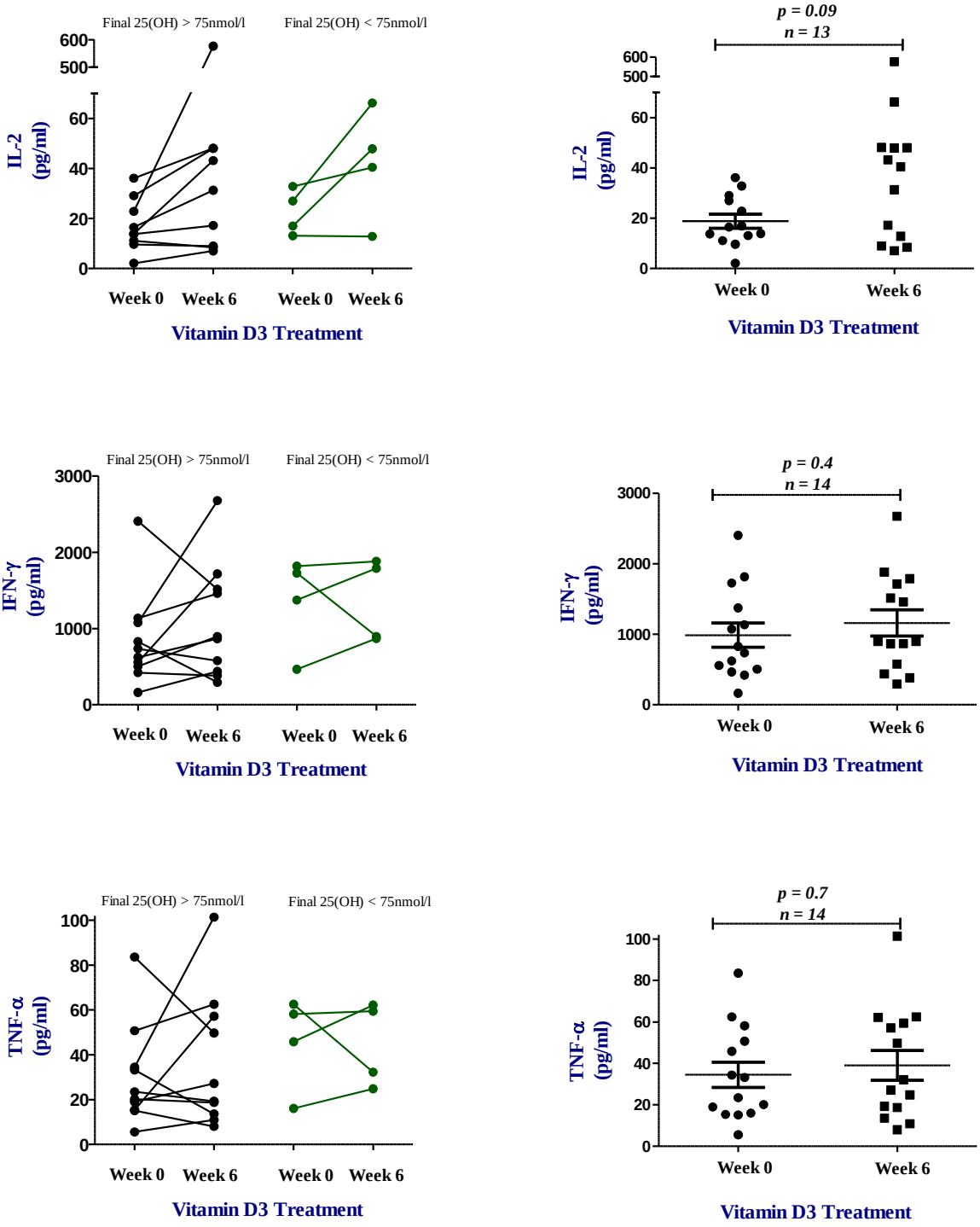


Figure 7-4: T helper 1 (Th1) secreted cytokines: IL-2, IFN-γ and TNF-α.
For illustration purposes, on the line graphs children were divided according to the final 25(OH)D reached. Green lines represent children who were still vitamin D deficient after supplementation; black lines represent children who were vitamin D sufficient after supplementation. Increases in serum IL-2, IFN-γ and TNF-α of study participants after six weeks of supplementation were not significant (p = 0.09, 0.4 and 0.7 respectively).

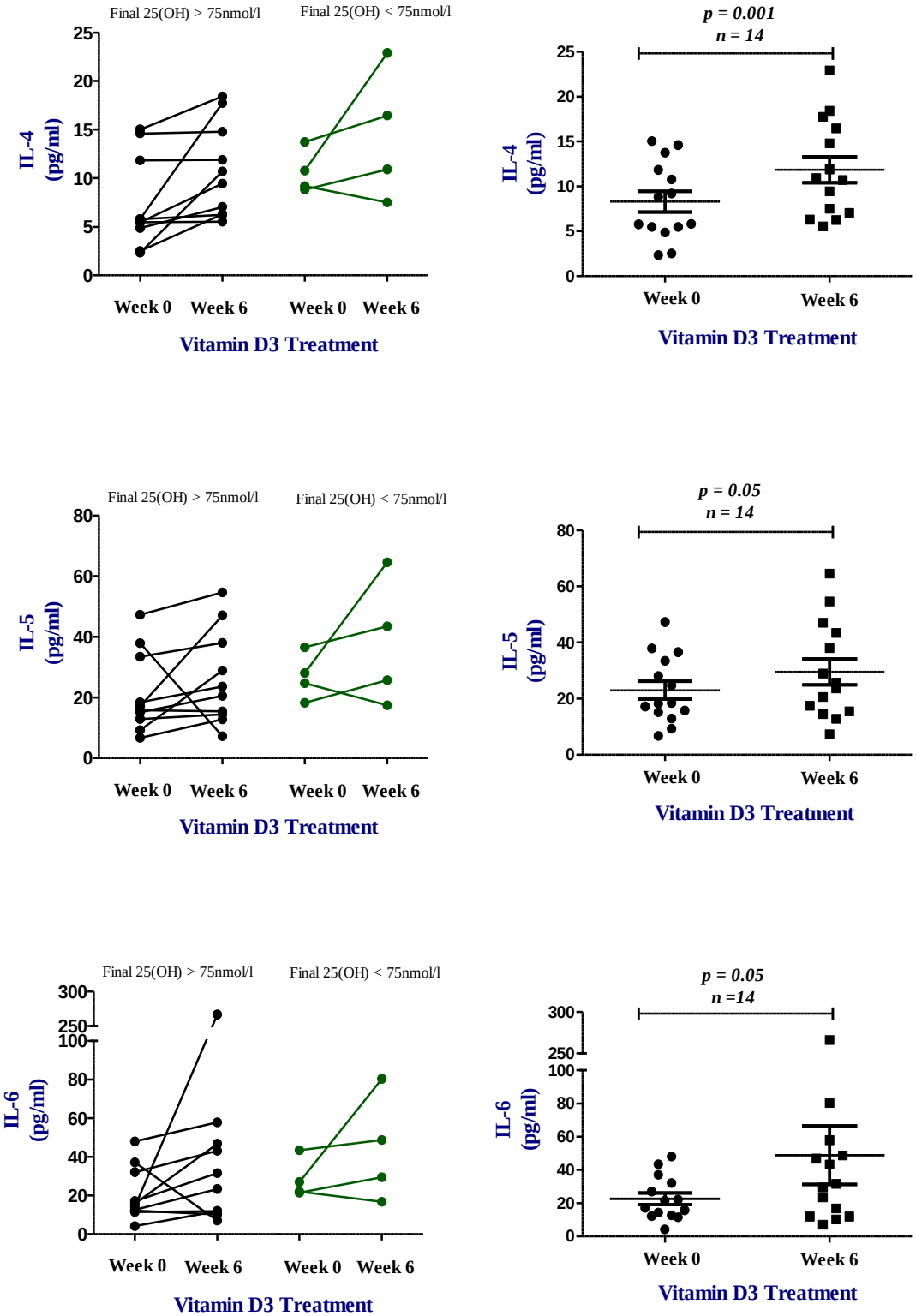


Figure 7-5: T helper 2 secreted cytokines (Th2); IL-4, IL-5 and IL-6.
For illustration purposes, on the line graphs children were divided according to the final 25(OH)D reached. Green lines represent children who were still vitamin D deficient after supplementation; black lines represent children who were vitamin D sufficient after supplementation. Significant increases in serum IL-4 level after six weeks of supplementation was noted (p = 0.001).

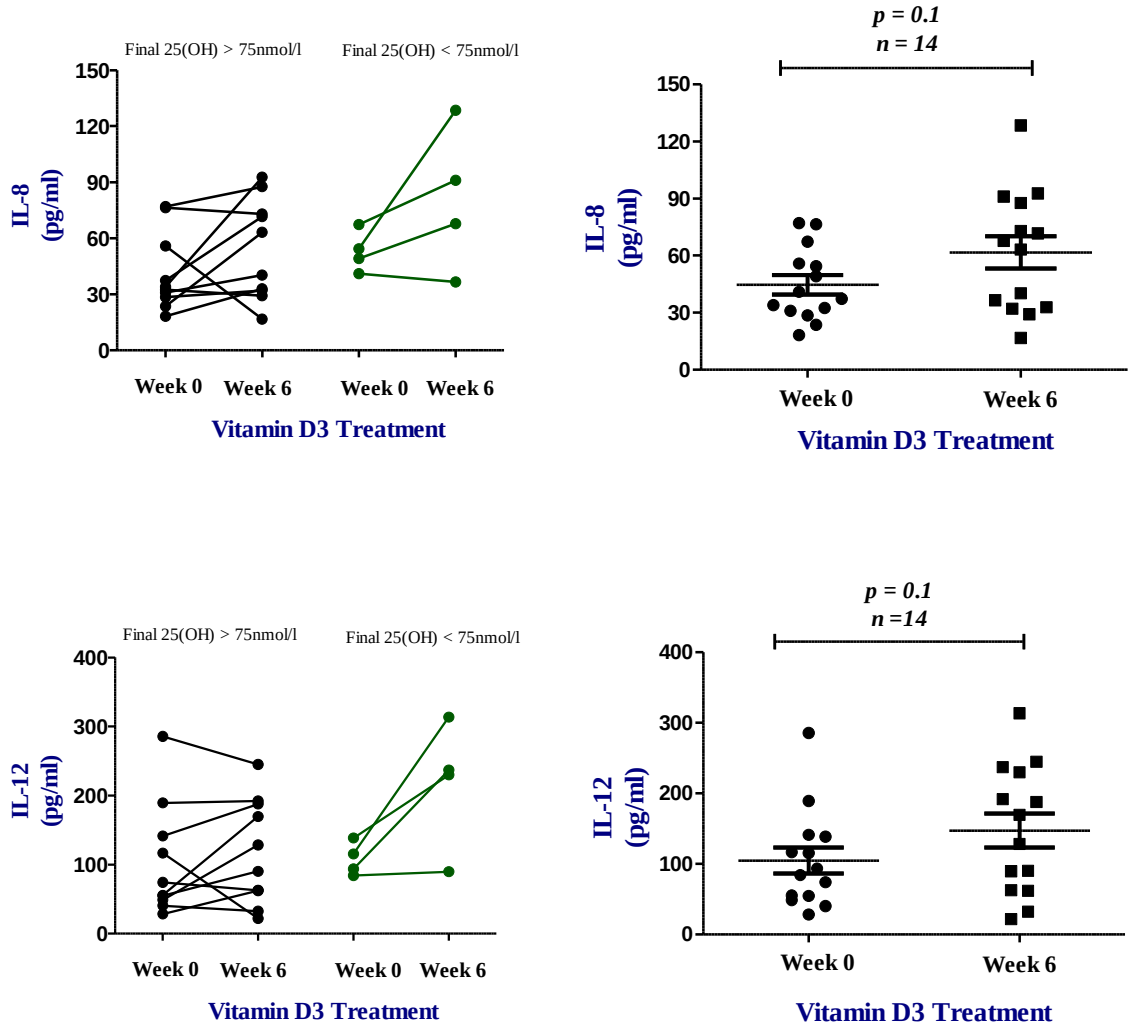


Figure 7-6: Cytokines involved in the development and function of Th1 effector cells IL-8 and IL-12.

For illustration purposes, on the line graphs children were divided according to the final 25(OH)D reached. Green lines represent children who were still vitamin D deficient after supplementation; black lines represent children who were vitamin D sufficient after supplementation. There was no significant change in serum IL-8 and IL-12 levels (p = 0.1 and 0.1 respectively).

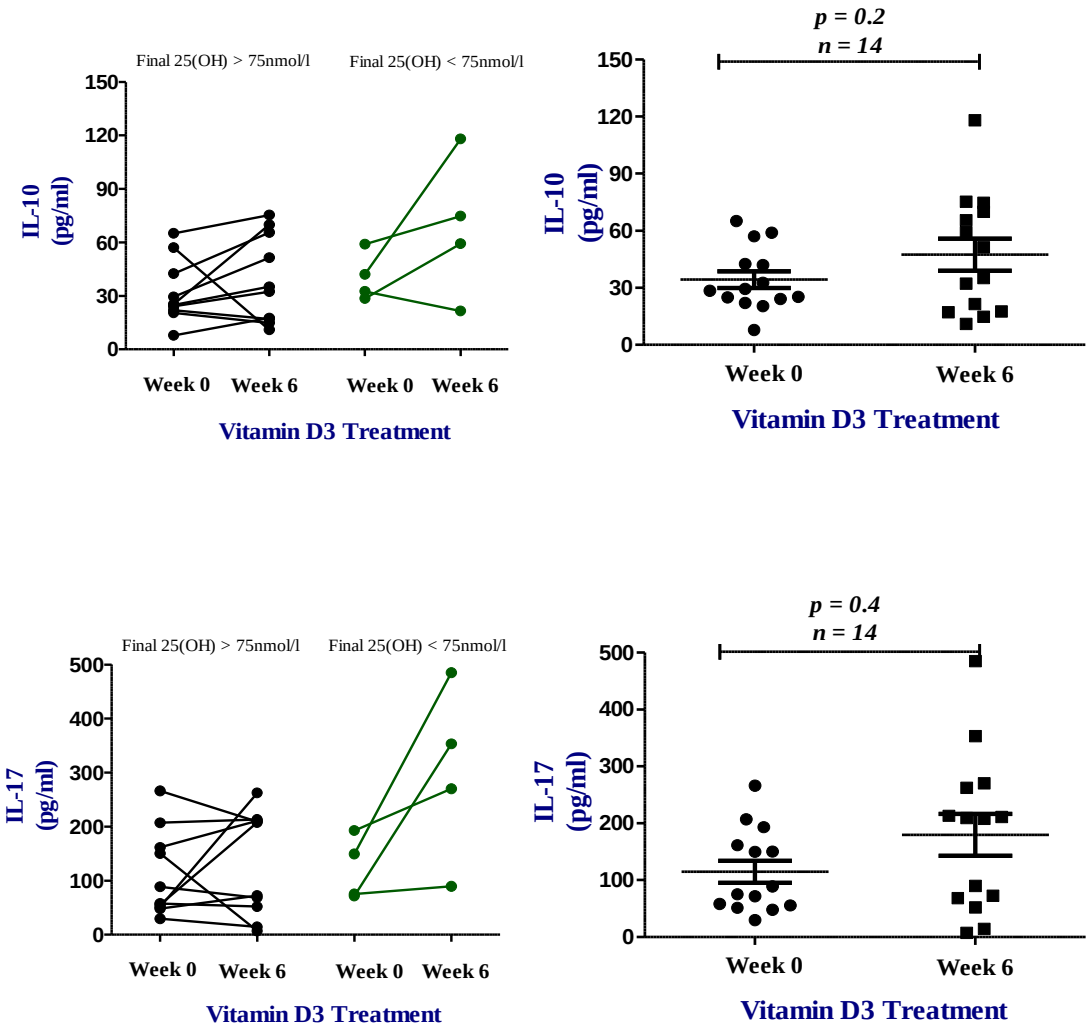


Figure 7-7: Cytokines involved in Th effector cell functions: IL-10 (Th2 and Treg secreted cytokine) and IL-17 (Th17 secreted cytokine).
For illustration purposes, the line graphs represent children divided according to the final 25(OH)D reached. Green lines represent children who were still vitamin D deficient after supplementation; black lines represent children who were vitamin D sufficient after supplementation. No significant change in serum IL-10 or IL-17 levels was noted (p = 0.2 and 0.4 respectively).

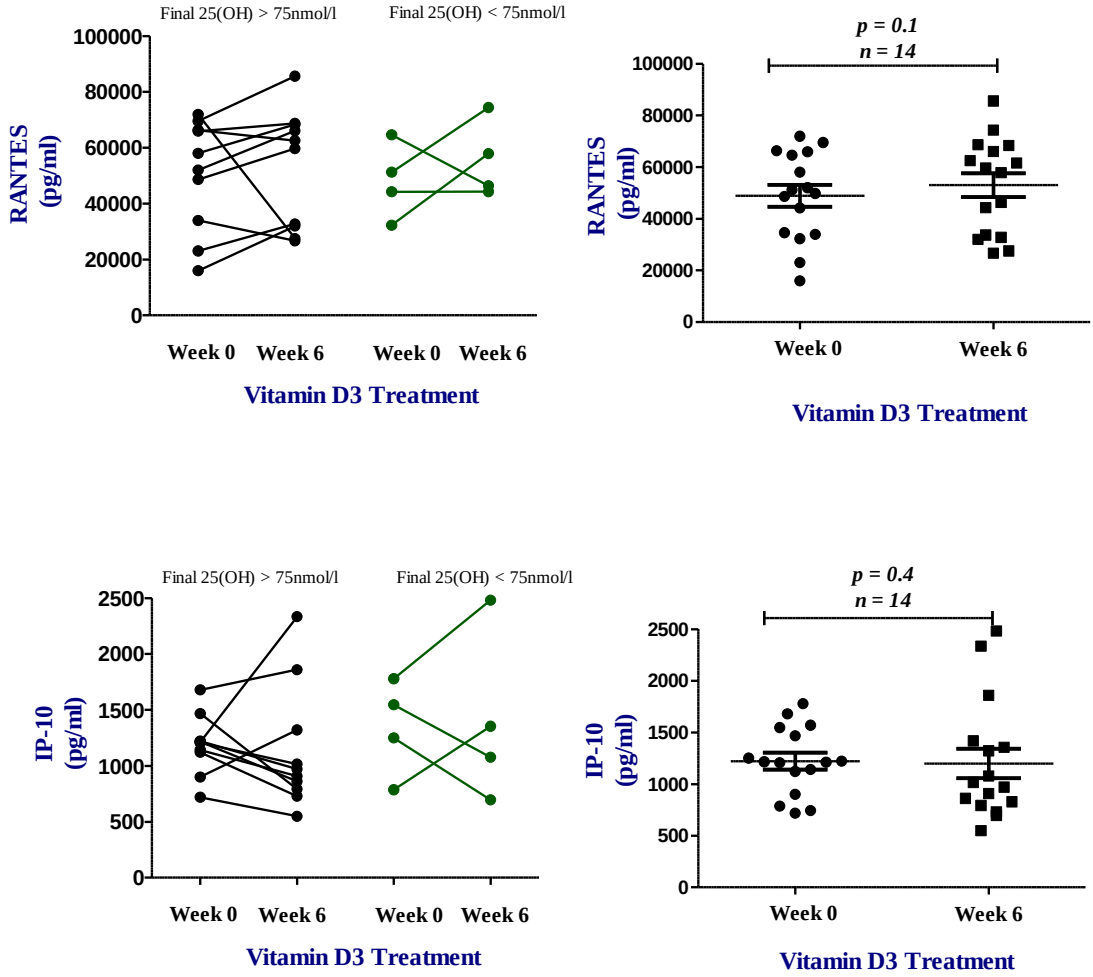


Figure 7-8: Other inflammatory chemokines are RANTES and IP-10 (associated with Th1 tissue recruitment and/or promoting Th1 differentiation).

For illustration purposes, the line graphs show the final 25(OH)D reached. Green lines represent children who were still vitamin D deficient after supplementation; black lines represent children who were vitamin D sufficient after supplementation. No significant change in serum RANTES and IP-10 was noted (p = 0.1 and 0.4 respectively).

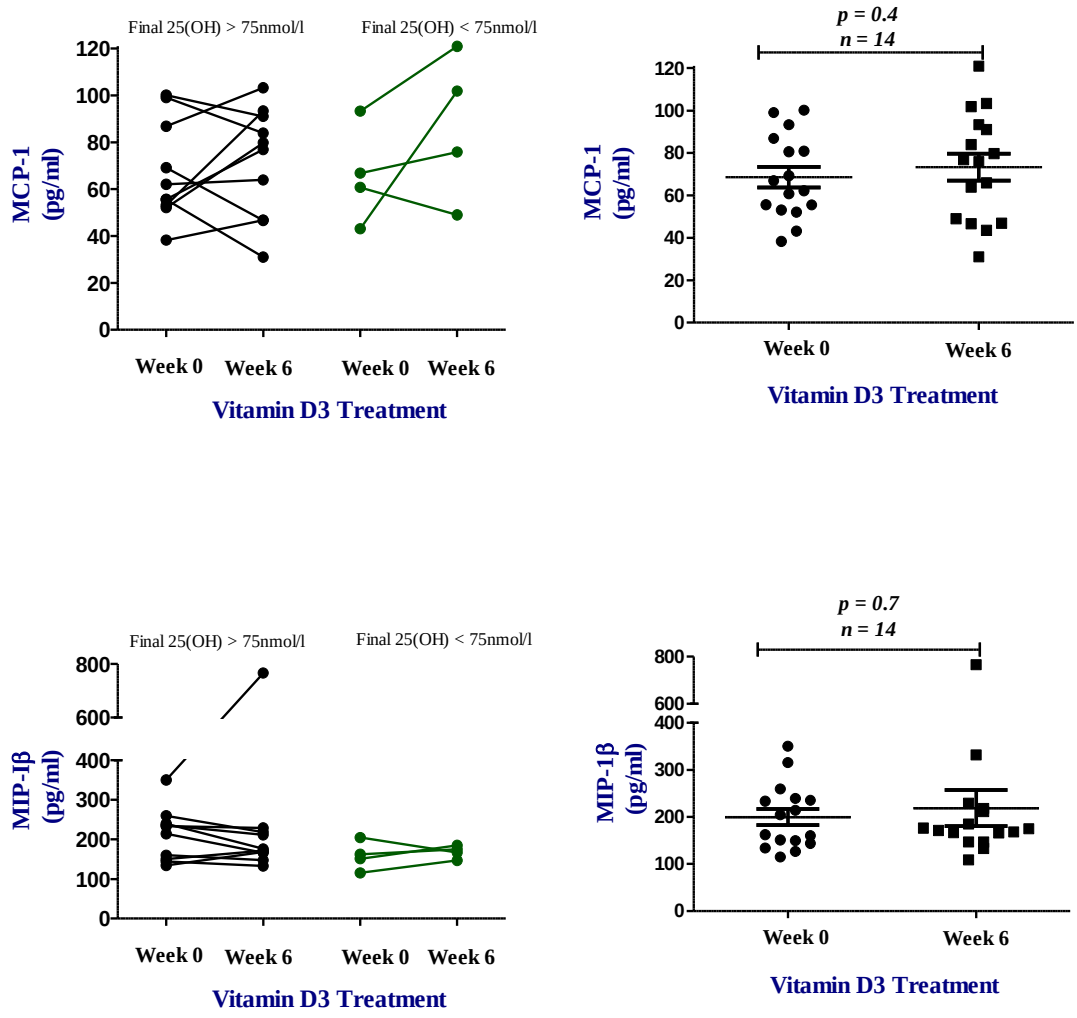


Figure 7-9: Other inflammatory chemokines: MCP-1 and MIP-1β (associated with Th1 tissue recruitment and/or promoting Th1 differentiation).
For illustration purposes, the line graphs show the final 25(OH)D reached. Green lines represent children who were still vitamin D deficient after supplementation; black lines represent children who were vitamin D sufficient after supplementation. There was no significant change in serum MCP-1 and MIP-1β levels (p = 0.4 and 0.7 respectively).

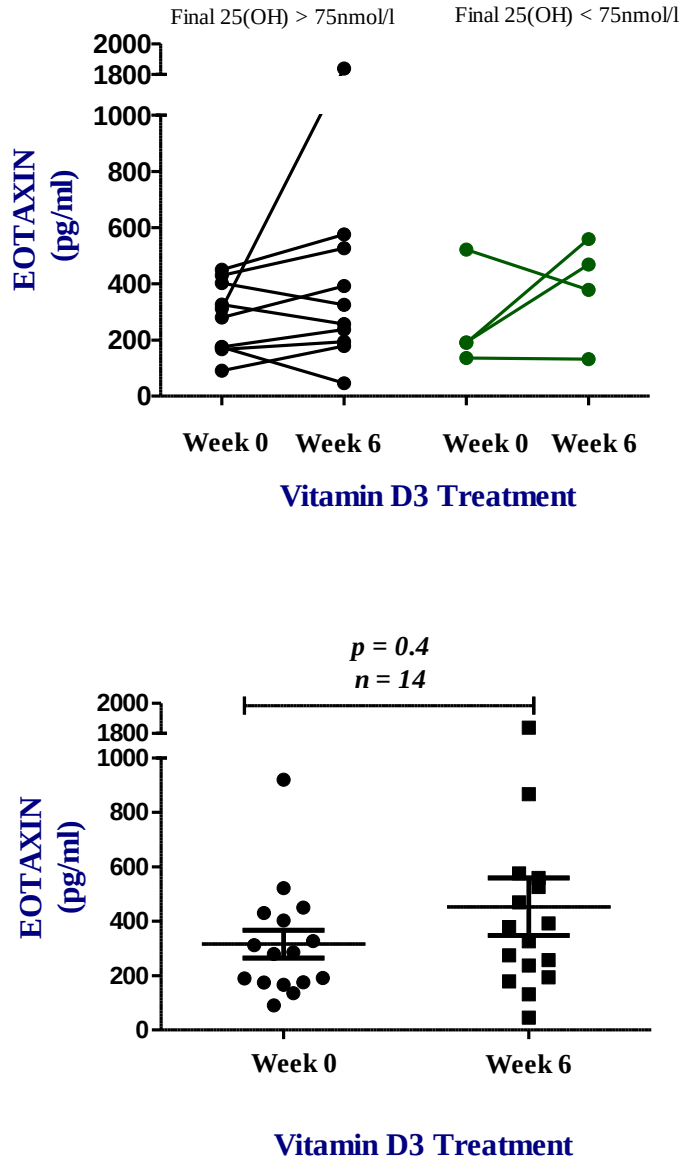


Figure 7-10: Other inflammatory chemokines: EOTAXIN (associated with Th2 tissue recruitment).

For illustration purposes, on the line graphs children were divided according to the final 25(OH)D reached. Green lines represent children who were still vitamin D deficient after supplementation; black lines represent children who were vitamin D sufficient after supplementation. No significant change in serum EOTAXIN was noted (p = 0.4).

Table 7-2: Summary statistics of differences/changes (week 6 – baseline) and percentage changes after six weeks of vitamin D3 supplementation.

Cytokines	Baseline value (pg/ml)	Week six value (pg/ml)	Percent change over six weeks (%)
Th1 secreted cytokines			
IL-2	16.6	40.5	65.2
INF-γ	779.3	895.8	29.4
TNF-α	28.2	29.6	12.7
Th2 secreted cytokines			
IL-4	7.3	10.8	22.9
IL-5	30.5	24.6	23.5
IL-6	19.2	30.5	36.1
Other cytokines/ chemokines			
IL-8	39.1	65.4	32.7
Il-12	88.7	148.9	48.9
IL-10	29.4	43.2	33.7
IL-17	81.9	208.5	25
EOTAXIN	235.8	352.1	25.1
IP-10	1213.8	993	-20.1
MCP-1	61.4	78.3	16.1
MIP-1β	183.6	173.2	-4.7
RANTES	51611.7	58790	20.2

Results are shown as median.

7.4.4 Changes in the lymphocyte subsets before and after vitamin D treatment:

Lymphocyte (T/B cells) and Treg analysis and gating are illustrated in Figure 7-11 and Figure 7-12. No significant changes were seen in the lymphocyte and Treg levels after vitamin D3 treatment (Table 7-3 and Table 7-4). However, an examination of the individual changes in those who responded well to vitamin D treatment shows a general trend towards an increase in both Treg and CD4 levels and a decrease in CD19 (see Figure 7-13).

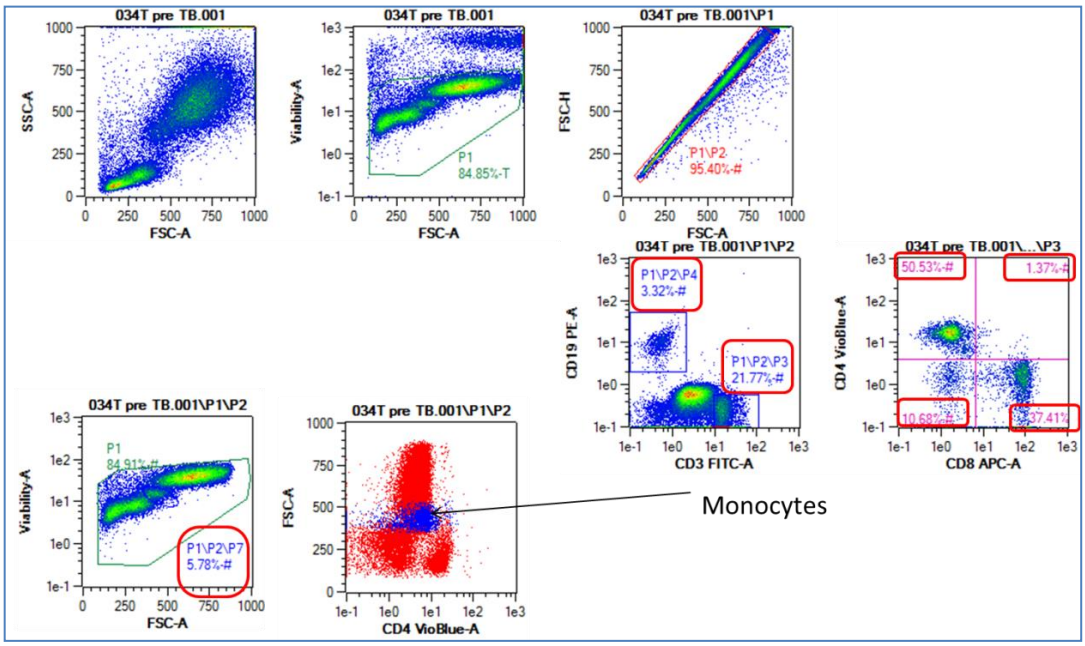


Figure 7-11: Total lymphocyte number and gating using flow cytometry.
Cells were isolated from fresh blood. Lymphocyte analyses (T/B cells) were labelled using CD19-PE, CD3-FITC, CD4-Vioblue and CD8-APC antibodies. Dead cells were labelled using DRAQ7. (Note: flow cytometry analysis was performed Dr Michelle Le Brocq, Institute of Infection, Immunology and Inflammation).

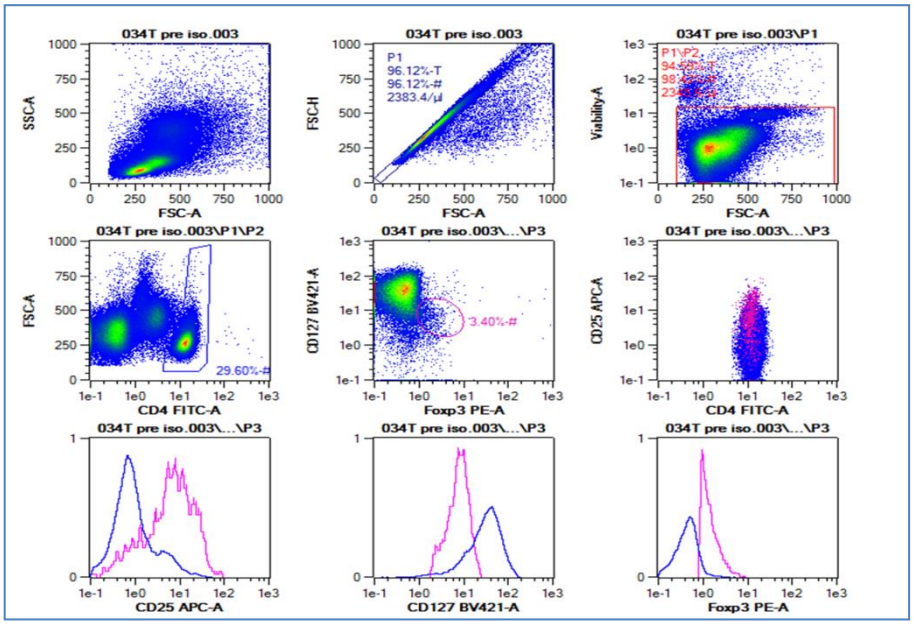


Figure 7-12: Isolation and counting of T regulatory cells (Tregs) using flow cytometry. Cells were isolated from fresh blood and stained with an immunofluorescent for Treg surface markers. (Note: flow cytometry analysis was performed Dr Michelle Le Brocq, Institute of Infection, Immunology and Inflammation).

Table 7-3: Effect of vitamin D3 treatment on peripheral lymphocytes.

Patient ID	Age (months)	Sex	Vitamin D Week 0 (nmol/l)	Vitamin D Week 6 (nmol/l)	Treg Week 0	Treg Week 6	CD19 Week 0	CD19 Week 6	CD3 Week 0	CD3 Week 6	CD4 Week 0	CD4 Week 6	CD8 Week 0	CD8 Week 6
27H	16	Male	27	37	6.5	6.3	16.4	21.5	23.8	36.4	56.8	56.1	33.3	37.2
28H	16	Female	23	117	8.4	10.6	11.6	6.8	23.8	14.2	45.5	48.5	46.7	40.2
29R	19	Male	26	106	13.4	13.3	9.8	6.6	58.2	56.5	66.2	68.2	29.3	26.6
30H	96	Male	49	93	2.9	6.6	4.7	2.5	34.2	21.8	45.2	46.5	41.4	38.6
34T	84	Male	33	122	3.4	5.1	3.3	2.5	21.7	9.2	50.5	52.8	37.4	38.1
Median	**19**		**27**	**106**	**6.4**	**6.5**	**8.5**	**5.71**	**23.8**	**24.9**	**53.6**	**54.5**	**35.3**	**37.6**
***P*-Value (wk 0 vs wk 6)**			**0.05**		**0.3**		**0.6**		**0.6**		**0.1**		**0.6**	

Table 7-4: Effect of vitamin D3 treatment in % change of peripheral lymphocytes.

Patient ID	Age (months)	Sex	% change vitamin D over six weeks	% change Treg over six weeks	% change CD19 over six weeks	% change CD3 over six weeks	% change CD4 over six weeks	% change CD8 over six weeks
27H	16	Male	37	-2.9	31.3	53.1	98.9	11.7
28H	16	Female	408	26.6	-41.3	-40	106.5	-13.9
29R	19	Male	307.6	-0.5	-32.9	-2.9	103	-8.9
30H	96	Male	89.7	121.8	-46.3	-36.2	102.8	-6.6
34T	84	Male	89.7	121.8	-46.3	-36.2	102.8	-6.6
Median	**19**		**269.6**	**13.3**	**-32.8**	**-19.6**	**103.7**	**-2.3**

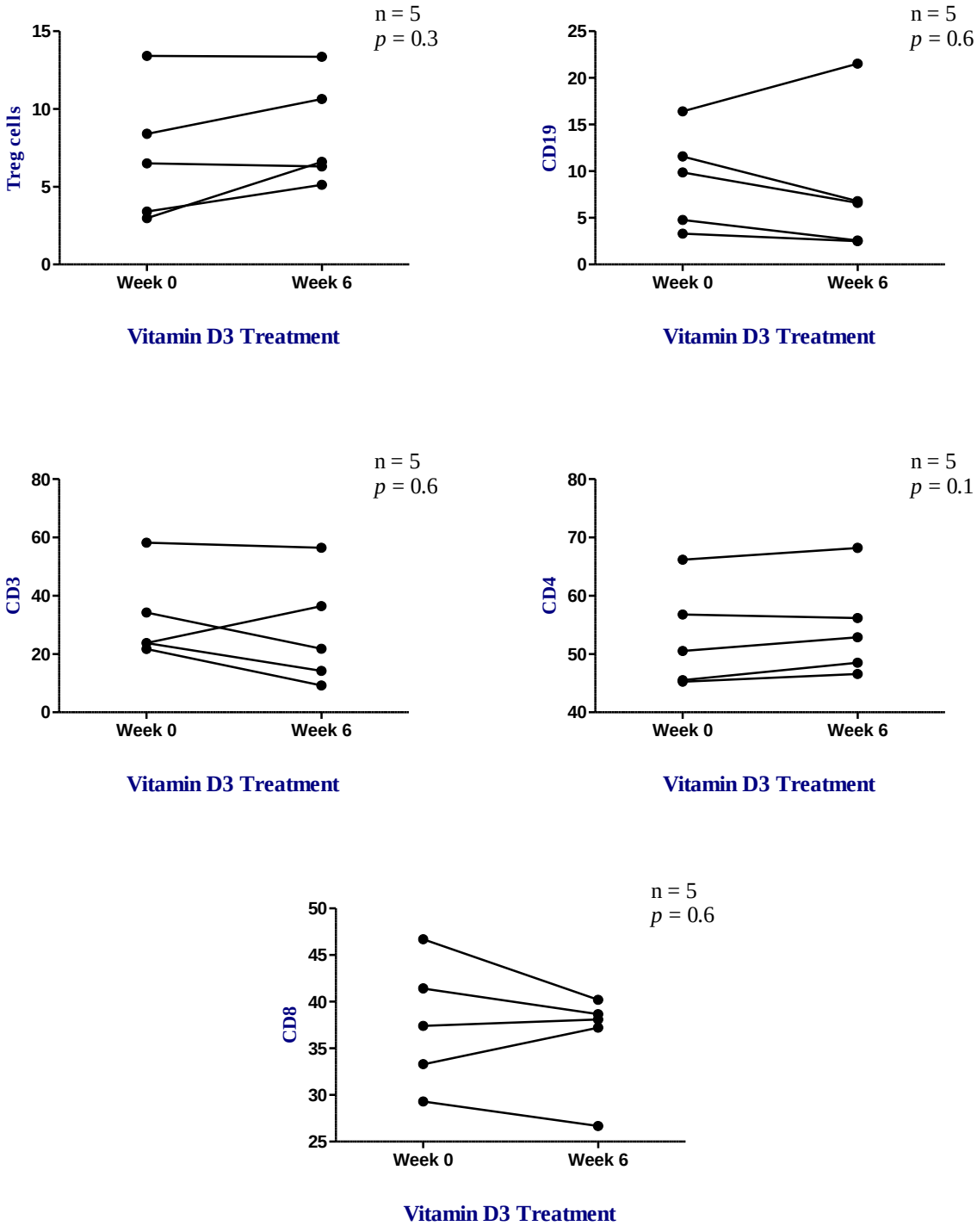


Figure 7-13: Individual change in peripheral lymphocytes following vitamin D3 supplementation from baseline to week six.

7.4.5 Cytokines and bone remodelling

The regulatory effects of vitamin D on the immune system and bone remodelling (as described in the introduction of this thesis) and the suggested role of cytokines as significant mediators of bone remodelling and bone cell metabolism may therefore directly and/or indirectly affect vitamin D function in vitamin D deficient children. This section examines the association between vitamin D, cytokines and bone remodelling markers for the 14 participants at the baseline and following six weeks of vitamin D3 supplementation.

7.4.5.1 Individual changes in bone remodelling markers

Individual details of markers of bone remodelling in the 14 participants included in the immunity study are summarised in Table 7-10. In these participants, median (range) serum 25(OH)D increased significantly after vitamin D3 supplementation from 24.5 (14, 49) nmol/l at the baseline to 103 (37, 191) nmol/l after six weeks ($p = 0.0001$). Serum PTH concentrations also decreased significantly from a median of 7.2 (3.6, 134) pmol/l at the baseline to 4.3 (2.7, 6.8) pmol/l after six weeks ($p = 0.006$) and serum ALP decreased significantly from a median of 238 (99.7, 2834) to 210 (64.9, 729) u/l, $p = 0.03$. Additionally, serum C- terminal telopeptide of type I collagen (CTX) decreased from a median of 2.2 (1.3, 3.1) ng/ml to 1.7 (0.8, 3.03) ng/ml. However, this reduction was not statistically significant ($p = 0.1$).

7.4.5.2 Association of bone remodelling markers and cytokines

Univariate correlation analysis at the week six point showed that 25(OH)D was significantly correlated only with INF-γ, ($p = 0.03$; r = -0.5), whereas no correlation was found with any cytokines at the baseline. Parathyroid hormone (PTH) was significantly correlated with IL-17 and IP-10 at baseline ($p = 0.02$; r = -0.5, $p = 0.01$; r = 0.6 respectively), and with Eotaxin at week six ($p = 0.003$; r = −0.7). C-terminal telopeptide of type I collagen (CTX) was found to be significantly correlated with IL-2, IL-5, IL-6, IL-8, IL-12, IL-10 and IL-17, ($p = 0.02$; r = -0.2, $p = 0.02$; r = -0.6, $p = 0.04$; r = -0.5, $p = 0.03$; r = -0.6, p = 0.01; r = -0.6, p = 0.04; r = -0.6, p = 0.01; r = -0.7 respectively) at the baseline. Finally, ALP was significantly correlated with IL-4, IL-5, IL-8 and IL-17, (p = 0.01; r = -0.6, p = 0.04; r = -0.5, p = 0.03; r = -0.6, p = 0.04; r = -0.5 respectively) at the baseline and with IL-2, IL-4, IL-5, IL-6, IL-8, IL-12, IL-10 and IL-17,(p = 0.02; r = -0.6, p = 0.01; r = -0.7, p = 0.001; r = -0.8, p = 0.001; r = -0.8, p = 0.0001; r = -0.8, P = 0.006; r =

-0.7, p = 0.002; r = 0.7, p = 0.02; r = -0.6 respectively) at week six (Table 7-5; Figures from 7-14 to 7-21). After Bonferroni adjustment , IL-5, IL-6 and IL-8 were only significantly correlated with ALP

Table 7-5: Association of bone remodelling markers and cytokines.

Baseline	**25 (OH)D (n = 14)**	**PTH (n = 14)**	**CTX (n = 12)**	**ALP (n = 12)**
		Correlation		
Th1 cytokines	**r (*p* value)**	**r (*p* value)**	**r (*p* value)**	**r (*p* value)**
IL-2	-0.1(0.5)	-0.1 (0.5)	-0.2(0.02)	-0.5 (0.07)
INF-γ	-0.1(0.5)	0.3(0.2)	-0.04(0.8)	0.03 (0.9)
TNF-α	-0. 2(0.3)	0.3(0.1)	-0.04(0.8)	0.1(0.7)
Th2 cytokines	**r (*p* value)**	**r (*p* value)**	**r (*p* value)**	**r (*p* value)**
IL-4	0.07(0.8)	-0.4(0.1)	-0.5(0.07)	-0.6(0.01)
IL-5	-0.2(0.4)	-0.3(0.1)	-0.6(0.02)	-0.5(0.04)
IL-6	-0.2(0.3)	-0.1(0.5)	-0.5(0.04)	-0.4(0.1)
Other chemokines	**r (*p* value)**	**r (*p* value)**	**r (*p* value)**	**r (*p* value)**
IL-8	-0.08(0.7)	-0.3(0.2)	-0.6(0.03)	-0.6(0.03)
IL-12	-0.2(0.4)	-0.4(0.07)	-0.6(0.01)	-0.5(0.06)
IL-10	-0.3(0.2)	-0.1(0.7)	-0.6(0.04)	-0.4(0.1)
IL-17	-0.1(0.6)	-0.5(0.02)	-0.7(0.01)	-0.5(0.04)
EOTAXIN	0.2(0.3)	-0.4(0.1)	-0.3 (0.2)	-0.5(0.07)
IP-10	-0.4(0.1)	0.6(0.01)	0.5(0.09)	0.4(0.1)
MCP-1	0.2(0.4)	-0.2(0.4)	-0.2(0.4)	-0.2(0.4)
MIP-1β	-0.07(0.7)	0.2(0.4)	-0.1(0.6)	0.1(0.6)
RANTES	-0.1(0.6)	-0.1(0.6)	-0.4(0.1)	-0.2(0.4)
Week six	**25 (OH)D (n = 14)**	**PTH (n = 12)**	**CTX (n = 12)**	**ALP (n = 12)**
		Correlation		
Th1 cytokines	**r (*p* value)**	**r (*p* value)**	**r (*p* value)**	**r (*p* value)**
IL-2	-0.08(0.7)	-0.2(0.5)	-0.2(0.4)	-0.6(0.02)
INF-γ	-0.5(0.03)	0.5(0.06)	-0.09(0.7)	0.09(0.7)
TNF-α	-0.4(0.1)	0.4(0.1)	-0.1(0.6)	0.1(0.6)
Th2 cytokines	**r (*p* value)**	**r (*p* value)**	**r (*p* value)**	**r (*p* value)**
IL-4	-0.2(0.4)	0.004(0.9)	-0.4(0.1)	-0.7(0.01)
IL-5	-0.1(0.5)	-0.07(0.8)	-0.4(0.1)	-0.8(**0.001***)
IL-6	0.004(0.9)	-0.2(0.4)	-0.3(0.3)	-0.8(**0.001***)
Other cytokines/ chemokines	**r (*p* value)**	**r (*p* value)**	**r (*p* value)**	**r (*p* value)**
IL-8	-0.2(0.3)	-0.04(0.8)	-0.2(0.3)	-0.8(**0.0001***)
IL-12	-0.2(0.2)	-0.1 (0.6)	-0.3(0.3)	-0.7(0.006)
IL-10	-0.2(0.3)	-0.06(0.8)	-0.4(0.1)	0.7(0.002)
IL-17	-0.3(0.1)	0.06(0.8)	-0.4 (0.1)	-0.6(0.02)
EOTAXIN	0.04(0.8)	-0.7(0.003)	0.01(0.9)	-0.4(0.1)
IP-10	-0.1(0.5)	0.1(0.6)	0.05(0.8)	-0.02(0.9)
MCP-1	-0.2(0.3)	-0.2(0.3)	-0.06(0.8)	-0.5(0.08)
MIP-1β	0.3(0.2)	0.2(0.3)	-0.1(0.6)	0.1(0.7)
RANTES	0.1(0.5)	-0.02(0.9)	-0.09(0.7)	-0.1(0.5)

* IL-5, IL-6 and IL-8 were significantly correlated with ALP
*Significance of P<0.001 after Bonferroni adjustment

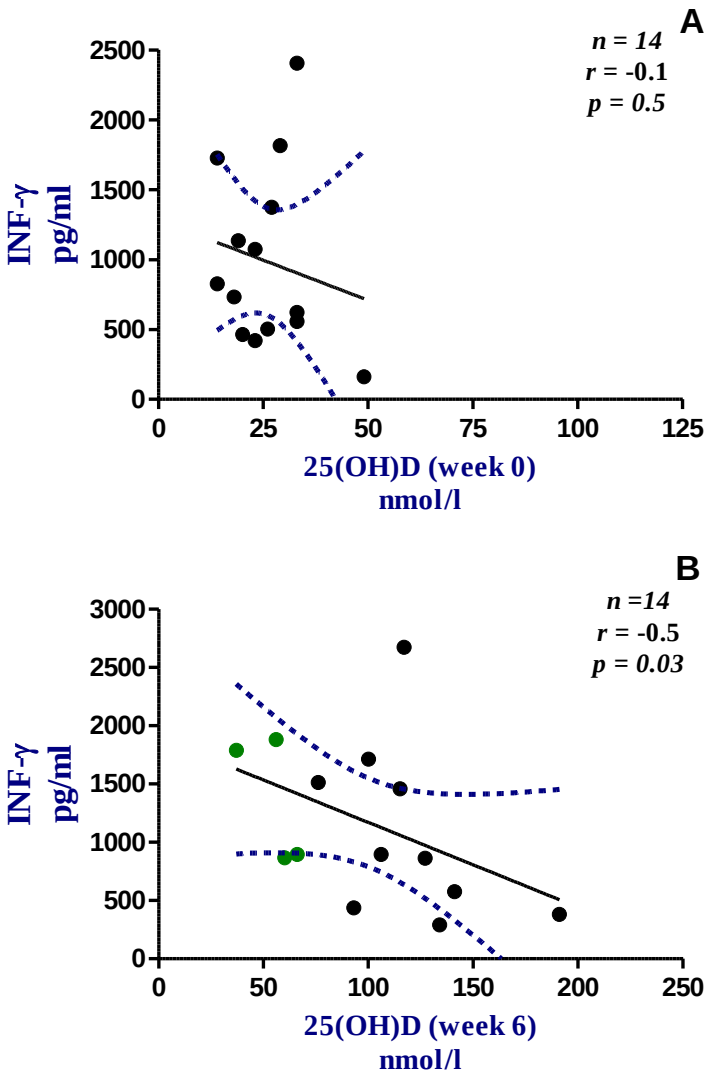


Figure 7-14: Relationship between serum 25(OH)D and INF-γ in 14 children presenting with vitamin D deficiency at baseline (A) and after six weeks (B) of high dosage vitamin D3 supplementation.

The green dots in graph B represent children who remained vitamin D deficient after six weeks. A significant association was found at week six (p = 0.03; r = -0.5).

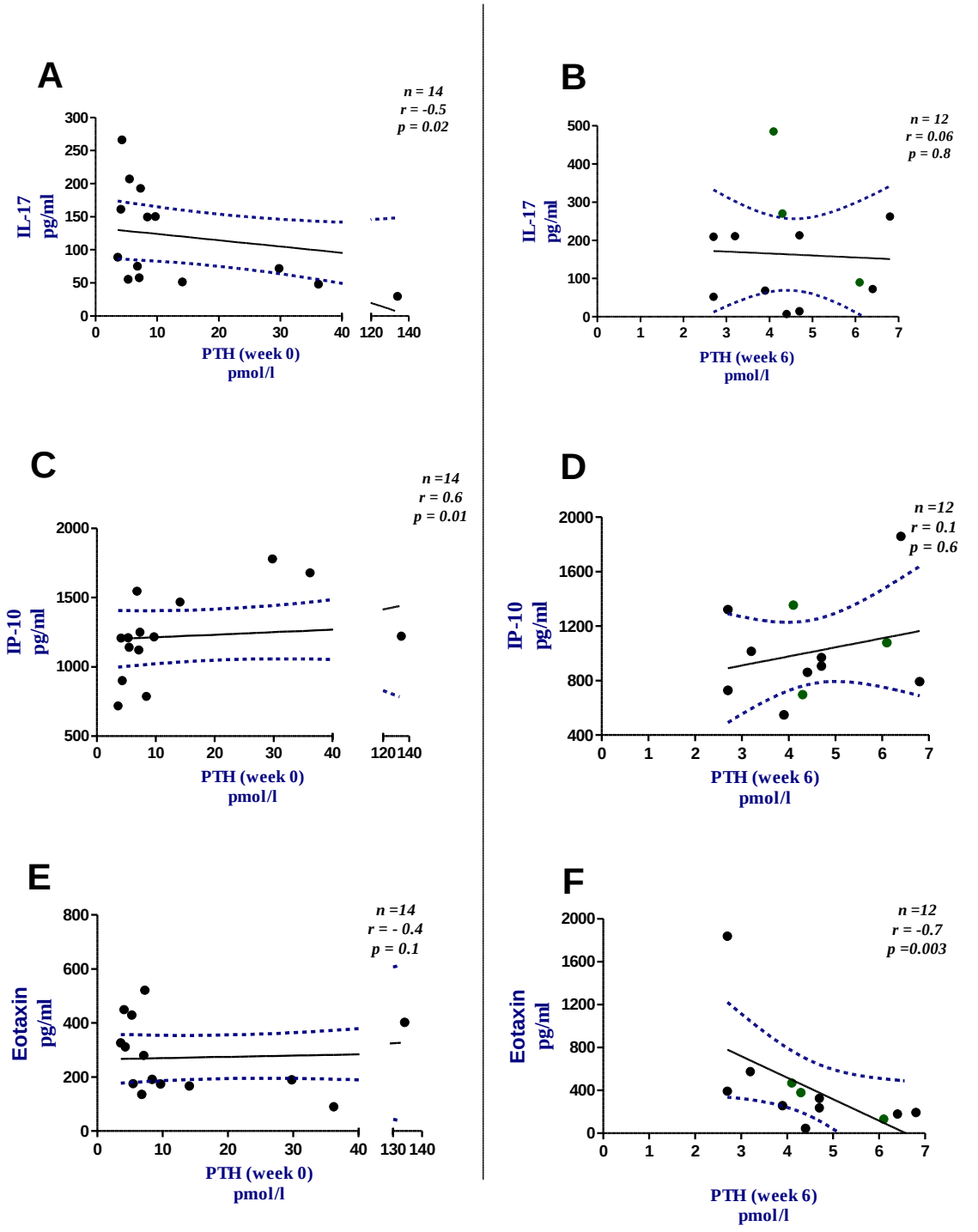


Figure 7-15: Relationship between serum PTH and IL-17, IP-10 and Eotaxin in 14 children presenting with vitamin D deficiency at the baseline (A,C and E) and in 12 children after six weeks (B,D and F) of a high dosage of vitamin D3 supplementation.
The green dots in the right-hand side panel represent children who remained vitamin D deficient after six weeks. A significant association was found at week 0 between PTH and both IL-17(p = 0.02; r = -0.5) and IP-10 (p = 0.01; r = 0.6), and at week six between PTH and Eotaxin (p = 0.003; r = -0.7).

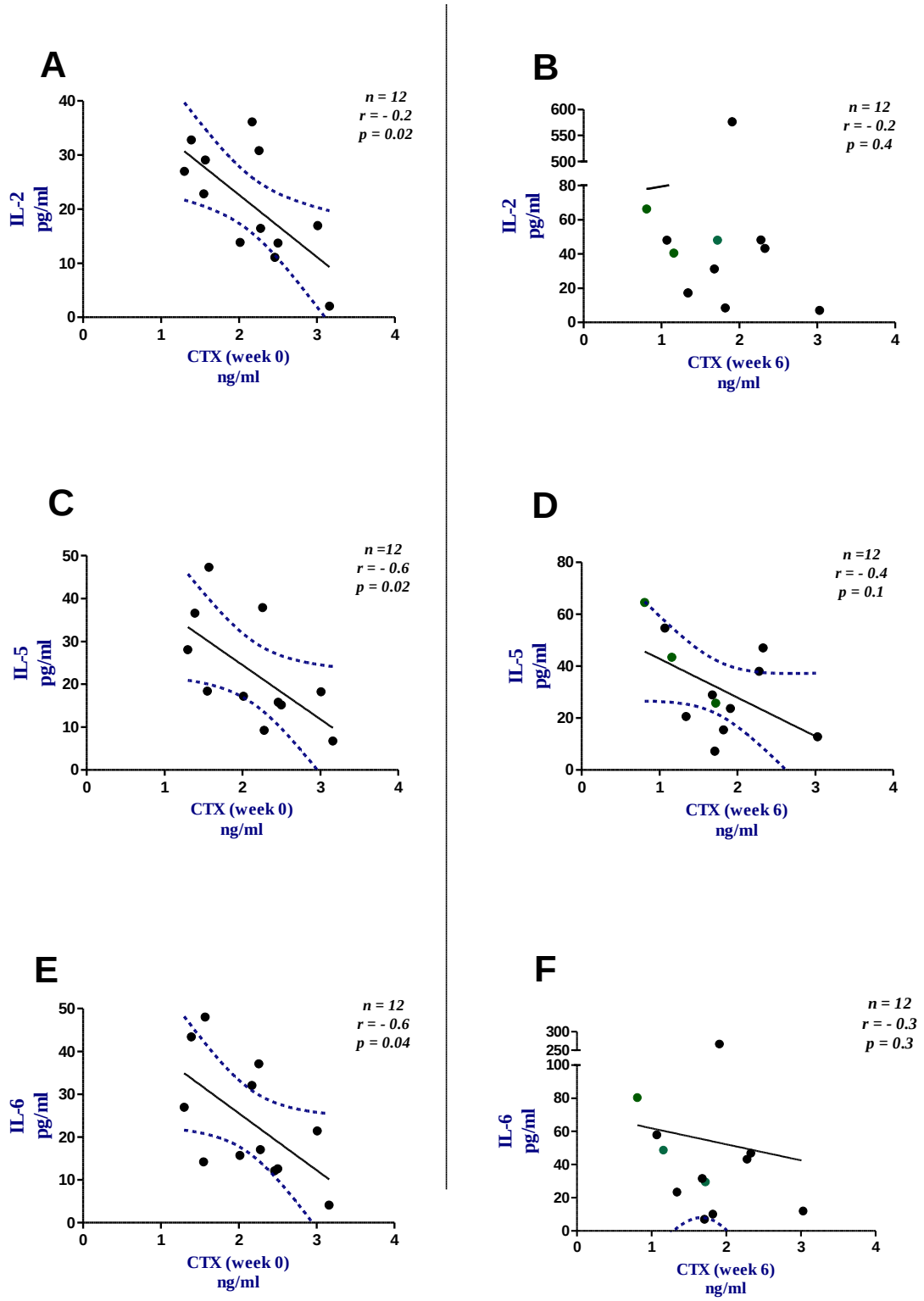


Figure 7-16: Relationship between serum CTX and IL-2, IL-5 and IL-6 in 12 children presenting with vitamin D deficiency at the baseline (A,C and E) and after six weeks (B,D and F) of high dosage vitamin D3 supplementation.

The green dots on the right-hand side panel represent children who remained vitamin D deficient after six weeks. A significant association was found at week 0 between CTX and IL-2 (p = 0.02; r = -0.2) and IL-5 (p = 0.02; r = -0.6), and IL-6 (p = 0.04; r = -0.6).

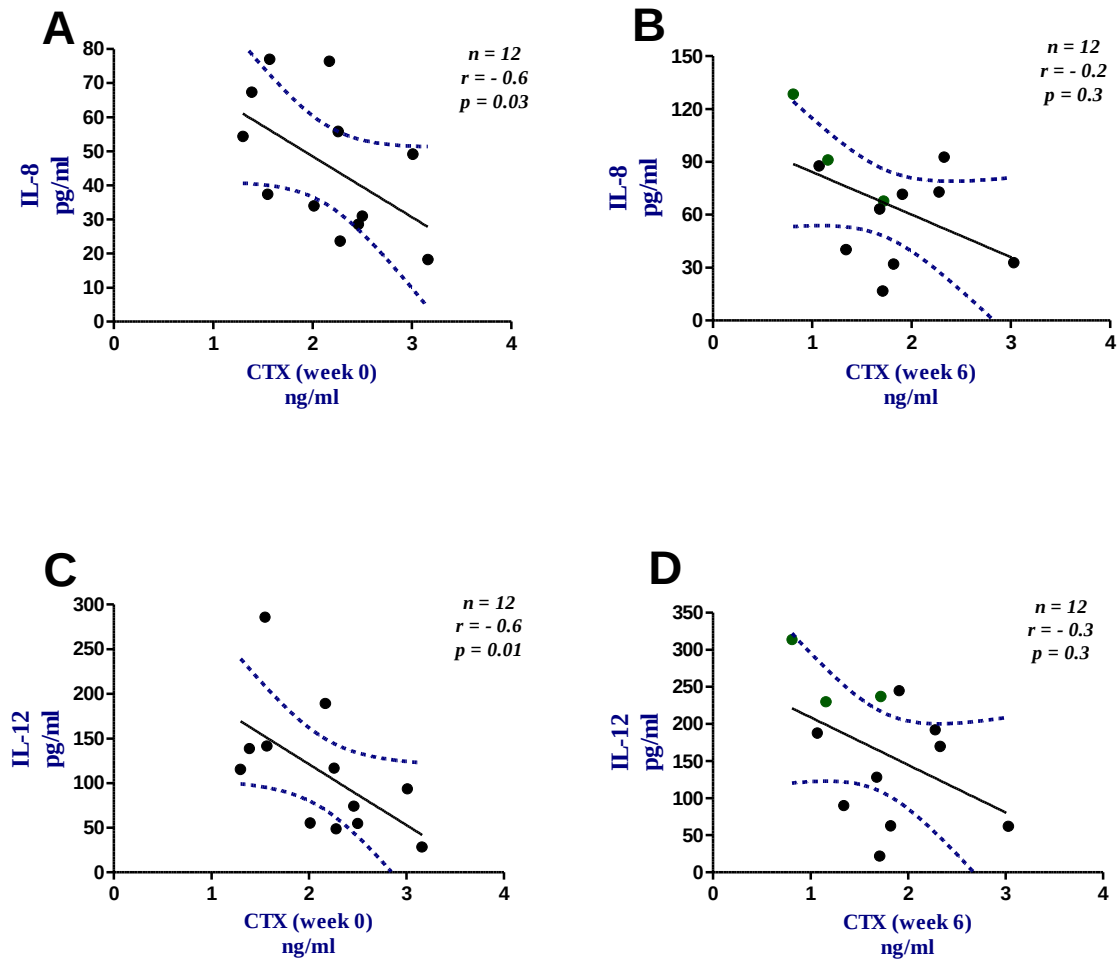


Figure 7-17: Relationship between serum CTX and both IL-8 and IL-12 in 12 children presenting with vitamin D deficiency at the baseline (A and C) and after six weeks (B and D) of high dosage vitamin D3 supplementation.

The green dots on the right-hand side panel represent children who remained vitamin D deficient after six weeks. A significant association was found at week 0 between CTX and IL-8 (p = 0.03; r = -0.6) and between CTX and IL-12 (p = 0.01; r = -0.6).

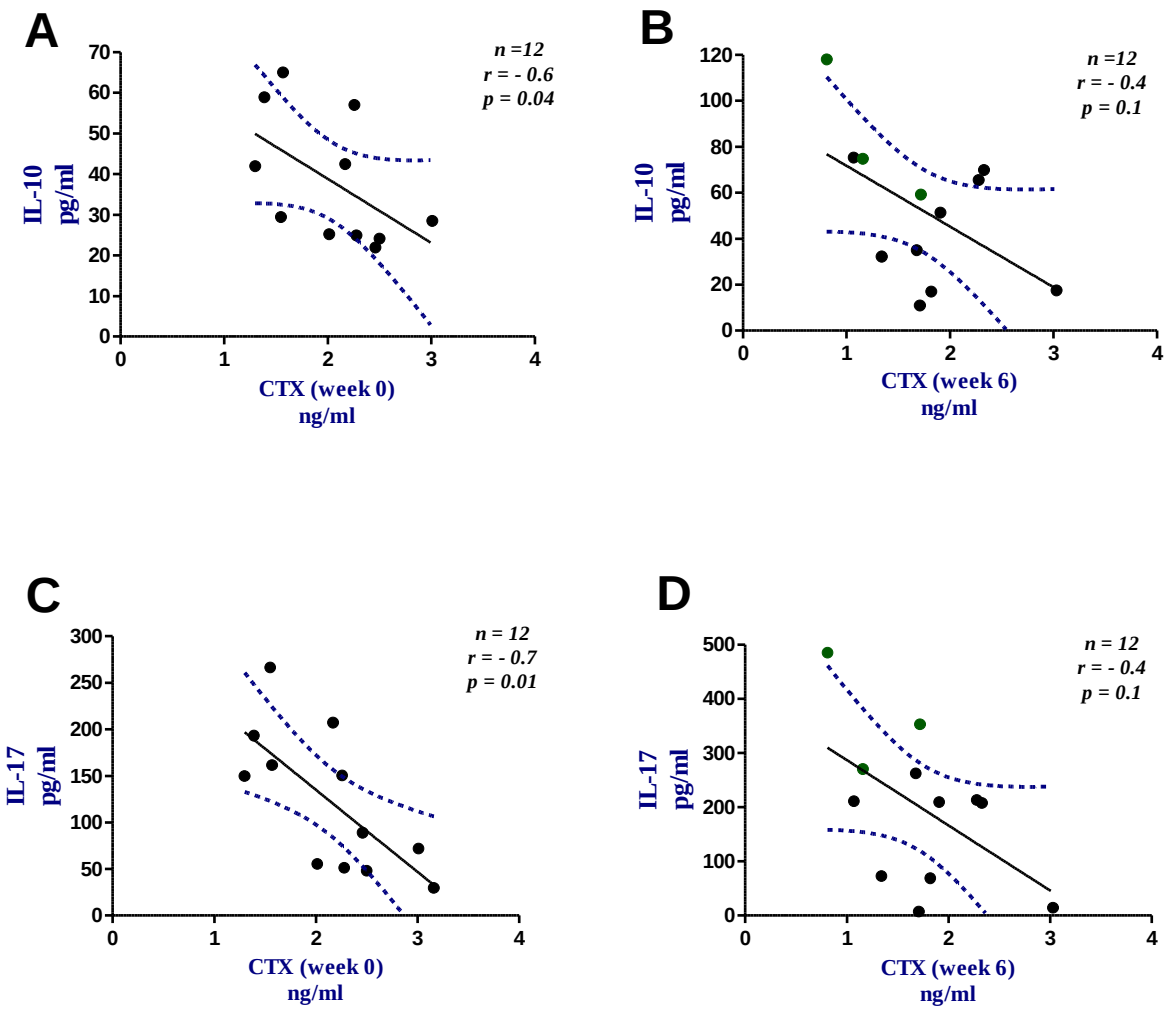


Figure 7-18: Relationship between serum CTX and both IL-10 and IL-17 in 12 children presenting with vitamin D deficiency at the baseline (A and C) and after six weeks (B and D) of high dosage of vitamin D3 supplementation.

The green dots on the right-hand side panel represent children who remained vitamin D deficient after six weeks. A significant association was found at 0 weeks between CTX and IL-10 (p = 0.04; r = -0.6) and between CTX and IL-17 (p = 0.01; r = -0.7).

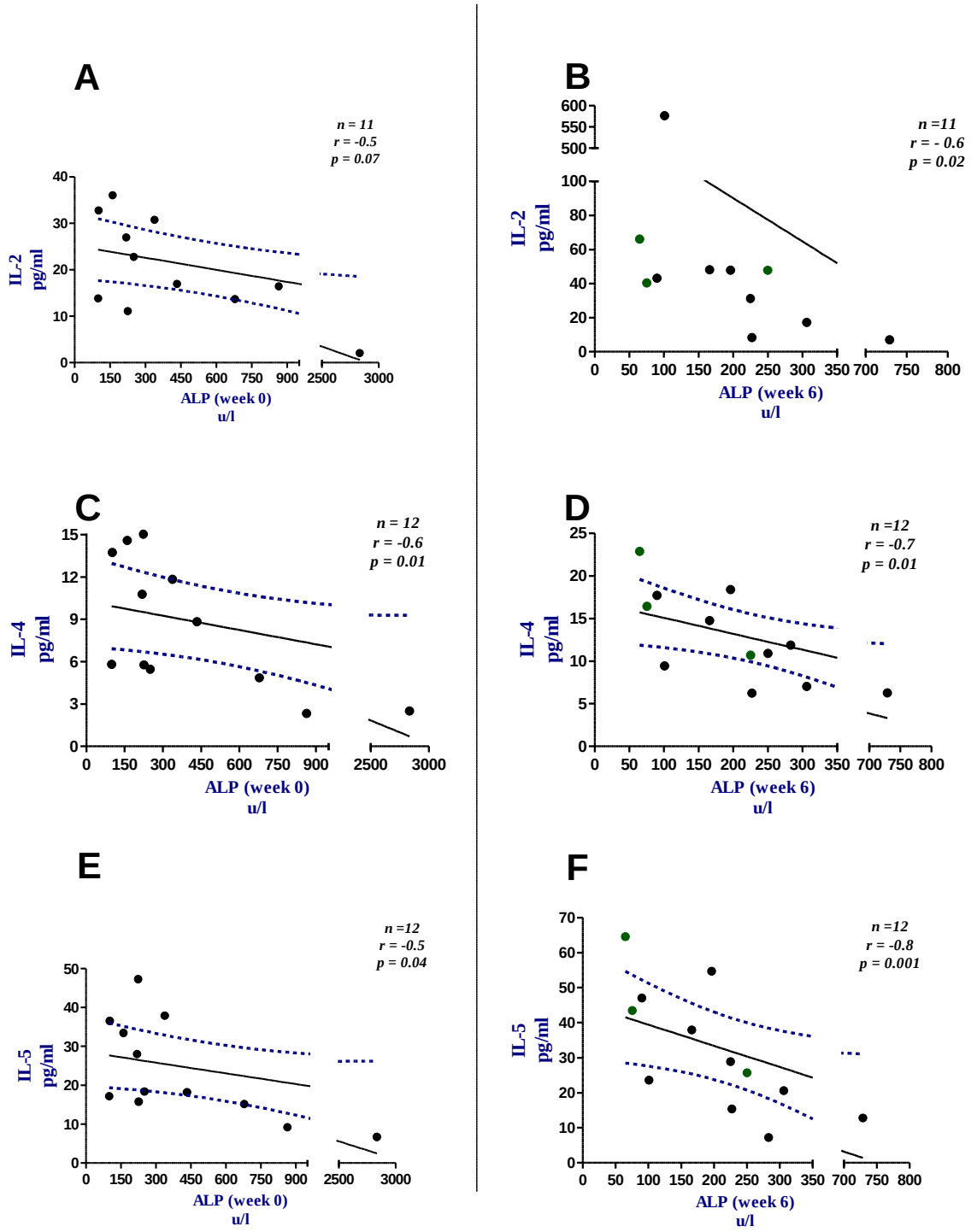


Figure 7-19: Relationship between serum ALP and IL-2, IL-4 and IL-5 in 12 children presenting with vitamin D deficiency at the baseline (A,C and E) and after six weeks (B,D and F) of high dosage vitamin D3 supplementation.

The green dots on the right-hand side panel represent children who remained vitamin D deficient after six weeks. A significant association was found at week 0 between ALP and IL-4 (p = 0.01; r = -0.6) and IL-5 (p = 0.04; r = -0.5). At week six, a significant association was found between ALP and IL-2 (p = 0.02; r = -0.6), IL-4 (p = 0.01; r = -0.7) and IL-5 (p = 0.001; r = -0.8).

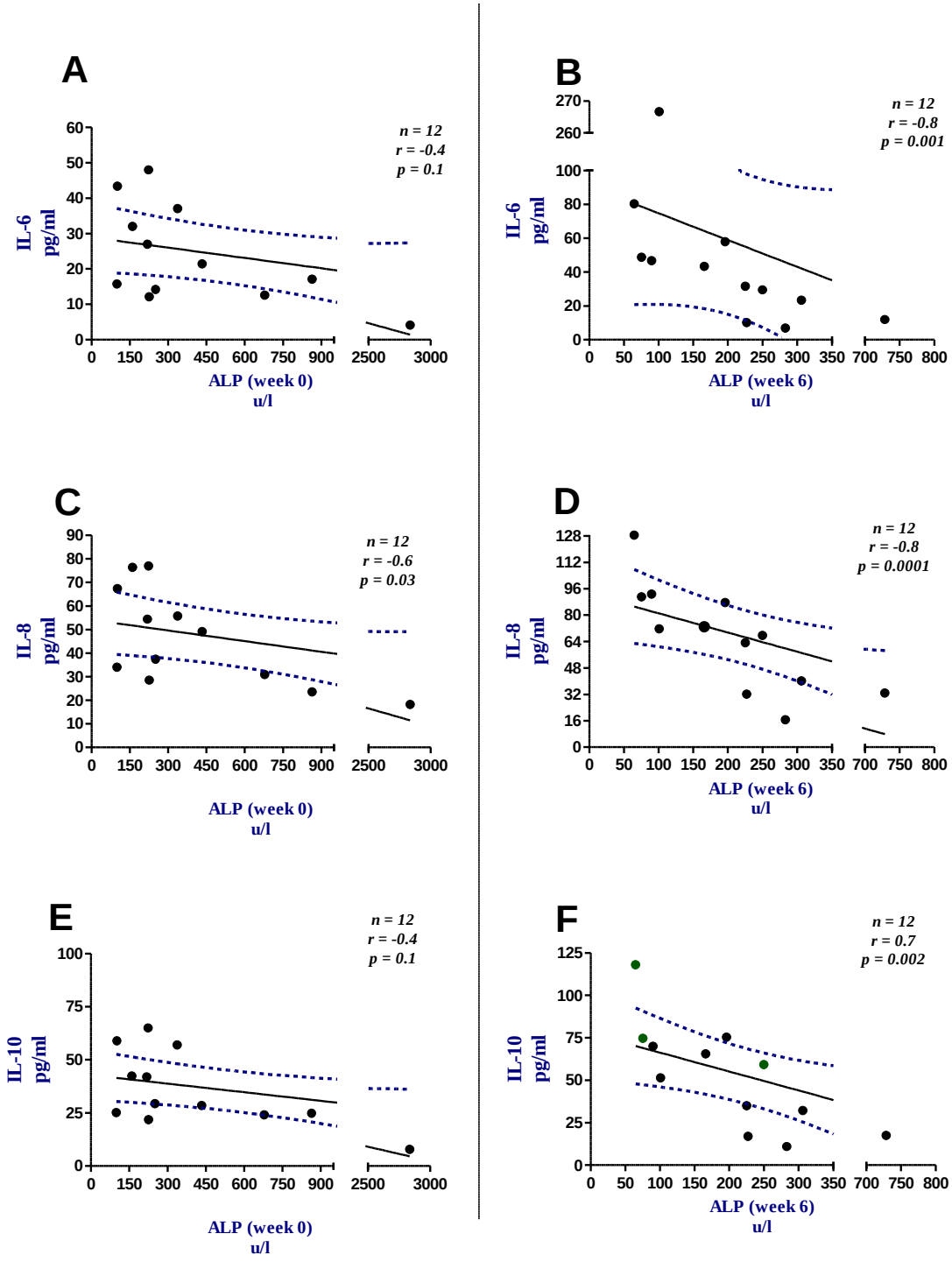


Figure 7-20: Relationship between serum ALP and IL-6, IL-8 and IL-10 in 12 children presenting with vitamin D deficiency at the baseline (A,C and E) and after six weeks (B,D and F) of high dosage vitamin D3 supplementation.

The green dots on the right-hand side panel represent children who remained vitamin D deficient after six weeks. A significant association was found at week 0 between ALP and IL-8 (p = 0.03; r = -0.6). At week six, a significant association was found between ALP and IL-6 (p = 0.001; r = -0.8) and IL-8 (p = 0.0001; r = -0.8) and IL-10 (p = 0.002; r = 0.6).

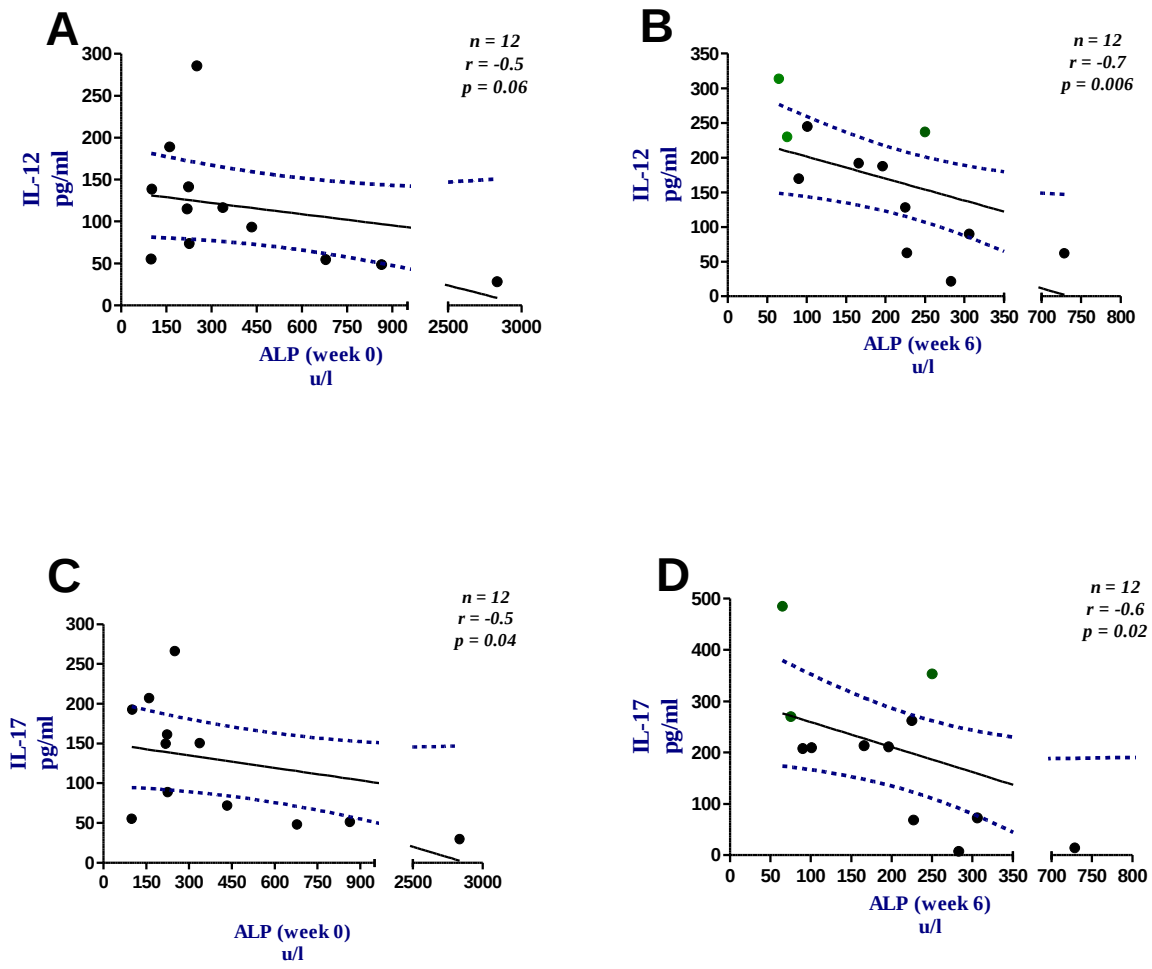


Figure 7-21: Relationship between serum ALP and both IL-12 and IL-17 in 12 children presenting with vitamin D deficiency at the baseline (A and C) and after six weeks (B and D) of high dosage vitamin D3 supplementation.
The green dots on the right-hand side panel represent children who remained vitamin D deficient after six weeks. A significant association was found at week 0 between ALP and IL-17 (p = 0.04; r = -0.5). At week six, a significant association was found between ALP and both IL-12 (p = 0.006; r = -0.7) and IL-17 (p = 0.02; r = -0.6).

7.4.5.3 Mixed Effects Model (MEM)

In order to investigate whether the serum levels of selected cytokines, IL-8, IL-12, INF-γ, TNF-α, IP-10 and IL-17, IL-4, IL-6 and IL-10, which have been suggested to play a role in the process of bone remodelling were independently related to vitamin D level and markers of bone remodelling, a Mixed Effects Model (MEM) was employed for further analysis. This type of analysis only takes into account data with available values at both time points of the study (no missed value).

Due to the low sample size (n = 14), it was not possible to perform an MEM analysis for each cytokine including all bone remodelling markers simultaneously. Therefore, in order to increase the sample size and perform an MEM analysis, the two children who were excluded from the study analysis due to coexistent chronic inflammatory conditions were included in the MEM. Similar results were obtained when the MEM analysis was performed using the whole cohort (n = 18).

The results of MEM using cytokine levels as dependent variables shows that both INF-γ and IL-6 are independently correlated with serum CTX, with *p* values of 0.006 and 0.03, respectively. Additionally, IL-17 was independently correlated with PTH with a *p* value of 0.01. Furthermore, this analysis shows that IL-8, IL-12, IL-17, IL-6 and IL-10 are independently correlated with ALP, with *p* values of 0.0001, 0.01, 001, 0.04 and 0.02, respectively (Table 7-6 and Table 7-7).

Table 7-6: Mixed model analysis to identify predictors of selected cytokines.

Variables	Estimate	SE	*p* value	95% CI (Lower, Upper)
Log IL-8				
Intercept	3.5	0.528655	**0.0001**	(2.4,4.6)
Time	0.03	0.081907	0.6	(-0.1,0.2)
Log vitamin D	-0.1	0.141928	0.4	(-0.4,0.2)
Log PTH	0.1	0.107403	0.2	(-0.09,0.3)
CTX	0.03	0.052775	0.4	(-0.07,0.1)
Log ALP	-0.7	0.149424	**0.0001**	(-1.04,-0.4)
Log IL-12				
Intercept	3.4	0.5	**0.002**	(2.05,4.8)
Time	0.05	0.09	0.5	(-0.1,0.2)
Log vitamin D	-0.1	0.1	0.4	(-0.4,0.2)
Log PTH	0.1	0.1	0.2	(-0.09,0.4)
CTX	0.04	0.05	0.4	(-0.06,0.1)
Log ALP	-0.7	0.2	**0.01**	(-1.2,-0.3)
Log INF-γ				
Intercept	4.1	0.7	**0.01**	(1.7,6.5)
Time	0.2	0.1	0.1	(-0.3,0.8)
Log vitamin D	-0.5	0.2	0.1	(-1.3,0.2)
Log PTH	-0.2	0.1	0.2	(-0.5,0.2)
CTX	-0.4	0.08	**0.006**	(-0.6,-0.2)
Log ALP	0.2	0.2	0.4	(-0.5,0.9)
Log Eotaxin				
Intercept	3.2	0.7	0.05	(-0.3,6.8)
Time	-0.06	0.1	0.7	(-1.4,1.3)
Log vitamin D	0.1	0.2	0.5	(-0.8,1.2)
Log PTH	0.2	0.1	0.3	(-0.8,1.4)
CTX	0.2	0.07	0.08	(-0.04,0.4)
Log ALP	-0.7	0.2	0.05	(-1.3,0.02)
Log IL-17				
Intercept	5.4	0.9	**0.005**	(2.8,8.1)
Time	0.2	0.1	0.3	(-0.2,0.5)
Log vitamin D	-0.5	0.3	0.09	(-1.1,0.09)
Log PTH	0.5	0.2	**0.01**	(0.1,0.97)
CTX	-0.01	0.09	0.8	(-0.2,0.2)
Log ALP	-1.2	0.3	**0.01**	(-2.02,0.4)

This table shows that INFγ is independently correlated with CTX. IL-8, IL-12 and IL-17 are independently correlated with ALP. IL-17 is independently correlated with PTH

Table 7-7: Mixed model analysis to identify predictors of selected cytokines (IL-4, IL-6 and IL-10).

Variables	Estimate	SE	*p* value	95% CI (Lower, Upper)
Log IL-4				
Intercept	2.1	0.5	0.4	(-1493.9,1498.2)
Time	-0.03	0.08	0.7	(-0.7,0.6)
Log vitamin D	0.10	0.1	0.6	(-18.4,18.6)
Log PTH	-0.0004	0.1	0.9	(-3.7,3.7)
CTX	-0.007	0.05	0.9	(-0.3,0.3)
Log ALP	-0.5	0.2	0.4	(-615.7,614.6)
Log IL-6				
Intercept	2.7	0.8	0.06	(-0.3,5.7)
Time	-0.1	0.1	0.4	(-0.6,0.3)
Log vitamin D	0.3	0.2	0.1	(-0.2,0.9)
Log PTH	0.4	0.3	0.2	(-0.3,1.09)
CTX	0.2	0.09	**0.03**	(0.01,0.4)
Log ALP	-1.03	0.3	**0.04**	(-2.01,-0.06)
Log IL-10				
Intercept	3.2	0.6	**0.003**	(1.6,4.8)
Time	0.1	0.1	0.3	(-0.1,0.3)
Log vitamin D	-0.2	0.2	0.1	(-0.6,0.1)
Log PTH	0.2	0.2	0.3	(0-.3,0.7)
CTX	0.07	0.06	0.2	(-0.05,0.2)
Log ALP	-0.7	0.2	**0.02**	(-1.2,-0.1)

This table shows that IL-6 is independently correlated with CTX and ALP. IL-10 is independently correlated with ALP

7.5 Discussion

The main finding of this study is that following six weeks of vitamin D3 supplementation of vitamin D deficient children, there was a significant increase in serum 25(OH)D and IL-4, and a closely approximating significant increase in IL-5 and IL-6 levels. In addition, a significant independent association between inflammatory cytokines and bone remodelling markers was suggested.

This study did not demonstrate an effect of vitamin D3 supplementation on the circulating levels of several cytokines. As indicated in experimental evidence, it was expected that the levels of Th1 cytokines TNF-α and INF-γ would decrease, while the levels of Th2 cytokines IL-5 and IL-6 were expected to increase following supplementation [526, 527, 538]. Additionally, with an exception of the week six significant negative association between 25(OH)D and INF-γ, cytokine levels/or changes in cytokine levels were not found to be associated with serum 25(OH)D levels. Moreover, lymphocyte subsets do not appear to significantly change.

Immunoglobulin E (IgE) antibodies are known as the main effector molecules of type I allergic diseases, such as asthma, food allergy and urticaria. Evidence has shown that IL-4, in addition to CD40L and IL-13, is the classical signal to enhance B cell class switching to generate IgE [539, 540]. Additionally, IL-5 produced by Th2 cells has been also suggested as a significant factor promoting the maturation and differentiation of eosinophils in humans [541]. It may, therefore, be reasonable to suggest that skewing towards a Th2 phenotype cell may be a significant contributor in the pathophysiology of allergic disease [542]. Interleukins-2 produced by naive CD4+ T cells following their activation by antigens have been suggested to be a mediator for CD4+ T cell differentiation into effector Th2 cells, and as an essential factor for the maintenance of the balance of both Th1/Th2 and Treg/Th17 cells [543, 544]. Finally, IL-6 has been shown to promote Th17 differentiation while blocking regulatory T cell (Treg) differentiation [545].

The results showed a significant increase in the main Th2 secreted cytokine IL-4 (p = 0.001) and a tendency for a significant increase in other Th2 secreted cytokines IL-5 (p = 0.05) and IL-6 (p = 0.05) following vitamin D3 supplementation. The results were therefore consistent with the experimental evidence that supports the role of vitamin D in shifting Th1/Th2 polarization towards the Th2 phenotype [301, 546, 547]. Furthermore, experimental evidence showed that naive CD4+ Th cells could differentiate into Th2 cells

if both cytokines IL-4 and IL-2 were present at the time of stimulation [548-550]. The results from this study support this proposal by showing a significant increase in IL-4 and a trend towards an increase in IL-2 levels.

Clinical trials on vitamin D-regulating serum cytokines in healthy individuals are very limited, with inconsistent results. Two studies conducted on healthy adults have failed to observe consistent changes in either circulatory Th1 or Th2 associated cytokines or their corresponding transcription factors following vitamin D supplementation. In a three-month placebo-controlled randomized trial of vitamin D3 supplementation in healthy vitamin D insufficient adults, 50 µg (2000 IU) vitamin D did have an effect in some circulating cytokines, including GM-CSF, IFN-γ, IL-4, IL-8, IL-10 and IL-13. On the other hand, no significant effect of vitamin D3 supplementation was observed in IL-2, IL-5, IL-6 and TNF-α cytokines [435, 440]. In another trial, 25 g (1000 IU) vitamin D3 supplementation of vitamin D-deficient adult patients with multiple sclerosis (MS) led to significantly increased serum levels of TGF-β1, while no effect on TNF-α, IFN-γ, and IL-13 was observed [404].

In children, a recent cross-sectional and interventional trial showed that children with atopic dermatitis had higher levels of circulating cytokines (except TNF-α) compared with a healthy control group. Following three months of 25 µg (1000 IU) vitamin D3 treatment, there was a significant reduction in the clinical severity of atopic dermatitis ($p < 0.001$) and a statistically significant reduction in cytokines, including IL-2, IL-4, IL-6 and IFN-γ ($p < 0.01$), but not TNF-α. The levels of these cytokines approached that of the healthy control group after supplementation [434]. The researcher is not aware of any available interventional study examining the effect of vitamin D3 supplementation on vitamin D-deficient healthy children.

The conflicting evidence from available clinical trials on both adults and children may be a result of several factors, mainly relating to study design. Firstly, most available trials used a relatively low dose of vitamin D3 of < 2000 IU, which may not be sufficient to stimulate innate immunity [434, 435]. Additionally, the duration of some studies may not have been sufficient to observe an effect. One trial involved a healthy young female given a relatively high dose of vitamin D. However, there were several limitations in this trial relating to sample size and the main study design, as it was specially designed for another primary object. Accordingly, measurement was only performed at the baseline and after six months of vitamin D3 treatment [440]. Secondly, most of available studies had either

basal 25(OH)D at an insufficient level (50-70 nmol/l) [432, 434, 435] or a non-significant change in final serum 25(OH)D compared with the basal level [435] (a final 25(OH)D concentration of < 75-100 nmol/l has been suggested as an ideal range for multiple health outcomes). Thirdly, the measurement of cytokines in most available trials was carried out only before and after vitamin D intervention and did not take into account the possible transient effect of vitamin D on circulatory cytokines. For example, a transient inverse relationship between vitamin D and inflammatory Th1 cytokines was reported in athletes during the period of exercise, suggesting that vitamin D deficient status contributes to the injury risk for this population [551]. Finally, most of the available interventional studies involved unhealthy populations with a variety of pathological conditions, such as multiple sclerosis and allergic or cardiac disease [404, 432, 434]. The response to vitamin D treatment in such populations may depend on the corresponding disease-specific original cytokine profile and the nature of immunological disorder. For example, MS is associated with the increased production of Th1-associated cytokines, including IL-2, INF-γ and TNF-α, whereas the T cell cytokines profile in adults with atopic dermatitis showed a heterogeneous alteration in Th1 and Th2 or Naive Th cell related cytokines [552, 553].

T regulatory cells (Tregs) are characterized as CD4+CD25++ T cells with expression of the FoxP3 and low or absent expression of CD127 [513, 554, 555]. Experimental evidence has shown the ability of the active form of vitamin D3 to up-regulate FoxP3 expression and inhibit the proliferation of human peripheral B cells. Additionally, Treg induction requires the presence of IL-2 [410, 528, 556]. Both IL-2 and IL-4 have been suggested to play a significant role in enhancing FoxP3 expression and maintaining CD4+CD25+ Treg development and function [557]. Clinically, in healthy adults, 140,000 IU of vitamin D3 supplementation at the baseline and after four weeks significantly increased the percentage of Treg (4.8 ±1.4 at the baseline to 5.6 ±1.4 at 8 week ($p < 0.001$) [403]. Furthermore, daily supplementation of HIV-infected patients (who already have underlying T cell dysfunction) with either 800 IU of vitamin D3 for three months or 25,000 IU weekly for two months showed a different effect. The higher dose (25,000) had a significant effect on skin-homing markers on Treg. On the other hand, vitamin D of 800 IU units has failed to either correct the vitamin D-deficient status or to exert any immunomodulatory effects in HIV-infected patients [433]. The results from the clinical trials described above may indicate the defending effects of vitamin D against T cell-related pathology and autoimmunity, which need further exploration in the clinical field. The researcher is not aware of any other studies that have investigated the effect of vitamin D on the Treg population as a primary target in younger children.

In this study, there were no consistent changes in peripheral blood lymphocyte analysis, namely B cells (CD19+), CD4/CD8 T cells and T regulatory cells (CD4+CD25+FoxP3+). This may have been due to the small sample size and wide ranges of final serum vitamin D response, in addition to the wide age range of the children involved. Only five patients' samples were available for peripheral blood lymphocyte subset analysis. Of those five patients, one was still vitamin D deficient after supplementation and no change in his T reg cell profile was observed. Most of the other four patients had an increase in their T reg cells after six weeks of vitamin D3 supplementation. A similar observation was noted in CD19 cells. The patient whose final vitamin D level was still low showed an increase in CD19 cells, whereas the other four showed a reduction in their CD19. Despite the low sample size, this study was therefore able to demonstrate some changes in Treg and CD19+ cells for each individual patient.

Osteoimmunology is the study of the influence of the immune system on skeletal dynamics and homeostasis, including study of the development of immune cells in bone marrow and the interaction between the immune and skeletal systems in health and disease. Cytokines secreted by lymphocytes and macrophages have been suggested to be powerful mediators that play a significance role in maintaining skeletal integrity and bone remodelling [558].

In this study, an independent correlation have been reported between ALP and pro-inflammatory cytokines including IL-8, IL-12, IL-17, IL-10 and IL-6, between CTX and both INF-γ and IL-6, and between PTH and IL-17. The univariate analysis demonstrated significant relationships between ALP and IL-5, IL-6 and IL-8 at the week 6 of vitamin D3 treatment. However, this is not a proof of causality.

The precise roles of IFN-γ and IL-17 in the bone remodelling process are controversial for several reasons, mainly related to the differences in study models and target cell types in experimental studies [559]. Interleukin-17 has been suggested to mainly act as a potent stimulator of osteoclastogenesis (bone resorption) by targeting the osteogenic differentiation of mesenchymal stem cells and osteoblast [560, 561]. Generally, based on the available evidence, IFN-γ can be considered to be a potent anti-osteoclastogenic agent through mechanisms that involve interfering with the receptor activator of NF-κB ligand (RANKL)-receptor activator of the NF-κB (RANK) signalling pathway, which mediates its anti-resorptive function [562, 563]. Similarly, IL-12 has been suggested to inhibit of osteoclast differentiation through indirect mechanisms mediated by IFN-γ [564].

In this study, the MEM did not reveal any independent association between serum levels of IFN-γ and 25(OH)D. In MEM, however, IFN-γ was found to be independently and negatively associated with CTX (a marker of bone resorption). Additionally, the result from MEM showed that IL-17 is positively and independently associated with PTH (which stimulates bone resorption by acting directly on osteoblasts/ stromal cells and indirectly via enhancing osteoclastogenesis) [565], and negatively independently associated with ALP (a marker of bone formation). Since VDR knockout mice showed up regulation of IL-17 and IFN-γ production more than wild-type mice [566], an indirect association between serum 25(OH)D and inflammatory cytokines may therefore be suggested. Additionally, the results from univariate correlation revealed the significant negative correlation between IL-12 and CTX in vitamin D deficient status (at the baseline), and between IL-12 and ALP after six weeks of vitamin D3 treatment.

Current evidence regarding the exact role of IL-6 in bone remodelling is inconsistent [567, 568]. Generally, it has been suggested that IL-6 may be regarded as a stimulator of osteoclastogenesis through a mechanism involving the stimulation of osteoblastic/stromal cell-mediated osteoclast differentiation. The result from MEM showed an independent negative association between IL-6 and ALP ($p = 0.04$). IL-10 has been suggested to suppress bone resorption by inhibiting osteoclastogenesis through mechanisms involved in the regulation of osteoprotegerin (OPG) expression, and down-regulation of RANKL and colony-stimulating factor-1 (CSF-1) expression [569]. Moreover, its potent anti-inflammatory effect enables IL-10 to indirectly inhibit infection-stimulated bone resorption, mediated through pro-inflammatory cytokines such as TNF-α (bone absorptive factors) [559]. Additionally, IL-10 has been suggested to promote osteoblastic differentiation, which promotes bone formation [570]. The result from MEM showed an independent negative association between IL-10 and ALP ($p = 0.02$). These results are inconsistent with the experimental evidence which support the role of IL-10 in promoting bone loss and suppressing bone formation.

In summary, results from correlation studies may indicate that circulatory cytokines play a role in maintaining the bone remodelling imbalance. Lower vitamin D status and an associated higher PTH level may lead to a high bone remodelling rate, which in turn depends on the integrated effects of a variety of local and systemic factors, including the cytokines. The inconsistency in the correlations between different cytokines and markers of bone remodelling, CTX and ALP, at the baseline and six weeks, may reflect the balance in the bone remodelling process during the vitamin D deficiency status. Additionally, the

results of this study show associations between most of the cytokines and either a decreased rate of bone formation or an increased rate of bone resorption. Several clinical studies have linked the systemic and/or local inflammatory pathological conditions with disturbance in the bone remodelling process, which in turn may contribute to the loss of bone mass. For example, pro-inflammatory cytokines, such as IL-6, IL-17 and IFN-γ, have been shown to be significant contributors to the development of bone-related diseases such as osteoporosis and rheumatic arthritis [571-573]. Finally, a small number of clinical studies have examined the association between circulatory cytokines and markers of bone remodelling. In one study of healthy adults, IL-6, IL-10, IL-17 and IFN-γ were found to be correlated with markers of bone remodelling, which are consistent with the observations made in this study [574].

The main strength of this study is that, to our opinion, it is the first interventional study examining the effect of vitamin D3 supplementation on vitamin D-deficient healthy children. However, there are several limitations to this study that may affect its ability to detect significant changes in other cytokines and lymphocytes following vitamin D3 supplementation. Firstly, 5000 IU /day may not be sufficient to stimulate innate immunity, and/or the duration of the study may not have been long enough to detect a significant effect of some cytokines and/or chemokines. Secondly, the results showed that following vitamin D3 supplementation in vitamin D-deficient children, only 68% achieved final vitamin D levels greater than 75nmol/l and the difference between baseline and six-week vitamin D levels may not be sufficient to exert an immunomodulatory effect for many cytokines. For example, Zhang et al. (2012) demonstrated that a level of 75 nmol/l was required to inhibit TNF-α and IL-16 [575]. Furthermore, the participants were illness-free at the time of study and the correlation between vitamin D and other cytokines/chemokines may be evident when the immune system is stimulated by sepsis or infection. Despite their good response to vitamin D3 treatment, some children in the study population showed an inconsistent cytokine profile compared with the other children. This inconsistency may suggest the possibility of an effect caused by ethnic diversity, minor unreported illnesses, different nutritional behaviours and the variety of the age groups. Moreover, only the circulatory level of cytokines was measured; it may be that changes for some cytokines are more evident at the tissue or organ level. For example, it has been reported that some of inflammatory cytokines are variably changed in serum and synovial fluid in patients with rheumatic arthritis [576]. Another limitation is that there was no control group who did not have any vitamin D supplementation which may have a critical impacts on the results. However, by confining the analysis to children with symptomatic vitamin D deficiency,

and testing the children pre- and post-treatment, each child may able to act as their own control to reduce the risk of confounding factors such as minor illnesses, different nutritional patterns and the variety of age groups, which may all affect serum cytokines levels [577-580]. Finaly, the sample size of this work must be considered as it may limits the power to properly control for confounding variables and determine true association between cytokiens and bone biomarkers.

In conclusion, increasing evidence from experimental and observational studies supports the role of vitamin D in human defence mechanisms. Theoretically, cytokines are multifunctional molecules secreted by human cells in response to immunological challenges and play direct roles in human responses to infection, inflammation, trauma, sepsis and cancer. These molecules involve in several pathophysiological conditions, and their measurement in the body fluids and /or circulation can be used as significant clinical and laboratory measures for monitoring diseases activity. Whilst this pilot work has shown that vitamin D3 treatment is associated with an alteration in multiple cytokines, it is unclear whether serum 25(OH)D in these children or response to vitamin D3 treatment are directly associated with a change in these cytokines. The results from our study suggest that vitamin D may modulate the immune system through a mechanism involving mainly the Th2 cytokines and there were independent correlations between systemic inflammation and markers of bone remodelling. Therefore, our findings support previous studies which indicated to the possible beneficial effects of supplementary vitamin D with respect to infectious and autoimmune diseases. However, the effect of vitamin D3 supplementation on the cytokines profile need further exploration in well-designed clinical trials involving a strictly controlled environment with either experimental animals and/or healthy adults.

Table 7-8: Pre-treatment levels of inflammatory and anti- inflammatory cytokines in the serum of 14 children presenting with vitamin D deficiency.

Patient ID	Baseline (week 0)														
	Th2 (Pro-inflammatory)			Other pro-inflammatory cytokines									Th1 (Anti-inflammatory)		
	IL-2	IFN-γ	TNF-α	IL-8	IL-12	IL-10*	IL-17	IP-10	Eotaxin	RANTES	MIP-β	MCP-1	IL-4	IL-5	IL-6*
1	13.84	556.33	15.36	33.99	55.25	25.19	55.47	1211.15	429.8	52005.61	233.4	86.83	5.81	17.18	15.72
6	26.95	1815.49	58.1	54.38	115.22	41.94	149.59	786.58	191.71	64660.54	204.94	93.29	10.77	28.03	26.97
7	36.1	2404.22	83.53	76.39	189.18	42.41	207.02	1141.39	175.6	48603.31	134.22	53.1	14.59	33.44	32.03
10	13.05	1726.21	62.45	40.88	84.02	32.54	75.09	1546.32	135.43	51217.93	115.23	60.78	9.19	24.69	22.01
11	22.8	825.88	33.15	37.36	285.51	29.41	266.18	900.3	311.87	66348.53	160.17	52.12	5.46	18.41	14.2
12	29.06	418.86	15.06	77.01	141.28	65.07	161.45	1207.86	449.94	58035.2	235.38	100.22	15.03	47.28	48.01
15	9.61	622.21	18.95	32.38	40.05	20.34	57.93	1121.46	280.06	65961.8	239.31	55.61	5.46	12.9	11.44
17	32.78	462.85	15.96	67.29	138.52	58.92	192.93	1250.54	521.91	44245.43	162.42	66.87	13.73	36.58	43.41
27	16.95	1373.66	45.74	49.16	93.47	28.47	71.83	1778.9	190	32257.51	150.97	43.12	8.83	18.24	21.44
28	13.71	1074.93	34.33	30.93	54.58	24.09	48.05	1679.4	90.63	69528.95	350.11	38.28	4.86	15.17	12.58
29	2.06	503.11	20.14	18.18	28.26	7.83	29.69	1221.84	403.24	23069.98	149.95	99.09	2.51	6.71	4.11
30	11.07	161.42	*5.50	28.5	73.93	21.91	88.86	717.97	326.49	33956.48	143.9	69.18	5.77	15.77	12.11
32	16.43	732.78	23.41	23.62	48.62	24.88	51.35	1468	166.82	15953.47	214.3	62.12	2.33	9.23	17.06
33	30.8	1133.99	50.69	55.8	116.58	57.03	150.38	1216.5	174.44	71917.53	259.69	55.53	11.83	37.9	37.09

*IL-6 can be considered to either block or promote inflammation and IL-10 as anti-inflammatory.

Table 7-9: Post-treatment levels of inflammatory and anti- inflammatory cytokines in the serum of 14 children presenting with vitamin D deficiency.

Patient ID	End of the study (week six)														
	Th2 (Pro-inflammatory)			Other pro-inflammatory cytokines									Th1 (Anti-inflammatory)		
	IL-2	IFN-γ	TNFα	IL-8	IL-12	IL-10*	IL-17	IP-10	Eotaxin	RANTES	MIP-β	MCP-1	IL-4	IL-5	IL-6*
1	43.17	1714.28	57.09	92.64	169.66	69.97	207.81	2333.39	526.42	66083.04	228.87	103.27	17.74	47.01	46.76
6	66.2	1880.7	59.41	128.49	313.56	118.1	485.18	1354.46	468.96	46369.72	166.7	120.98	22.9	64.54	80.29
7	48.13	1513.16	49.68	72.94	191.98	65.55	213.28	907.63	237.15	59688.87	168.12	93.36	14.79	37.95	43.22
10	12.79	895.86	32.12	36.56	89.41	21.44	89.67	1077.69	131.7	74364.99	146.71	49	7.49	17.42	16.68
11	576.35	291.1	13.56	71.69	244.85	51.36	209.37	1321.55	1838.65	62538.82	147.17	79.75	9.44	23.61	266.64
12	48.01	381.31	7.96	87.64	187.78	75.36	210.93	1015.51	576.37	68335.27	211.52	91.05	18.4	54.64	57.9
15	8.94	864.11	27.11	29.15	32.18	14.72	52.17	729.28	391.74	68698.46	176.29	76.91	5.54	14.46	11.92
17	40.5	867.29	24.74	91.08	229.97	74.72	270.06	696.14	378.71	44312.7	175.07	75.86	16.44	43.45	48.68
27	47.89	1788.75	62.16	67.76	237.05	59.24	353.28	2481.83	559.53	57891.2	184.76	101.85	10.91	25.71	29.45
28	17.2	2674.81	101.34	40.24	90.09	32.23	72.65	1859.52	178.5	85646.67	765.88	46.83	7.04	20.56	23.34
29	7.04	895.86	18.65	32.87	61.9	17.53	14.33	970.57	325.49	32791.25	171.47	83.9	6.28	12.78	11.92
30	8.4	435.83	10.84	32.06	62.57	17.06	68.57	549	256.96	26691.67	133.16	46.66	6.24	15.41	10.11
32	31.29	576.17	19.25	63.21	128.22	35.04	262.31	792.78	193.98	32015.85	218.27	63.92	10.7	28.87	31.55
33	*	1458.72	62.45	16.69	21.76	10.96	7.32	860.42	45.54	27536.32	165.99	31	11.88	7.22	6.97

*IL-6 can be considered to either block or promote inflammation and IL-10 as anti-inflammatory.

Table 7-10: Markers of bone remodelling for 14 children enrolled in the immunity study.

			Bone markers/ Baseline				Bone markers/ Week six			
Patient ID	**Sex**	**Age (months)**	**Vitamin D mmol/l (n=14)**	**PTH pmol/l (n=14)**	**ALP u/l (n=12)**	**CTX ng/ml (n=12)**	**Vitamin D mmol/l (n=14)**	**PTH pmol/l (n=12)**	**ALP u/l (n=12)**	**CTX ng/ml (n=12)**
1	M	43	33	5.3	99.79	2.014	100	*	89.9	2.33
6	F	36	29	8.4	219	1.30	56	4.1	64.9	0.81
7	F	115	33	5.5	161.1	2.17	76	4.7	166	2.28
10	M	79	14	6.8	*	*	66	6.1	*	*
11	M	115	14	4.3	251	1.55	134	2.7	101	1.91
12	F	33	23	4.1	224	1.57	191	3.2	196	1.07
15	F	51	33	7.1	*	*	127	2.7	*	*
17	F	71	20	7.3	102	1.39	60	4.3	75.3	1.16
27	F	16	27	29.8	434	3.01	37	*	250	1.72
28	F	16	23	36.2	679	2.50	117	6.4	306	1.34
29	M	18	26	134	2834	3.16	106	4.7	729	3.03
30	M	96	49	3.6	226	2.46	93	3.9	227	1.82
33	F	18	18	14.1	864	2.28	141	6.8	225	1.68
32	M	10	19	9.7	338	2.26	115	4.4	283	1.71

Chapter 8

Discussion, Conclusion and Recommendations for Future Research

8.1 An electronic surveillance system for monitoring nutritional vitamin D deficiency in children in Scotland

Despite the national recommendations and the interventional measures in the UK to address the problem of vitamin D deficiency during early childhood, anecdotal reports still showing that there are increasing numbers of symptomatic cases of vitamin D deficiency [5, 105]. There is a need to institute a reliable system to prospectively collect data and monitor the incidence of this preventable condition. Accordingly, a monthly survey of symptomatic vitamin D deficiency was launched using the established electronic ScotPSU methodology, with two aims. The first aim was to estimate the current incidence of vitamin D deficiency in children in Scotland. The second aim was to validate the electronic surveillance system and to look at the range of clinical presentations of vitamin D deficiency in Scotland.

Over the two years of the survey, the reported incidence of vitamin D deficiency in non-white children was greater in Glasgow at 7/1000 compared with 2.6/1000 in Edinburgh ($p = 0.009$). However, it is likely that the true incidence of vitamin D deficiency was underestimated, due to the significant numbers of unreported cases identified from the regional laboratory data in the cross-validation study. In Glasgow, 82 cases were identified through ScotPSU services over the two years of the study, while only 16 cases were identified in Edinburgh during the same period. The increasing incidence of symptomatic vitamin D deficiency in Glasgow suggests that current public health measures have not yet had a beneficial effect on prevention of vitamin D deficiency. However, during the second year only two cases had been reported in Edinburgh whereas 40 cases were reported in Glasgow during the same period, which may simply reflect a change in enthusiasm for notification in the second year in Edinburgh.

As expected, children with Asian and Sub-Saharan African ethnic origin have a much higher risk for profound symptomatic vitamin D deficiency with 76 (74%) cases reported through ScotPSU during the two years. The high prevalence of profound symptomatic vitamin D deficiency in these ethnic groups was also reported previously in Glasgow [5]. In addition to that, the results from our survey showed that symptomatic vitamin D deficiency was also prevalent among other populations such as Caucasian children. A recent survey conducted through the British Paediatric Surveillance Unit (BPSU) confirmed our observations by reporting 91 confirmed or probable cases of hypocalcaemic seizures secondary to vitamin D deficiency in children in the UK and Ireland, including 11 (12%) children of Caucasian origin [581]. In our survey, skeletal or bone mineral-based clinical suspicion of vitamin D deficiency represented the main presenting features of vitamin D deficiency, which is consistent with previous observations in Glasgow [5]. Hypocalcaemic seizure, which is one of the most serious complications of nutritional rickets, was reported in four (4%) children in our survey. A considerable proportion of children had unusual presentations such as concerns about nutritional status and recurrent constipation. In summary, vitamin D deficiency is very common in Scotland due to the variations in ethnic demography and low vitamin D intake, in addition to insufficient sunlight exposure. Although it is a preventable condition, people seem to be unaware of, or disregard, government advice regarding vitamin D supplementation for high-risk groups. The results from this survey show that ScotPSU may provide reliable data for monitoring symptomatic vitamin D deficiency and may be helpful for assessing the effectiveness of public health measures.

8.2 Effect of vitamin D supplementation on bone status, glucose homeostasis and immune function in children with symptomatic vitamin D deficiency

Three different studies were conducted in an attempt to explore the effects of vitamin D3 supplementation on bone remodelling, glucose homeostasis and immune function. In summary, the main objectives of these studies were:

- To study the effect of six weeks of vitamin D supplementation on bone remodelling markers. To study the baseline and post-treatment relationships between serum 25(OH)D levels and the bone remodelling markers.

- To investigate the effect of vitamin D3 supplementation on glycaemic status and insulin resistance in vitamin D deficient healthy children.
- To assess the effect of short-term vitamin D3 supplementation on the main Th1 and Th2 cytokines, Th17 cytokine, T regulatory cytokine (Treg) and chemokines linking the balance between the Th1 and Th2 subsets.

Generally, our recruitment summary showed that symptomatic vitamin D deficiency is common among breastfeeding infants and older children of non-Caucasian ethnic origin. The increasing number of overall study participants also raised questions about public awareness about the problem of vitamin D deficiency.

8.2.1 Effect of vitamin D3 supplementation on bone status

The effect of six weeks of vitamin D3 supplementation on bone remodelling markers was explored in chapter 5. The results of this study suggest that short-term vitamin D3 supplementation significantly increases serum 25(OH)D levels, and may affect bone remodelling in children, possibly by suppressing PTH levels. Both PTH level and ALP decreased significantly with treatment. However, no significant response emerged following supplementation, effecting either CTX or OCN

A recent review of the laboratory assessments of vitamin D status, vitamin D dosing and various supplementation options for the treatment of vitamin D deficiency in the paediatric population, recommended doses of vitamin D ranging between 1000 to 5000 IU daily for infants 1 to 12 months old, and a daily dose of 5000 IU vitamin D for patients over 12 months old to correct nutritional vitamin D deficiency. The study also advised this regime for a period of 4–8 weeks, although the radiological evidence of healing occurs within 2 to 4 weeks of treatment [582].

In our study there were individual differences in treatment responses. A daily dose of 5000 IU oral vitamin D3 was insufficient to increase the serum 25(OH)D level to a normal reference range of 35% in our study participants. However, this part of the study has several limitations, as detailed previously in Chaper 5. A better designed study with a longer duration of high-dose vitamin D treatment, involving multiple doses at measurable intervals, is advised for future studies.

As discussed in Chapter 5, short-term vitamin D3 supplementation significantly reduced the overall median level of PTH, which was most significant in children aged 10 months–3 years and 3–6 years. This younger group also showed a lower 25(OH)D level, higher basal PTH levels, higher basal ALP and lower basal serum calcium compared to other older groups. This may indicate that infants and young children are at an increased risk for nutritional rickets than the older children due to prolonged breast feeding, indoor activity, age and race, for example. Therefore, it is also recommended that future studies measure patient compliance, and take into account the nutritional status, indoor activity and differences in the rate of bone remodelling biomarkers in different age groups and races.

The regression analysis suggested that PTH could have an independent role in the bone remodelling process . Furthermore, although some of the children in the present study were already on a low dose of multivitamin supplementation before starting the higher dose vitamin D treatment, their basal ALP and PTH showed persisting abnormalities, indicating that a high dose of vitamin D is necessary for better skeletal response. Few studies have assessed the effects of vitamin D supplementation on serum ALP, osteocalcin, and CTX in healthy children [464, 465]. The results from our study, as well as previous studies, did not find any significant response in either CTX or OCN and concluded that ALP is a more reliable marker for diagnosing and monitoring patients with nutritional vitamin D deficiency in children than CTX and OCN. However, further studies are required to explore the the dynamics of biochemical bone markers during vitamin D supplementation.

8.2.2 Effect of vitamin D3 supplementation on glucose homeostasis

Lower circulating 25(OH)D status has been correlated with an increased risk of diabetes mellitus. Interventional studies, however, are inconclusive, and the effect of improving vitamin D status on glucose homeostasis in healthy children remains unclear. This study aimed mainly to investigate the effect of short-term vitamin D replacement on glucose homeostasis parameters in vitamin D deficient healthy children. Homeostatic model assessment (HOMA IR) was employed to assess insulin resistance from fasting glucose and fasting insulin measurements.

The participants in this study were normal, healthy and mostly non-obese children. It was expected that their blood glucose parameters were within the normal range. Any

abnormality in HOMA IR measurement was therefore likely to be due to an abnormality in insulin concentration rather than hyperglycaemic status. Accordingly, the high basal HOMA IR measurements which were noted in a few participants in this study were attributed to either a reduction in insulin sensitivity or increased insulin resistance rather than a defect in insulin secretion. Consistent with some other studies conducted in obese and/or high risk populations, this study also observed that vitamin D3 supplementation in vitamin D deficient healthy children was associated with an improvement in insulin resistance, as indicated by HOMA IR [266, 283]. This reduction in fasting insulin following vitamin D supplementation suggests that the abnormality in HOMA IR was due to the reduction in insulin sensitivity, and there may have been an improvement in insulin uptake following vitamin D supplementation. Furthermore, given the fact that most of our study population was not at risk of diabetes and did not have any predisposing factors such as obesity, abnormal lipid profiles or abnormal glycaemic indices, the improvement in vitamin D status could therefore be one of the contributors to the improvement in HOMA IR following vitamin D3 supplementation. This was further supported by the observation that the children whose final serum 25(OH)D was higher than 75nmol/l had a significant improvement in HOMA-IR compared with those who were still deficient/insufficient following supplementation. Consistent with this observation, Von Hurst et al. (2010) reported the greatest improvement in insulin sensitivity and/or insulin secretion following 4000 IU vitamin D3 supplementation occurred when endpoint serum 25(OH)D reached 80 nmol/l or higher. However, the study group in this study was completely different, and consisted of South Asian women at high risk of type 2 diabetes mellitus [279].

The remarkable difference we observed in serum insulin and insulin resistance, when children were grouped according to their final serum 25(OH)D may also indicate the significance of a vitamin D dose in obtaining desirable outcomes. Additionally, the lack of significant effects of vitamin D3 supplementation (which we observed in the obese child with abnormal lipid profiles and a family history of hypertension and hypercholesterolemia) may suggest a need for a larger dose and/or longer duration of treatment in high risk children. Unlike studies conducted in adult populations, no changes and/or associations were noticed in this study between serum 25(OH)D level and other pre-diabetic parameters such as lipid profile, fasting glucose, HbA1c or SHBG. It could be hypothesised that the relationship between vitamin D status and these measurements are dependent on other factors such as age, production of sex hormone and diabetic risk, and the lack of significant association between 25(OH)D and these measurements is due to the

nature of study participants: pre-pubertal non-obese children at lower risk of diabetes mellitus. In summary, six weeks of vitamin D3 supplementation of vitamin D deficient children does have a favourable effect on key glucose homeostasis parameters, HOMA IR in normal-weight, low-diabetic-risk children. A further study of a longer duration, involving multiple vitamin D doses and a larger sample size is required.

8.2.3 Effect of vitamin D3 supplementation on immune function

One key mechanism by which vitamin D influences human immune responses may be related to the regulation of specific cytokines and chemokines produce by antigen presenting cells (APCs). Additionally, some reports suggest that vitamin D may play a central role in the alteration of different T-helper-cell subset balances. At present there are no published paediatric studies assessing the effect of vitamin D supplementation in cytokines in healthy children. Therefore, we assessed the effect of short term vitamin D3 supplementation on main T helper 1 (Th1) and T helper 2 (Th2) cytokines, Th17 cytokine, T regulatory cytokine (Treg) and chemokines linking the balance between Th1 and Th2 subsets. In addition, we determined whether these cytokines were associated with markers of bone remodelling before and after vitamin D3 supplementation.

The main finding from this pilot work is that six weeks of vitamin D3 supplementation in vitamin D deficient children is associated with a significant increase in the main Th2 secreted cytokine, IL-4 ($p = 0.001$) and a tendency towards a significant increase in other Th2 secreted cytokines, IL-5 ($p = 0.05$) and IL6 ($p = 0.05$). However, it is unclear if serum 25(OH)D in these children or their response to vitamin D3 treatment are directly associated with a change in these cytokines. This finding from the present study was consistent with the experimental evidence which suggested that IL-4 may stimulate naive Th cell response to differentiate towards the Th2 effector phenotype. T helper 2 (Th2) cells in turn can also secrete more IL-4 and IL-5 [550]. This proposal is further supported by other evidence which indicates that APCs such as dendritic cells (can also secrete IL-4) are able to express vitamin D receptor (VDR), and the active form of vitamin D is a potential target for these cells [374]. Finally, Th2 response and Th2- related cytokines have been correlated with the incidence of allergic airway diseases which are further connected with low vitamin D status [409]. For example, low levels of IL-4 have been found in vitamin D deficient asthmatic patients compared with healthy controls ($p = 0.003$) and lower vitamin D status was correlated with the severity of asthma [583]. Finally, our results suggest that vitamin

D may associated with a skewing of the immune response to a Th2 phenotype, with an increase in IL-4 secretion and pointed to the significant association between systemic inflammation and markers of bone remodelling. These finding may further support the possible beneficial effects of supplementary vitamin D with respect to infectious and autoimmune diseases.

8.3 Limitations and general conclusion: Effect of vitamin D supplementation on bone status, glucose homeostasis and immune function in children with symptomatic vitamin D deficiency

The main strength of this thesis is the prospective and interventional nature of our work, in addition to its unique population. However, there are general limitations in our study. Firstly, it was not a randomized controlled trial and we are unable to confirm whether any of observed changes were due to the intervention. Secondly, the sample size was relatively small which may limit our ability to detect significant changes in some biomarkers. Thirdly, for ethical reasons, collection of blood samples for the study was performed at the same time as the clinical samples, which limited our ability to carry out sufficient investigation in many cases. The fourth limitation was the variability in age distribution among study participants. Furthermore, it was very difficult to assess compliance for the treatment among study participants; compliance was assessed verbally from parents and may not reflect the actual intake. Finally, sun exposure, physical activity and food intake were not measured in our study. Due to the multi-ethnic population of our study, food intake may be one of the significant contributors to the lower vitamin D status.

Our work was performed as a pilot study, since no prior research existed regarding the impact of high dose of vitamin D supplementation on bone biomarkers, glucose homeostasis and cytokines profile in children when we embarked on this project.
It was therefore difficult to calculate the sample size adequately. However, now that we have undertaken this preliminary work, we now able to calculate the numbers required to design a sufficiently powered study in the future. For instance, based on our results in the glucose study (chapter 6), a sample of 149 children would provide 80% power to detect a treatment difference of 1.04 in mean change of insulin resistance (HOMA IR) in healthy, vitamin D deficient children which would be significant at $p<0.05$.

In summary, the result from the second part of this thesis were consistent with the findings in our ScotPSU study in the first section which revealed a high prevalence of vitamin D deficiency and insufficiency among children in Glasgow. The current work also provided some evidence that vitamin D deficiency and insufficiency among healthy children may be associated with a disturbance in immune and glucose profiles, in addition to a well-established musculoskeletal effect. Therefore, six weeks of daily supplementation with 5000 IU vitamin D3 to healthy children led to a significant increase in median serum 25(OH)D, significant decreases in serum PTH and ALP, a significant improvement in insulin resistance and a possible improvement in cytokines profile.

8.4 Recommendations for future research

1. Investigation of the long term effect of vitamin D3 supplementation on bone remodelling, glucose homeostasis and immune function using multiple point measurements, to further explore the effect of improving vitamin D status on glucose and immune profile measurements.
2. The effect of improving vitamin D status on bone remodelling, glucose homeostasis and immune function should be further investigated in a larger population to control for potential bias such as age, ethnicity, diet and BMI.
3. More studies are needed in this research area, and measurement of sun exposure, and vitamin D and calcium intake, should be considered in any future study. It would be helpful to investigate whether these confounders have any effect on vitamin D level.
4. Basal serum 25(OH)D concentration and/or the end point of serum 25(OH)D achieved should be considered as a significant contributor for the study outcomes.
5. In immunity studies, the effect of vitamin D3 supplementation in cytokine profiles is still contradictory and may need further exploration. Well-designed clinical trials under strictly controlled environments involving experimental animals and/or healthy adults may be needed in order to minimize the risk of confounders such as the effect of age variability, minimal illness and physical activity.

Appendices

Appendix A-1: ScotPSU/ Case Report Form.

Appendix B-1: Vitamin D deficiency and its potential effect on bone status, immune and glucose homeostasis among children in Scotland/ Case Report Form.

Appendix C-1: Child information sheet.
Appendix C-2: Concent form for the child.
Appendix C-3: Parent information sheet.
Appendix C-4: Concent form for the parent.

Appendix D-1: Clinic check list for eligible participant for the study.
Appendix D-2: A letter for a general practitioner (GP).

Appendix A–1- ScotPSU/ Case Report Form

Symptomatic Vitamin D Deficiency In Scotland
Case Report Form

ScotPSU Register Number:

REPORTING CLINICIAN

1. Dr's Name (please print) ……
2. Month/Year of Report ☐☐ / ☐☐
3. Date diagnosis considered ☐☐ / ☐☐ / ☐☐

CHILD'S DETAILS

4. First Letter of Forename ☐
5. Month and year of birth ☐☐ / ☐☐☐☐
6. Gender M ☐ F ☐
7. Postal code ☐☐☐☐☐☐
8. Gestation (wks) ☐☐

BIRTH MOTHER'S DETAILS

9. First Letter of MAIDEN SURNAME ☐
10. Current Age …….
11. Country of Birth
12. Ethnic Origin: ☐ South Asia ☐ North -African ☐ Other Africa
    ☐ Middle- Eastern ☐ Chinese ☐ Caucasian
    ☐ Other……

PRESENTATION

Date of Clinic: Date of referral: Hospital:

Source of referral:
- ☐ A&E
- ☐ GP
- ☐ Inpatient Medical
- ☐ OPD Medical
- ☐ Orthopaedic
- ☐ Other……

Reasons for referral:
- ☐ Gait disturbance
- ☐ Bowed leg
- ☐ Known Rickets
- ☐ Pain
- ☐ Fractures
- ☐ Swollen Wrists
- ☐ Other
- ☐ Delayed walking
- ☐ FHx of Vit D def
- ☐ Seizure
- ☐ Abnormal blood results
- ☐ Dietary Concerns
- ☐ Abnormal X ray

X-Ray Abnormal findings……

Blood abnormal findings……

MATERNAL VITAMIN D STATUS

Antenatal supplementation: Name: Start Month/Yr: Dose:

Recommended By: ☐ HV ☐ GP ☐ Other

Other……

Current supplementation: Name: Start Month/Yr;
Dose:

Recommended By: ☐ HV ☐ GP ☐ Other

Other……

Known Mat Vit D Def: ☐ Yes ☐ No

Maternal Clothing that limits sun exposure: ☐ Yes ☐ No

Parental Understanding of Vit D: ☐ None ☐ Some ☐ Complete

CHILD'S NUTRITIONAL & VITAMIN D STATUS

Prior Vitamin supplementation: Name: Duration: Dose:

Recommended by: ☐ HV ☐ GP ☐ Other

Other……

Breast feeding duration:

Age at weaning:

Diet at presentation:
☐ Breast Milk (BM) only
☐ Formula milk (FM) only
☐ BM + FM
☐ BM + solids
☐ FM + solids
☐ Other

Other……..

Dietary problems:
☐ None
☐ Yes

Details……

Coexisting conditions:……..

CHILD'S EXAMINATION FINDINGS AT PRESENTATION

Age at walking: ☐ Not walking yet

Weight: Height: OFC:

☐ Bowed Legs ☐ Abnormal gait ☐ Wrist Swelling ☐ Frontal Bossing

☐ Rachitic rosary ☐ Abnormal Dentition ☐ Bone pain ☐ Weakness

☐ Patent Ant Fontanelle Other……

RESULTS OF FIRST INVESTIGATIONS

Biochemistry	**Ca**	**PO**	**PTH**	**ALP**	**25(OH)D**
Date					
Result					
Reference Range					
Measurement unit					

X-rays	**Specify site**	**Clear Rickets**	**Healing Rickets**	**No Rickets**
Site 1				
Site 2				
Site 3				

TREATMENT OF VITAMIN D DEFICIENCY

Drug	**Name**	**Start Date**	**Dose**	**Administration Method**	**Stop Date**
Drug 1					
Drug 2					
Drug 3					
Other					

In Case Of Any Queries About Completing This Form, Contact:
Dr S Faisal Ahmed 0141 201 0571 or s.f.ahmed@clinmed.gla.ac.uk
Dr Nagla E lfakhri : n.el-fakhri.1@research.gla.ac.uk.

Appendix B–1- Vitamin D deficiency and its potential effect on bone status, glucose homeostasis and immune function among children in Scotland/Case report form

Vitamin D deficiency and its potential effect on bone status, immune and glucose homeostasis among children in Scotland.

Case Report Form

Hospital Number:
Study Number:
Date of first visit:

REPORTING CLINICIAN

1. Dr's Name (please print) ……
2. Month/Year of Report ☐☐ / ☐☐
3. Date diagnosis considered ☐☐ / ☐☐ / ☐☐

CHILD'S DETAILS

4. First Letter of Forename ☐
5. Month and year of birth ☐☐ / ☐☐☐☐
6. Gender M ☐ F ☐
7. Postal code ☐☐☐☐☐☐
8. Gestation (wks) ☐☐

BIRTH MOTHER'S DETAILS

9. Current Age …….
10. Country of Birth
11. Ethnic Origin: ☐ South Asia ☐ North -African ☐ Other Africa
    ☐ Middle- Eastern ☐ Chinese ☐ Caucasian
    ☐ Other……

PRESENTATION

Date of Clinic: Date of referral:

Source of referral:
- ☐ A&E
- ☐ GP
- ☐ Inpatient Medical
- ☐ OPD Medical
- ☐ Orthopaedic
- ☐ Other……

Reasons for referral:
- ☐ Gait disturbance
- ☐ Bowed leg
- ☐ Known Rickets
- ☐ Pain
- ☐ Fractures
- ☐ Swollen Wrists
- ☐ Other
- ☐ Delayed walking
- ☐ Family history of Vit D def
- ☐ Seizure
- ☐ Abnormal blood results
- ☐ Dietary Concerns
- ☐ Abnormal X ray

Abnormal X-Rays findings……
Abnormal blood findings……

MATERNAL VITAMIN D STATUS

Antenatal supplementation: Name: Start Month/Yr: Dose:

Recommended By: ☐ HV ☐ GP ☐ Other

Other……

Current supplementation: Name: Start Month/Yr Dose:

Recommended By: ☐ HV ☐ GP ☐ Other

Other……

Known Mat Vit D Def: ☐ Yes ☐No

Maternal Clothing that limits sun exposure: ☐ Yes ☐ No

Parental Understanding of Vit D: ☐ None ☐ Some ☐Complete

PARENT CLINICAL DETAILS

Asthma Mother ☐ Father ☐

Skin disease Mother ☐ Father ☐

Allergy Mother ☐ Father ☐

Hypertension Mother ☐ Father ☐

Diabetes Mother ☐ Father ☐ Other……..

CHILD'S NUTRITIONAL & VITAMIN D STATUS

Prior Vitamin supplementation: Name: Duration:

Dose:

Recommended by: ☐ HV ☐ GP ☐Other

Other……

Breast feeding duration: …….

Age at weaning:

Diet at presentation: ☐ Breast Milk (BM) only
☐ Formula milk (FM) only
☐ BM + FM
☐ BM + solids
☐ FM solids
☐
Other……..

Dietary problems: ☐ None
☐ Yes*

*Details……

Coexisting conditions: ----------

CHILD'S CLINICAL DETAILS

Asthma ☐
Skin disease ☐
Allergy ☐
Diabetes ☐ Others……..

CHILD'S EXAMINATION FINDINGS AT PRESENTATION

Age at walking: ☐ Not walking yet

Weight: Height: OFC:

☐ Bowed Legs ☐ Abnormal Dentition ☐ Wrist Swelling ☐ Weakness
☐ Rachitic rosary ☐ Patent Ant Fontanelle ☐ Bone pain ☐ Abnormal gait
☐ Frontal Bossing Other…… ……

TREATMENT RECEVING OF VITAMIN D DEFICIENCY

Drug	**Name**	**Start Date**	**Dose**	**Administration Method**	**Stop Date**
Drug 1					
Drug 2					
Drug 3					
Other					

RESULTS OF INVESTIGATIONS BEFORE VITAMIN D TREATMENT

Biochemistry	**Ca**	**PO**	**PTH**	**ALP**	**25(OH)D**
Date					
Result					
Reference Range					
Measurement unit					

Biochemistry	**Glucose**	**Insulin**	**HbA1c**	**SHBG**
Date of blood taken				
Result				
Reference Range				
Measurement unit				

Biochemistry	**Total cholesterol**	**HDL**	**LDL**
Date of blood taken			
Result			
Reference Range			
Measurement unit			

X-rays	**Specify site**	**Clear Rickets**	**Healing Rickets**	**No Rickets**
Site 1				
Site 2				
Site 3				

Vitamin D deficiency and its potential effect on bone status, immune and glucose homeostasis among children in Scotland.

Six Week visit

Hospital Number:

Study Number:

Date of the visit:

Weight ……. Height ……..

Any change in the medical history
……
……
Any medication
……
……
Adverse effect
……
……

RESULTS OF INVESTIGATIONS AFTER VITAMIN D TREATMENT

Biochemistry	**Ca**	**PO**	**PTH**	**ALP**	**25(OH)D**
Date					
Result					
Reference Range					
Measurement unit					

Biochemistry	**Glucose**	**Insulin**	**HbA1c**	**SHBG**
Date of blood taken				
Result				
Reference Range				
Measurement unit				

Biochemistry	**Total cholesterol**	**HDL**	**LDL**
Date of blood taken			
Result			
Reference Range			
Measurement unit			

Appendix C–1- Child information sheet

Department of Child Health
Royal Hospital for Sick Children
Yorkhill, Glasgow
G3 8SJ, United Kingdom
faisal.ahmed@glasgow.ac.uk

A STUDY LOOKING AT GLUCOSE CONTROL AND IMMUNE FUNCTION IN CHILDREN WITH VITAMIN D DEFICIENCY

NEW PATIENT

INFORMATION SHEET FOR CHILDREN (6-9 YRS)

- You are being asked if you would like to take part in some work which we are doing at Yorkhill hospital.
- This work is looking at the effects of something called 'Vitamin D'.
- Because your Vitamin D level is low you need some blood tests and some treatment.
- These tests will be done when you start Vitamin D and then after a few weeks to check the treatment.
- We would like to take an extra blood sample when you come for these tests.

Please read this sheet and talk to your family about it. Take your time and ask us if anything is not clear.

- If you do take part, please make sure you do not eat or drink any milk or juice before coming for your tests.
- If you do take part, we will take a sample at the same time as your other tests. You will not have an extra jag.
- We will use special cream for the jag to make sure you don't feel any pain.

You do not need to take part in this work if you don't want to.

Please ask your parents, the doctors or nurses anything you want to know about the study.

THANK YOU FOR READING THIS

Appendix C–2- Child consent form

Study Number:

Patient Identification Number:

CONSENT/ASSENT FORM FOR CHILDREN 6-9 YEARS

A STUDY LOOKING AT GLUCOSE CONTROL AND IMMUNE FUNCTION IN CHILDREN WITH VITAMIN D DEFICIENCY

Name of Researcher: Dr ……

(Please initial boxes)

I have read the Information Sheet (Please take a copy with you to keep)	
I have asked questions about the study	
My questions have been answered	
I have spoken to the following people about the study (Please add name below) …… ……	

☐ I agree to for an addition blood sample to be taken.

Name of patient	Date (Day / Month / Year)	Signature (Childs)ASSENT
Name of parent / guardian 1	Date (Day / Month / Year)	Signature (parent guardian)
Name of parent / guardian 2 (if applicable)	Date (Day / Month / Year)	Signature(parent guardian)
Investigator	Date (Day / Month / Year)	Signature (investigator)
Name of person taking consent (if different from investigator)	Date (Day / Month / Year)	Signature(researcher if different from investigator

THANK YOU FOR YOUR HELP

Investigator to keep original form; 1 copy to be given to patient; 1 copy to be kept with hospital notes

Appendix C–3- Parent information sheet

Department of Child Health
Royal Hospital For Sick Children
Yorkhill, Glasgow
G3 8SJ, United Kingdom
faisal.ahmed@glasgow.ac.uk

A STUDY LOOKING AT GLUCOSE CONTROL AND IMMUNE FUNCTION IN CHILDREN WITH VITAMIN D DEFICIENCY

PARENT INFORMATION SHEET FOR NEW CHILDREN

We are asking you and your child's help with a study. This leaflet explains the study, so please read it carefully and asks any questions. You can discuss the leaflet with anybody you want. If you have any difficulty understanding the information, we can arrange for an interpreter to help you.

What Is The Purpose Of This Study
There is some suggestion that Vitamin D may help how the body controls the sugar levels in blood. Vitamin D may also be helpful in fighting infection and how the body copes with allergies. We would like to find out whether children with Vitamin D deficiency have any of these features. We would also like to know whether these features change with treatment.

Why Has My Child Been Chosen?
There is a very strong suspicion that your child has Vitamin D deficiency and your child needs treatment with Vitamin D. Your child will be attending the hospital for tests and for the first dose of Vitamin D.

What Will Happen To My Child & I In The Study?
We will first discuss the study with you when you bring your child to the clinic. We will provide you with the information sheet and go through it with you in the clinic. When you and your child come to the hospital in two weeks for a blood test and treatment for Vitamin D deficiency, we will take some extra blood sample, if you are agreed to take part. The amount of sample will be about two tea-spoons. For the study, we are requesting that, on the day of the blood test, you bring your child to the hospital in the morning before your child has the first meal or milk feed. We will do this again when your child returns for a check of Vitamin D treatment.

What Do I Need To Do In The Study?
If you agree to participate, we will collect extra blood sample from your child when your child attends for the test. On the day of the tests, you need to make sure that your child does not have the morning meal or feed. Please remember, that if you decide not to take part in the study, your child will still have the tests and the Vitamin D. We will just not take the extra sample for the study.

Will The Study Cause Any Discomfort Or Inconvenience
You and your child will need to attend the ward first thing in the morning; we will arrange transport if you have any difficulties in attending and will discuss this with you beforehand. Your child may feel hungry in the morning when attending the ward but will be able to eat as soon as the blood test is completed. You may have to wait to have the tests done but we will make sure that they get done as soon as possible. For the blood your child will need a jag and we will make sure that these procedures are as painless as possible by using a local anaesthetic cream.

What Will Happen To My Child & I After The Study?
After the study, you will continue to come to the routine clinic at the hospital until there is a clear improvement in your child's condition. Your child may need further xrays, tests or scans but this will be decided by the doctors in the clinic. When the results of the study are published, you or your child's name will not be shown, so there is no way of anyone knowing that you have taken part unless you tell them.

What Will Happen To My Child If Any Of The Study Tests Show Any Abnormality?
We are not expecting that any of the study tests will show any abnormality that requires any action. We think that if there will be any abnormalities than they will be very mild. The team of doctors who are doing the research are specialists and will discuss any important results with you in the clinic.

Are There Any Benefits To My Child Of Taking Part In The Study?
There are no obvious benefits of taking part in the study as your child will still have the treatment whether your child takes part or does not take part in the study.

Do I Have To Take Part?

YOU DO NOT HAVE TO SAY 'YES' to taking part in this study if you do not want to, and you can come out of the study at any time if you are unhappy.

What If Something Goes Wrong?
We will take every care in the course of this study but we are not expecting any problems. However, if you are not happy about anything about this study, talk to other members of your family, your family doctor and ask them to speak to us. If your child is harmed due to someone's negligence, then you may have grounds for a legal action but may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way your child has been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you. Taking part in this study would not affect your child's legal rights.

Will My Taking Part In This Study Be Kept Confidential?

All information which is collected about your child during the course of the research will be kept strictly confidential. Any information about you that leaves the hospital will have name and address removed so that your child cannot be recognised from it.

Who Is Organising The Study?

The study is being organised by a group of doctors who look after children with Vitamin d deficiency at Yorkhill. You will find the main doctors' details below.

Who Is Helping These Doctors With The Study?

This study is being performed as an educational project by a doctor who is doing her PhD. She is supported by an educational grant from the Government of Libya.

What Will Happen To The Results Of The Study?

The results of this study will be discussed with other doctors who work with children. They will be discussed at meetings and written up as articles. Do let us know if you would like to see a copy of these results. The results will also be used to design future studies of Vitamin d deficiency.

Who Has Looked At his Study?

The study has also been checked and approved by a number of organisations including the:-

- NHS Research Ethics Committee in Glasgow
- NHS Research & Development Dept in Glasgow
-

Who Can I Contact For Further Information?

If you want to discuss the study further with one of the research team, please contact:
Professor SF Ahmed - 0141 201 0571, faisal.ahmed@glasgow.ac.uk
Dr Nagla El-Fakhri - 0141 201 0502, n.el-fakhri.1@research.gla.ac.uk
If you want to discuss the study with a doctor who is not directly linked to the study, please contact: **Dr MG Shaikh- 0141 201 1889, guftar.shaikh@nhs.net**

A copy of this information sheet and consent form will be given to you to keep. If you have any further questions about the study, please feel free to ask the doctor or nurse at the clinic your child attends at Yorkhill.

THANK YOU FOR READING THIS

Appendix C–4- Parent consent form

Study Number:

Patient Identification Number:

PARENT / LEGAL GUARDIAN CONSENT ASSENT FORM

A STUDY LOOKING AT GLUCOSE CONTROL AND IMMUNE FUNCTION IN CHILDREN WITH VITAMIN D DEFICIENCY

Name of Researcher: Dr ……

(Please initial)

I have read the Parent / Guardian Information Sheet (Please take a copy with you to keep)	
I have had the opportunity to discuss the study and ask questions	
I have received satisfactory answers to all my questions	
I have received enough information about the study	
I have spoken to the following people about the study (Please add name below) ……	
I understand that my child can withdraw from the study at any time without giving a reason and without affecting their medical care	
I give permission for my child's family doctor to be told of my child's involvement in the study	

☐ I agree for an additional blood sample to be taken

Name of patient	Date (Day / Month / Year)	Signature
Name of parent / guardian 1	Date (Day / Month / Year)	Signature
Name of parent / guardian 2 (if applicable)	Date (Day / Month / Year)	Signature

Investigator	Date (Day / Month / Year)	Signature
Name of person taking consent (if different from investigator)	Date (Day / Month / Year)	Signature

THANK YOU FOR YOUR HELP

Investigator to keep original form; 1 copy to be given to patient; 1 copy to be kept with hospital notes

Appendix D–1- Clinic check list for eligible participant for the study

Bone status, glucose homeostasis and immune function in children with vitamin D deficiency

Clinic check list for eligible participant for the study

1. Exclusion and inclusion criteria ☐
2. Information sheet handed ☐
3. Verbal explanation ☐

4. Investigation requested:

5. A: Routine (Hospital laboratory)

☐

U&E's
PTH
Bone profile
Full blood count
25(OH) D

4. B: Blood sugar profile (Hospital laboratory)

☐

Fasting blood sugar
HbA1c
SHBG

4. C: Department of child health (DCH)

☐

Lipid profile
Insulin
Urine
Immunie study
Cytokines
Lymphocytes

6. Participant needs x- ray and/or echo ☐
7. Vitamin D prescription prepared ☐
8. Appointment for the result discussion ☐
9. Patient notified 4 days before the date of the first test ☐
10. Alert the nurses ☐

Appendix D–2- A letter for a general practitioner (GP)

Royal Hospital for Sick Children, Dalnair Street, Glasgow, G3 8SJ

Endocrine Services

Consultants: *Dr A Mason Professor S F Ahmed Dr M G Shaikh Dr H McDevitt*
Specialist Nurses: *Ethel McNeill Vicky Campbell*
Occupational Therapist: *Liz Dougan*
Dietician: *Jill Morrison*

*Contact Details
☎ 0141 201 0000 (Main Switchboard)
☎ 0141 201 0767 (Professor Ahmed's Secretary)
☎ 0141 201 0571 (Professor Ahmed direct line)

✉kathleen.gibson2@ggc.scot.nhs.uk
☎ 0141 201 0245 (Specialist Endocrine Nurses)
☎ 0141 201 0149 (Occupational Therapist)
☎ 0141 201 9368 (Dietitian)

Fax: +44 (0) 141 201 0562

Date----------
GP name----------
GP address---------

Dear Dr ----------

Child name -----, DOB ---/--/--, Address----------, CHI--------

The above patient has been participating in the Vitamin D - Metabolic and Immune study. A summary of this patient's involvement in the study is outlined below.

First study visit date: 15/04/2014

1. Clinical samples collected – YES
2. Study samples collected – YES
3. Vitamin D (cholecalciferol) - Solgar 5000IU daily for 6 weeks- YES

Second study visit date: 29/05/2014

1. Clinical samples collected – YES
2. Study samples collected – YES
3. Multivitamin prescription-YES

Follow Up Clinic Consultant: ---------- **Date:** --/---/---

Yours sincerely

Nagla El-fakhri
PhD student, Child Health
Cc Doctor ---------- Consultant Paediatrician

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DE GRUYTER DOI 10.1515/jpem-2013-0175 — J Pediatr Endocr Met 2013; x(x): xxx–xxx

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An electronic surveillance system for monitoring the hospital presentation of nutritional vitamin D deficiency in children in Scotland

Abstract

Background: Routine surveillance would be valuable for vitamin D deficiency as symptomatic vitamin D deficiency may be common in Scotland.
Aim: To assess the effectiveness of an electronic surveillance system to determine the current incidence of hospital-based presentation of childhood vitamin D deficiency in Scotland.
Methods: Active surveillance was performed for 2 years as part of an electronic web-based surveillance programme by the Scottish Paediatric Surveillance Unit. Notifications were followed by completion of a questionnaire. To further examine the validity of the system, cases with severe vitamin D deficiency in Glasgow and Edinburgh were identified from the regional laboratory and their clinical details were checked against those identified through the surveillance system.
Results: Between September 2009 and August 2011, 109 cases of vitamin D deficiency were notified. The majority of cases (n=82) were reported in Glasgow with an annual incidence of 41 cases per year. Fourteen cases were reported in Edinburgh during the first year of the study and two cases during the second year. At the time of clinical diagnosis, the median age of the children was 2 years (range 3 months–16 years). Cross-validation of data showed that among symptomatic cases that had a measured serum vitamin D of <14 nmol/L, 89% of eligible cases had been reported in Glasgow and 33% of cases had been reported in Edinburgh.
Conclusion: The incidence of vitamin D deficiency remains high in Scotland. An electronic surveillance system can provide data for studying the epidemiology of vitamin D deficiency but may underestimate the number of positive cases.

Keywords: rickets; surveillance.

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Introduction

Vitamin D deficiency, which is associated with nutritional rickets, has been historically recognized as a widespread health problem in the UK (1, 2). The increase in the occurrence of vitamin D deficiency among the population in Scotland has been attributed mainly to its northerly location, insufficient dietary intake and population mix (3). As a consequence, a number of preventative measures have been introduced in an attempt to decrease the prevalence of vitamin D deficiency, especially among vulnerable groups such as ethnic minorities, pregnant and lactating women. A Greater Glasgow Health Board campaign was launched in 1979 targeting vitamin D supplementation among the Asian community (4, 5). Although guidelines for vitamin D supplementation were issued in the UK in 1994, a survey carried out in 2001 showed that vitamin D deficiency still exists, raising concern over the ineffectiveness of public health measures (6). As a result, the Healthy Start Scheme was implemented in 2006, but recent surveys indicate that symptomatic vitamin D deficiency is still prevalent among children in the UK (7, 8). Anecdotal reports suggest that vitamin D deficiency occurs across Scotland, but the relative geographical incidence is unclear (9, 10). The Scottish Paediatric Surveillance Unit (ScotPSU) has recently been developed as an electronic version of the British Paediatric Surveillance Unit (BPSU) which has been very successful at monitoring the incidence of rare childhood conditions in the UK (11). The aims of the current study were two-fold: firstly, to study

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Mini Review

HORMONE RESEARCH IN PÆDIATRICS

Horm Res Paediatr 2014;81:363–378
DOI: 10.1159/000357731

Received: September 18, 2013
Accepted: December 4, 2013
Published online: April 26, 2014

Vitamin D and Its Effects on Glucose Homeostasis, Cardiovascular Function and Immune Function

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Key Words
Vitamin D · Extraskeletal benefits · Immune function · Cardiovascular function · Glucose homeostasis

Abstract
In recent years there has been increasing interest in the non-skeletal effects of vitamin D. It has been suggested that vitamin D deficiency may influence the development of diabetes, cardiovascular dysfunction and autoimmune diseases. This review focuses on the current knowledge of the effects of vitamin D and its deficiency on cardiovascular function, glucose homeostasis and immune function, with a particular focus on children. Although, there is good evidence to show that there is an association between vitamin D deficiency and an abnormality of the above systems, there is little evidence to show that vitamin D supplementation leads to an improvement in function, especially in childhood.

Vitamin D Overview

Most plants and animals that are exposed to sunlight have the ability to produce vitamin D. In humans, the active vitamin D metabolite, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), has been well recognized for its role in calcium and phosphate homeostasis [1]. However, intensive research over the last two decades has indicated that vitamin D may also be a critical modulator of several non-skeletal systems and related diseases. Vitamin D refers to two biological precursors – ergocalciferol (D_2) and cholecalciferol (D_3). Vitamin D_3 constitutes around 80–90% of the circulating metabolites and is synthesized mainly in the skin by the action of ultraviolet (UV) light (280–315 nm). Vitamin D_3 can also be obtained exogenously from animal sources such as fish oils or fortified dairy products and vitamin supplements. Sun-dried mushrooms, UV-B irradiation of the yeast sterol ergosterol, as well as vitamin supplements are considered as main sources of vitamin D_2 [2]. Vitamin D (D_2, D_3) has no biological activity without a two-step hydroxylation process. The first step, in the liver, requires P450 enzymes such as CYP2R1 and CYP27A1 (25-hydroxylases) to form the major circulating form 25(OH)D. The second step, in the kidneys, requires the P450 enzyme, CYP27B1 (1α,25-hydroxylase) to form the main active metabolite – 1,25(OH)D or calcitriol. The second hydroxylation reaction is stimulated mainly by parathyroid hormone (PTH), and inhibited by calcium, phosphate and fibroblast growth factor-23 (FGF-23) [3]. The biological action of $1,25(OH)_2D$ is mediated through the vitamin D receptors (VDRs) and this receptor as well as CYP27B1 are expressed widely in several tissues [4] (fig. 1).

Vitamin D deficiency has been defined by the Institute of Medicine (IOM) as a measured serum 25(OH)D of <30 nmol/l (12 ng/ml), vitamin D insufficiency when the se-

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1663–2818/14/0816–0363$39.50/0

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