Vitamin D and Skeletal Health: Across the Life Course

The University Of Sheffield.

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Dedication

Guru Brahma Guru Vishnu
Guru Devo Maheshwaraha II
Guru Saakshaat ParaBrahma
Tasmai Sri Gurave Namaha II

The Teacher (Guru) is like Brahma, Vishnu and Shiva

He creates, sustains knowledge and destroys the weeds of ignorance

The teacher is indeed the Primordial Omnipotent God and

My salutations to a Teacher so venerable

Dedicated to

My parents who are my first Gurus

Shri G Kothandapani and Smt K Pankajam

My teachers and mentors

Professor Nick J Bishop and Professor Leena Patel

My Patients, Study Participants and their Parents

My husband Gopal from whom I learn the meaning of a beautiful life
&
My girls Shreya and Shrada from whom I learn the meaning of my life

Declaration

In accordance with the guidance issued by the University of Sheffield this thesis includes published works and works submitted for publication in which am the first and the corresponding author.

I have included the written statements from all the co-authors, and my supervisor Prof Nick J Bishop is aware of all my correspondence from all co-authors attesting to my contribution to the joint publications.

The contribution statements from all the co-authors were obtained and submitted to the journals.

Acknowledgements

Finally, the odyssey has ended! It gives me immense pleasure to thank all my teachers, mentors, colleagues, friends, family and funders for shouldering me to successfully complete my research projects and submit this thesis. The list is endless and to name a few….

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Attribution Statement

I conducted the literature review on Vitamin D and Bone and wrote the introduction chapter 1. Prof Bishop revised and approved the final version of this chapter.

I am the principal investigator for the Vitamin D and Vibration [VIVID] Study (Chapter 2), and Vitamin D Dosing study (Chapter 4). Both these studies are prospective single centre clinical trials. Prof Bishop proposed the study design(s) and provided me with the academic and research supervision. I secured open-competition grants for both these trials. I conducted the literature review, wrote the original protocols for both the studies and the subsequent amendments based on the reviewer's comments. I liaised with Research and Development (R&D), Clinical Research Facility (CRF) at Sheffield Children's Hospital, and Royal Hallamshire Hospital, pharmacy, biochemistry laboratory, genetics and clinical laboratory at SCH in order to set-up the studies.

I conducted a patient and public involvement (PPI) event for the VIVID study (chapter 2) and amended the protocol based on the comments received from the PPI event. I wrote the participants' Information Sheets (PIS), consent forms, advertising flyers and all other supporting documentation for both the studies. I applied for and obtained the Research Ethics Committee (REC) approval and the Health Research Authority (HRA) approvals and made amendments requested by REC and HRA. I also acquired the Research and Development (R&D) approval for the study. I advertised the studies, recruited participants based on the inclusion criteria, arranged for their study visits to take place at clinical research facility for Vitamin D dosing study and at their homes for the VIVID study. I purchased and distributed the gift vouchers for my study participants in recognition of their time and dedication. I trained parents of VIVID study participants how to use the vibration platform for the VIVID study. I collected and entered the data in SPSS, conducted preliminary statistical analysis and interpretation for both the studies. I maintained the site management files and conducted the overall management

of the studies. I wrote letters to the GP's of my vitamin D dosing study participants with abnormal vitamin D results for appropriate management. Prof Rigby conducted the final statistical analysis. I wrote the initial draft of the manuscripts; the other co-authors revised the manuscripts. Prof Bishop contributed to the data analysis, revised and approved the final version of the manuscripts. I am the corresponding author for both the manuscripts. I have submitted the VIVID study (chapter 2) manuscript to the Journal of Musculoskeletal and Neuronal Interactions [JMNI]. I published the vitamin D dosing study (chapter 4) manuscript in the Clinical Endocrinology journal and was responsible for all the communications with the journal. I drafted the rebuttal to the reviewer's comments and submitted the amended version of the manuscript.

I am the co-investigator for the Vitamin D and Fracture study (Chapter 3), Dr Amaka C Offiah proposed and contributed to the study design, implementation of the project, scoring of the radiographs as observer 1, analysis of results and to the writing of the manuscript. Dr Amaka C Offiah provided me with the research supervision for this project and Prof Bishop provided me with the general supervision. I contributed to scoring of the radiographs as observer 2, statistical analysis, drafting the manuscript and designed the tables and figures. Ms Elaine Pang contributed to designing the spreadsheet and preliminary data collection. Dr Alan Sprigg contributed as observer 3 in arbitrating the discrepancies in radiographic scoring between observer 1 and 2 and to the writing of the manuscript. Prof Bishop and Prof Mughal critically reviewed the manuscript.

For all the three studies, bone profile, parathyroid hormone and urinary calcium creatinine ratio were performed in the biochemistry laboratory at SCH. Vitamin D was measured by the biochemistry laboratory at Bristol Royal Infirmary. Vitamin D binding protein genotypes were performed by the genetic lab at Sheffield Children's hospital for vitamin D dosing study and bone turnover markers were measured by the metabolic bone department at the Royal Hallamshire Hospital for both Vitamin D dosing study and VIVID study.

Presentations, Prizes & Publications

Chapter 2: Vitamin D and Vibration (VIVID) study

Title	**Maternal pregnancy vitamin D supplementation increases offspring bone formation in response to mechanical loading: Findings from a MAVIDOS Trial sub-study**
Grants	Patient and Public involvement grant (£564) Researchers Night exhibition grant (£62.50) Experimental Arthritis Treatment Centre [internal] grant (£6000)
Presentations: Local	**30th Sep 2016, Researchers' night: Faculty wide outreach event:** I organised an 'exhibition stall' where members of the public had the opportunity to experience the whole body vibration (WBV) platforms, used for this study.  **24th Feb 2016, Patient and Public Involvement (PPI) focus group:** I conducted a PPI focus group meeting and adapted my study protocol based on the feedback invited from mothers with young children. **28th Oct 2015, Vibration Vibe Forum:** I organised regular lunchtime meetings, for my fellow researchers and colleagues where we shared our ideas, learn from, support each other, discuss, and debate on relevant literature to advance our research skills on projects that involve the use of whole body vibration as a means to assess bone responsiveness to mechanical stimulation.
International	Oral poster, 23rd Jun 2019, 9th International Conference on Children's Bone Health, Salzburg, Austria
Abstract Publications	Gopal S, Rigby A, Moon R et al. (2019) Response of bone to mechanical stimulation in the offspring of MAVIDOS study mothers in a single centre; the effect of antenatal vitamin D supplementation. Bone Abstracts. doi: 10.1530/boneabs.7.p5
Journal Publications	Gopal-Kothandapani, J. S., Rigby, A. S., Harrison, R., Eastell, R., Moon, R. J., Curtis, E. M., Cooper, C., Harvey, N. C., & Bishop, N. (2020). Maternal pregnancy vitamin D supplementation increases offspring bone formation in response to mechanical loading: Findings from a MAVIDOS Trial sub-study. Journal of musculoskeletal & neuronal interactions, 20(1), 4–11.

Chapter 3: Vitamin D Fracture study

Title	**Unexplained Fractures in Infants & Young Children: (Ir)relevance of Serum Vitamin D**
Grant	Paediatric Endocrine Society (PES) travel grant £500
Presentations: National	Oral, 7th April 2017, British Paediatric and Adolescent Bone Group (BPABG) spring meeting, London, UK Poster, 25th Nov 2017, BRIC [BAPIO RESEARCH AND INNOVATION COMPETITION] conference, Manchester, UK
International	Oral, 28th Nov 2015, Radiological Society of North America (RSNA) Conference, Chicago, USA Poster, 16th Sep 2017, 10th International Meeting of Paediatric Endocrinology, Washington, USA
Prizes	**Best poster prize - BRIC [BAPIO RESEARCH AND INNOVATION COMPETITION] conference, Manchester, UK** 
Abstract Publications	Gopal-Kothandapani J, Pang E, Sprigg A, Offiah A (2017) Unexplained Fractures In Infants And Young Children: (Ir) Relevance Of Serum Vitamin D.10th International Meeting of Pediatric Endocrinology: Hormone Research in Paediatrics 88:1-628. doi: 10.1159/000481424
Journal Publications	In progress

Chapter 4: Vitamin D Dosing study

Title	**Effect of vitamin D supplementation on free and total vitamin D: A comparison of Asians and Caucasians**
Grants	Sir Halley Stewart Trust, £20,000 Internis pharmaceuticals, £5000
Presentations: Local	Oral, 11th May 2015, The University of Sheffield
Regional	Oral, 4th Dec 2015, The 6th Annual Mellanby Centre Research Day, Sheffield
National	Oral, 25th Nov 2017, BRIC [BAPIO RESEARCH AND INNOVATION COMPETITION] conference, Manchester, UK E-poster, 26th Nov 2015, British Society of Paediatric Endocrinology and Diabetes (BSPED) conference, Sheffield, UK
International	Poster, 1st Oct 2015, European Society of Paediatric Endocrinology (ESPE) conference, Barcelona, Spain Poster, 15th May 2016, European Calcified Tissue Society (ECTS) conference, Rome, Italy
Abstract publications	https://www.endocrine-abstracts.org/ea/0039/ea0039ep13.htm http://abstracts.eurospe.org/hrp/0084/hrp0084lbp-1266.htm http://www.bone-abstracts.org/ba/0005/ba0005p97.htm
Journal Publications	Gopal-Kothandapani J, Evans L, Walsh J et al. (2018) Effect of vitamin D supplementation on free and total vitamin D: A comparison of Asians and Caucasians. Clinical Endocrinology 90:222-231. doi: 10.1111/cen.13825

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aBMD	areal Bone Mineral Density
BMI	Body mass index
BMC	Bone Mineral Content
BMD	Bone Mineral Density
BMI	Body mass index
BMU	Bone Multicellular Unit
Bone ALP	*Bone Alkaline Phosphatase*
BPABG	British Paediatric and Adolescent Bone Group
BTM	Bone turnover markers
CI	Confidence interval
CK	Creatinine kinase
COMA	Committee on the Medical Aspects of Food Policy
COPD	Chronic obstructive pulmonary disease
CP	Cerebral palsy
CSF-1	Colony Stimulating Factor-1
CTX	Cross-linked C-terminal telopeptide of type I collagen
CV	Co-efficient of variation
CYP	Cytochrome *P450 enzymes*
DBP	Vitamin D Binding Protein
DH	Department of Health
DHCR7	7-dehydrocholesterol reductase
DMD	Duchenne Muscular Dystrophy
DPD	Deoxypyridinoline
DXA	Dual energy X-ray absorptiometry
eSS	Extra-cellular Steady State
GMFM	Gross Motor Function Measurement
HNF1	Hepatocyte Nuclear Factor1
HOMA-IR	Homeostatic model assessment of insulin resistance
ICTP	Carboxy-terminal telopeptide of type I collagen
IOM	Institute of Medicine
iSS	Intracellular Steady State
ITW	Idiopathic Toe Walking
IU	International Unit
kDA	Kilo Dalton

LM	Lean Mass
MAF	Macrophage Activating Factor
MAS	Modified Ashworth Scale
MRCT	Multicentre, double-blind, randomised, placebo-controlled trial
MAVIDOS	Maternal Vitamin D Osteoporosis trial
MCP-1	Monocyte Chemoattractant Protein-1
METs-	Metabolic equivalents
NADSYN1	NAD synthetase 1
NDNS	The National Diet and Nutrition survey
NF-κB	Nuclear Factor - kappa light chain enhancer of activated B cells
NHS	National Health Service
NICE	National Institute for Health and Clinical Excellence
nmol/L	Nanomole Per Litre
NTX	Cross-linked N-terminal telopeptide of type I collagen
OGTT	Oral Glucose Tolerance Test
OI	Osteogenesis Imperfecta
OPG	Osteoprotegerin
OR	Odds ratio
OSI	Overall Stability Index
P1NP	N-terminal propeptide of type I procollagen
PTH	Parathyroid hormone
PYD	Pyridinoline
RANKL	Receptor activator of nuclear factor kappa-B ligand
RDNI	Recommended Daily Nutrient Intake
RGD	Tri-amino acid sequence, Arginine-Glycine-Aspartate
SACN	Scientific Advisory Committee on Nutrition
SMA	Spinal Muscular Atrophy
SNPs	Single Nucleotide Polymorphisms
TRACP	Tartrate resistant acid phosphatase
UK	United Kingdom
US	United States of America
UV	Ultraviolet
VDBP	Vitamin D Binding Protein
VDD	Vitamin D deficiency
VDI	Vitamin D insufficiency
VDR	Vitamin D receptor
Vitamin D2	Ergocalciferol

Vitamin D3	Cholecalciferol
vTBMD	volumetric Trabecular BMD
WBV	Whole Body Vibration
$1,25(OH)_2D$	1, 25-dihydroxyvitamin D
25OHD	25 hydroxyvitamin D
6MWT	Six-minute walking test

Chapter 1: Introduction

1.1 Vitamin D

Vitamin D is not only important for skeletal health but also for immune function, insulin sensitivity, muscle strength and cardiovascular function (1). As sunlight is the main source of vitamin D in the UK (2) we are dependent on our body stores and dietary sources of Vitamin D during winter months (3). The Committee on the Medical Aspects of food policy(COMA) [DH 1991] (4) and National Institute for Health and Clinical Excellence (NICE) [2012] (5) issued evidence based guidelines on vitamin D supplementation for at risk groups; however these recommendations were not fully implemented and there has been a resurgence of rickets in UK [Figure 1.1] (6) (7) (8) (9).

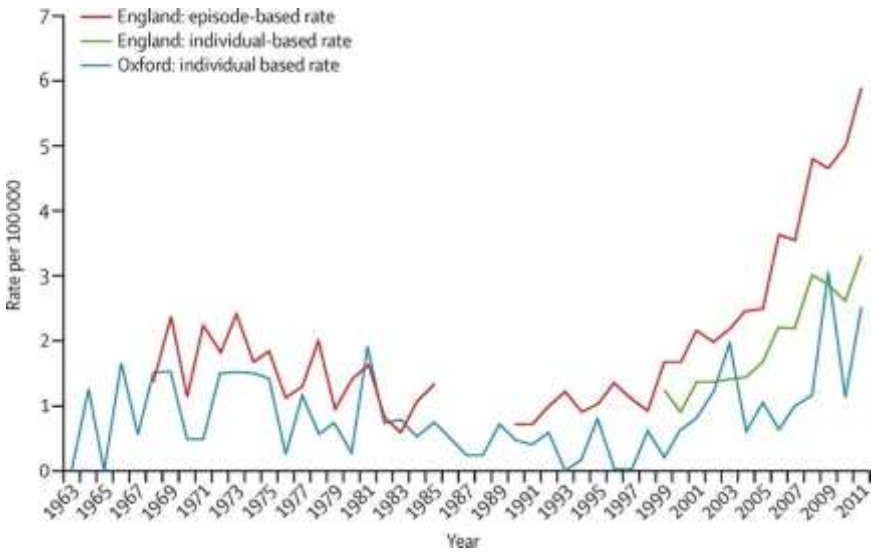

Figure 1.1 Increased rate of hospitalisation with Rickets in children younger than 15 years. Red: Episode-based rates (hospital discharges) for England, 1968–2011. Green: individual-based rates for England, from 1999. Blue: individual-based rates for Oxford, from the Oxford record linkage study.
Reproduced with permission from Goldacre et al, (8).

The Scientific Advisory Committee on Nutrition (SACN) has reported that around 30-40% of the UK population had deficient levels of Vitamin D (serum total 25OHD <25 nmol/L) in the winter compared to 2-13% in the summer (10). And 53% of South Asian women in Southern England and 29% of pregnant women in North West England did not attain a serum total 25OHD concentration ≥ 25 nmol/L. And the annualised mean serum total 25OHD concentration was much lower in Asian (20.5 nmol/L) and African (27.7 nmol/L) adults (≥ 16 years) when compared to white adults (45.8 nmol/L). The age groups with the highest frequency of low circulating vitamin D levels are infants, older teenagers/young adults and the elderly (10).

The recommended serum total 25OHD concentration at any time of the year to protect musculoskeletal health for all individuals in the UK is >25 nmol/L. In order to achieve that, SACN has issued guidance on the recommended daily nutrient intake (RDNI) of Vitamin D as 400 IU during the winter months. Safe intakes (10) recommended for infants and children under the age of four years was between 340 and 400 IU per day [Table 1.1]

<table>
<tr><th rowspan="2">Population Groups</th><th colspan="2">RDNI of Vitamin D per day</th></tr>
<tr><th>Winter (Oct - Mar)</th><th>Summer (Apr - Sep)</th></tr>
<tr><td>General population (4 years and above)</td><td rowspan="2">Not routinely recommended</td><td rowspan="2">400 IU</td></tr>
<tr><td>Pregnant and breast-feeding women</td></tr>
<tr><td>All breastfed and formula fed infants (0-1 years) receiving <500 mls of formula per day</td><td colspan="2">340 - 400 IU</td></tr>
<tr><td>All children aged 1- 4 years</td><td colspan="2" rowspan="3">400 IU</td></tr>
<tr><td>Little or no sunshine exposure</td></tr>
<tr><td>Minor ethnic groups with dark skin – African, African-Caribbean and South Asian</td></tr>
</table>

Table 1.1 Recommended Daily Nutrient Intake of Vitamin D

There is an increasing evidence in the literature that the circulatory levels of 25-hydroxyvitamin D (25OHD) is not only determined by factors such as age, season, sunlight, sunscreen, clothing, diet and

vitamin supplements but also by inherited characteristics such as ethnicity, skin colour, vitamin D binding protein (VDBP) level, VDBP genotype and other factors such as adiposity (11).

1.2 Vitamin D synthesis and metabolism

Vitamin D is a 9, 10 secosteroid and so far six forms of Vitamin D have been identified (12). Of the six forms, Vitamin D2 and Vitamin D3 [Figure 1.2] are of prime importance in human nutrition (13).

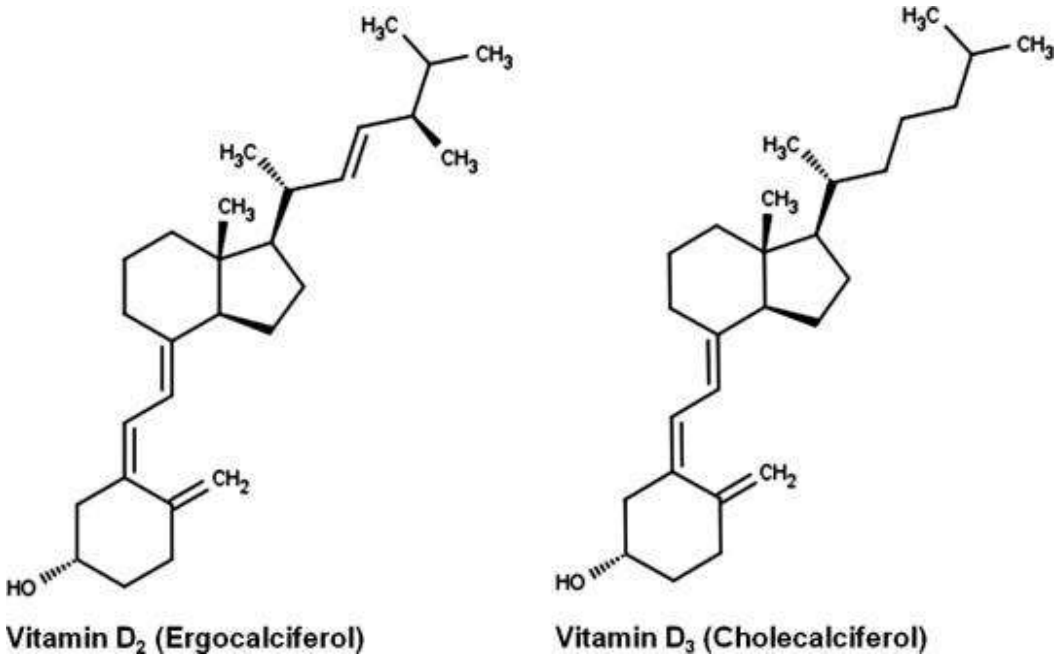

Figure 1.2 Comparison of structures of vitamin D2 and D3.
Reproduced with permission from Simon et al, 2013 (13)

Vitamin D2 also known as Ergocalciferol, predominantly originates from dietary (plant) sources such as fungi and yeast. It is manufactured by the conversion of Ergosterol to Ergocalciferol on exposure to ultraviolet (UV) radiation (14). Vitamin D3 also known as Cholecalciferol, originates from animal sources. It is manufactured in the skin by the conversion of 7 dehydrocholesterol to pre-vitamin D3 on exposure to UV radiation of wavelength 280-315 nm (15). [Figure 1.3]

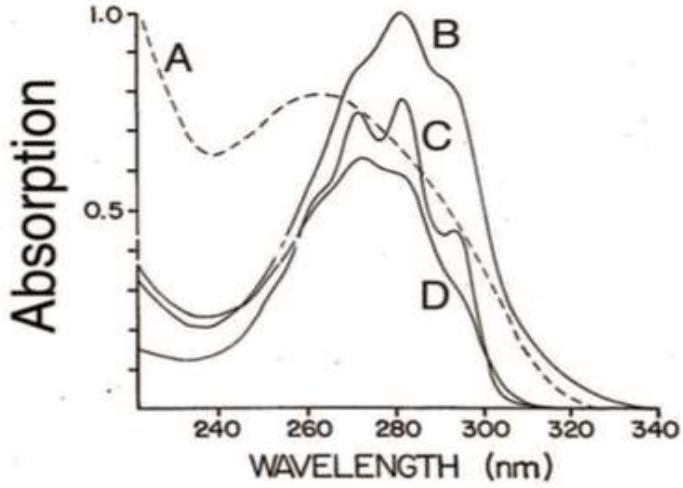

Figure 1.3 UV absorption spectra for (A) Pre-vitamin D3 (B) Tachysterol (C) Pro-vitamin D3.
Reproduced with permission from Matthias Wacker & Michael F. Holick, 2013 (16)

Pre-vitamin D3 is then rapidly converted to D3 by isomerisation (17). Both vitamin D2 and D3 termed together as "vitamin D", is fat-soluble (18).

Vitamin D binds to vitamin D binding protein (VDBP) and carried in the circulation to the liver (19). Vitamin D is hydroxylated into 25OHD in the liver by the cytochrome P450 enzyme CYP2R1 also known as 25-hydroxylase, (20). 25OHD is the major circulating form of vitamin D, the level of which is routinely measured to assess the vitamin D status (21). However this form is inactive and needs further conversion mainly in the distal tubules of the kidneys but also in brain, placenta and few other tissues, (22) to its active form 1α, 25-dihydroxyvitamin D (1,25 $(OH)_2D$) by the CYP27B1 enzyme, also known as 1 α hydroxylase (1 alpha-OHase),(23) (24) [Figure 1.4]. Both 25OHD and 1, 25$(OH)_2D$ is degraded by the enzyme CYP24A1 (24 hydroxylase) to 24,25(OH)2D and 1,24,25(OH)3D respectively and subsequent production of the final degradative product, Calcitroic acid (25).

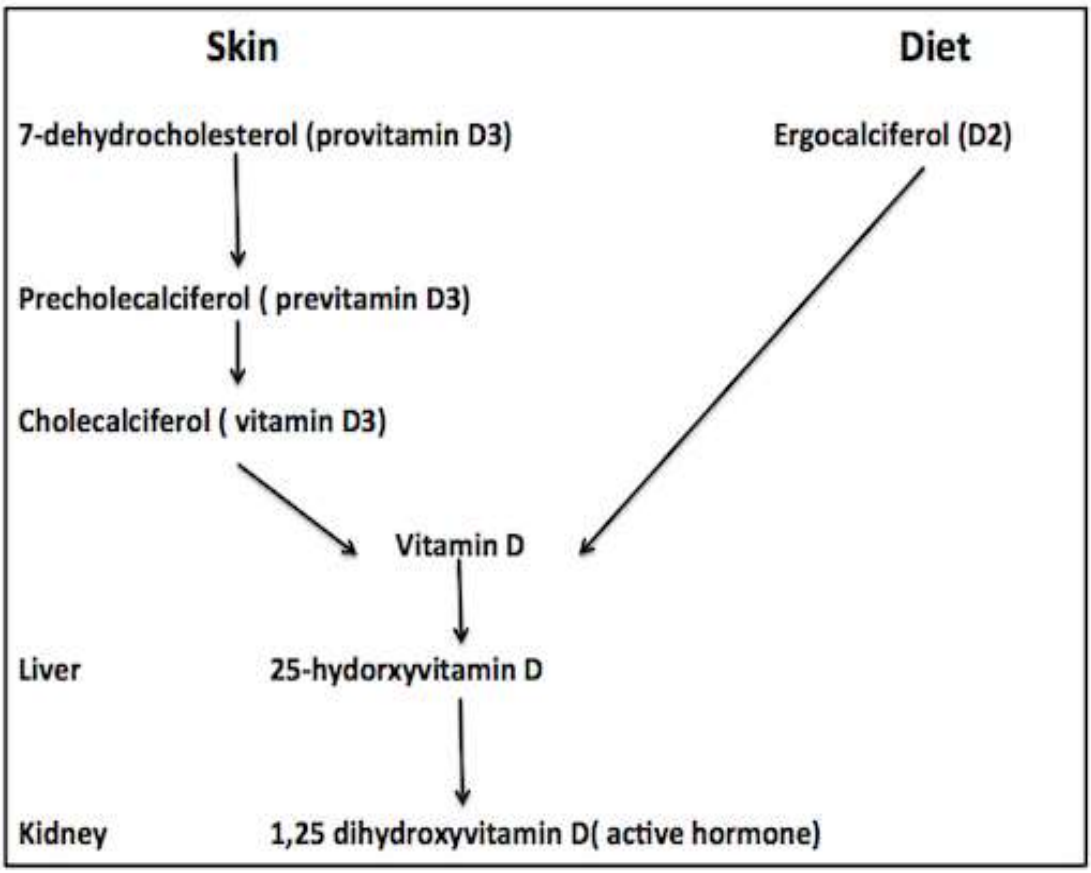

Figure 1.4 Vitamin D synthesis and metabolism

1.3 Vitamin D function:

1,25 $(OH)_2D$ is the active form of Vitamin D and has 3 main functions (i) facilitates the intestinal absorption of calcium, (ii) promotes the mobilization of calcium from the bones and (iii) increases the tubular reabsorption of calcium from the kidneys. 1,25 $(OH)_2D$ binds to vitamin D receptor (VDR), which is expressed in all the cells involved in calcium homeostasis such as enterocytes, osteoblasts, distal renal tubule, and parathyroid cells [Figure 1.5]. It exerts its action in synchronous with parathyroid hormone (PTH) and calcitonin to maintain the plasma calcium concentration at an optimum level (23).

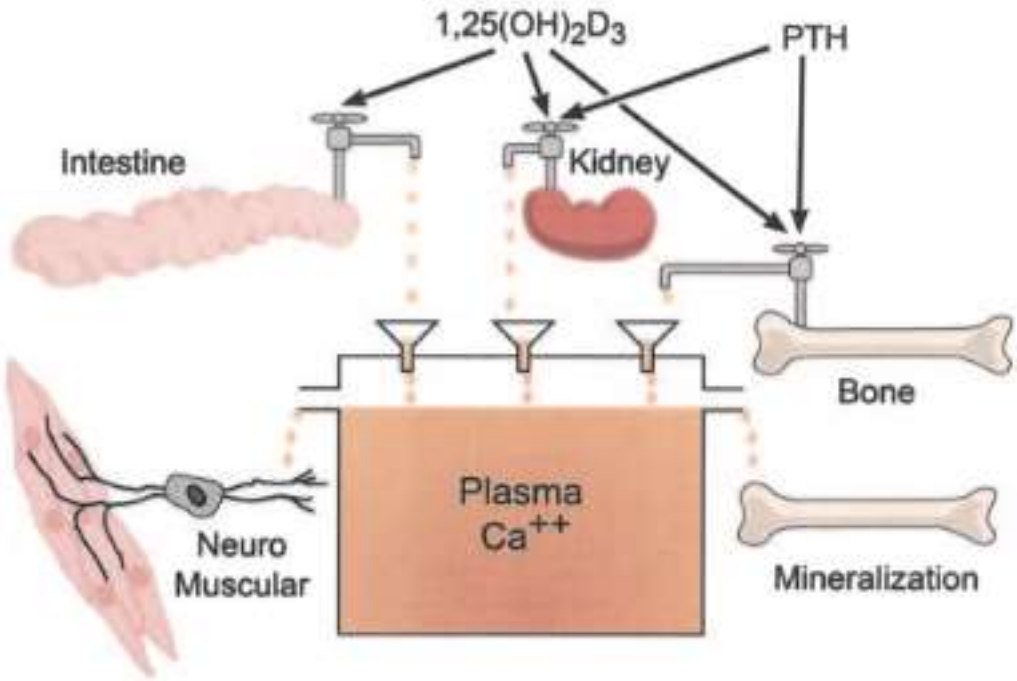

Figure 1.5 Functions of Vitamin D.
Reproduced with permission from De Luca, 2004 (23)

1.4 Vitamin D status:

The most reliable indicator of the vitamin D status is the measurement of total 25OHD levels in blood. A cut-off of 25nmol/L has been used by the Department of Health, 1998 to define the lower limit of adequacy of vitamin D status. There are a number of guidelines and consensus statements available to define vitamin D status and a few that are routinely used in clinical practice in UK are listed in Table 1.2.

Guideline	Serum 25OHD concentration (nmol/L)		
	Deficient	Insufficient	Sufficient
Global consensus Recommendations on Prevention and Management of Nutritional Rickets (26)	< 30	30 - 50	≥ 50
Institute of Medicine (IOM) (27)	< 30	30 - 50	≥ 50
National Osteoporosis Society (UK) (28)	< 30	30 - 50	≥ 50
British Paediatric and Adolescent Bone Group (29)	< 25	25 - 50	≥ 50
Endocrine Society (30)	<50	50- 75	≥ 75

Table 1.2 List of guidelines and consensus statements on vitamin D status

1.5 Factors affecting circulating levels of Vitamin D

1.5.1 Age

Aging can affect the intestinal absorption of dietary Vitamin D but does not alter the absorption of vitamin D supplements (31). The amount of 7-dehydrocholesterol in the skin also begins to decline at around 70 years of age at which time the cutaneous production of vitamin D falls to 25% of the levels made by 20 year olds (32) (24).

1.5.2 Season

A seasonal variation in 25OHD concentration is observed in UK being situated at a latitude of 50-60^0 N (33). The prevalence of Vitamin D deficiency among the general population in UK is higher during the winter and spring when compared to summer months, (34). The National Diet and Nutrition survey (NDNS) in 2000 reported that serum 25OHD concentration is at its peak between July and September and troughs between January and March (35). Holick reported in 2004 that an increased serum concentration of 25OHD during summer is due to amount of skin and time exposed to UV radiation (32). Hence the UK population is dependent largely on body stores and supplements to maintain the optimum levels of serum 25OHD during the winter months.

1.5.3 Sunshine

Solar UVB irradiation accounts for nearly 90% of the vitamin D production in the skin (36). The amount of Vitamin D synthesised by sun exposure is influenced by a multitude of factors including aging, season, weather, and time of the day, altitude and latitude (16). Excessive clothing and sunscreen use can block the synthesis of Vitamin D3 in the skin. There are evidences for and against the 'use of sunscreen causing vitamin D deficiency' (36).

1.5.4 Altitude & Latitude

UV levels are higher when closer the equator and higher the altitude. The sunrays travel a shorter distance when closer to the equator, hence much of the harmful rays are filtered through the atmosphere. In mid latitude countries like UK, cutaneous production of vitamin D is highest during the summer months and between 10am-2pm (solar noon) of the day when there is direct sunshine to the earth. The production of vitamin D is lowest during winter months and early morning or late evening, when sun rays crosses the atmosphere at an angle (37).

1.5.5 Skin colour & ethnicity

Several studies have shown that people of South Asian, Afro –Caribbean and middle eastern origin are at increased risk of vitamin D deficiency defined by serum 25 OHD <25nmol/L (9, 38, 39).

The same amount of vitamin D synthesis requires increased sunlight exposure in dark skinned compared with light skinned individuals (16). Studies have recommended a 15 min unshaded noon time sunlight exposure 3 times a week with 35% skin exposure is adequate for improving 25OHD concentrations in white population whereas this is inadequate in South Asian participants (40). Even a three-fold increment in sun exposure failed to achieve sufficiency in more than half of the South Asian cohort, suggesting that sun exposure advice needs to be tailored based on the degree of skin

pigmentation (41-43). Similarly, simulated UV exposure has resulted in a higher 25OHD concentration in white and East Asians when compared to black and South Asians (44).

1.5.6 Serum 25OHD levels and genes

The three candidate genes found to be associated with serum 25OHD levels are 7-dehydrocholesterol reductase / NAD synthetase 1 (DHCR7/NADYN1), 25-hydroxylase (CYP2R1), and the vitamin D binding protein (VDBP gene - GC) (45).

1.5.7 Obesity

Vitamin D being a fat-soluble vitamin is stored in the fatty tissues. In obese individuals' concentration of vitamin D in the sub cutaneous fat tissues positively correlated with the serum fat concentration. The serum total 25OHD concentration was correspondingly low in those individuals as demonstrated by Blum and his colleagues, (46). A study conducted recently in US involving 6-18 years old children has shown the prevalence of vitamin D deficiency as nearly 29% in overweight children and 34% in obese children (47). Theories that are proposed to link vitamin D deficiency and obesity are (i) sequestration of vitamin D in abundant fat cells, (48) (ii) limited sun exposure and skin synthesis of vitamin D due to sedentary life style (49).

1.6 Vitamin D binding protein

Vitamin D binding protein (VDBP) also known as group-specific component (gc globulin), is a 52-59 kDA multifunctional plasma protein and is encoded by the *GC* gene, on chromosome 4 (19), (50).

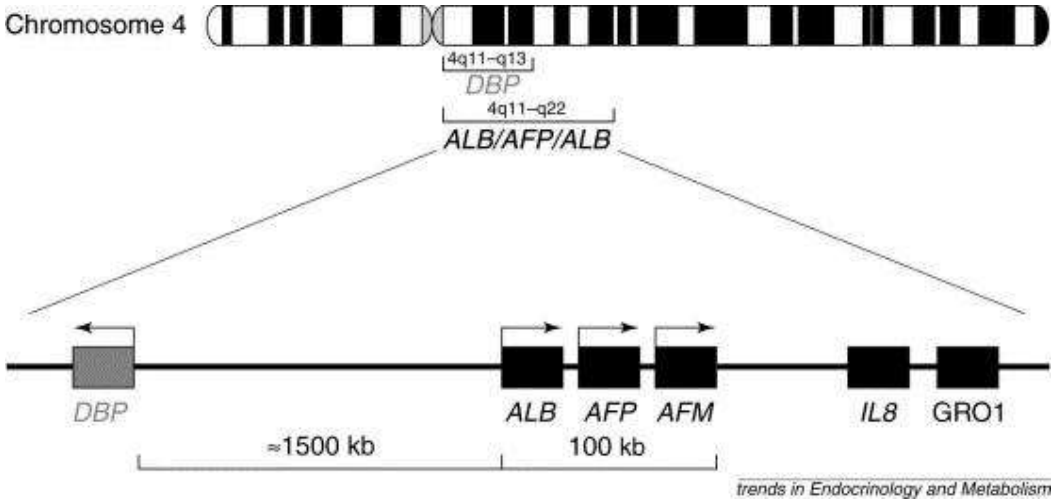

Figure 1.6 VDBP gene and its localisation.
Reproduced with permission from White and Cooke, 2000 (51)

VDBP and the other albumin family members are linked on chromosome 4 and sub localised to bands 4q11 – q13 is shown (51). It is synthesised in the liver, and acts as the major carrier protein for vitamin D and its metabolites in the circulation (52). VDBP gene expression is regulated by three HNF1 (hepatocyte nuclear factor) binding sites – HNF1 alpha, HNF1 beta and HNF2. HNF1 alpha acts as a trans-activator of VDBP, found in high levels in liver thus highly VDBP is highly expressed in liver. HNF1 beta acts as a trans-dominant inhibitor of VDBP, found in high levels in kidney accounting for very low VDBP expression in kidneys. HNF1 is absent in brain hence VDBP is not expressed in brain cells (51).

VDBP is highly polymorphic, has nearly 120 variants and the three most common isoforms are Gc1f, Gc1s and Gc2, (53). Nearly 85 -90% of 25OHD & 1,25,OHD is transported in the circulation bound to vitamin D binding protein (VDBP), 10-15% bound to albumin and chylomicrons, (54, 55) and <1% in

the 'free' or unbound form (19). Huge variation in VDBP levels and its binding properties have been reported in humans, (56-59). The half-life of VDBP is very short (2.5-3 days) when compared to 25OHD (2-3 weeks) (60).

The circulatory levels of VDBP is 20 times higher than the circulating 25OHD levels, (61) and is not only found in serum but also in urine, milk and cerebrospinal fluid (62). The normal plasma concentration of VDBP is 4-6umol/L (2-300mg/L) (63). VDBP binds to 88% of 25OHD with a higher affinity constant of 5x10^8 M^{-1}(64) and 85% of 1,25$(OH)_2$D with a ten-fold lower affinity constant of 4x10^7 M^{-1} (19).

When measurements of vitamin D are undertaken, the measured value is a composite of both the bound and the free (i.e. unbound) metabolites in serum. It is thought that the variations in this binding protein affect the availability to tissues of both 25OHD, the widely used measure of vitamin D sufficiency and 1,25$(OH)_2$D, the active metabolite of vitamin D. VDBP therefore has the potential to substantially affect calcium homeostasis. Lower levels of VDBP will allow greater "free or bioavailable" vitamin D metabolites. If VDBP is low, a lower measured " serum total 25OHD level" may not then reflect reduced delivery of vitamin D metabolites to tissue (65).

1.6.1 Factors influencing VDBP levels

VDBP levels are found to be increased (i) during an inflammatory process (66) (ii) in the last trimester of pregnancy (67, 68) and (iii) decreased in cirrhosis due to reduced protein synthesis (64) (iv) decreased in obese individuals with a positive correlation between serum 25OHD and VDBP levels (69) and (v) in individuals with type 2 diabetes and insulin resistance (70).

Bouillon and his colleagues reported that there was no correlation between circulating levels of VDBP and serum 25OHD (52). Bolland et al. also reported that there was no correlation between age, body weight and BMI but was with gender with a higher VDBP concentration in females (71). However, Ashraf et al. reported that VDBP concentration is influenced by age, race, gender, BMI, insulin, serum

total, free & bio available 25OHD levels. The authors reported a strong positive correlation between VDBP and total, free & bioavailable 25OHD concentrations (70). VDBP levels are found to be low in African Americans when compared to white Caucasians (72, 73).

1.6.2 Physiological actions of VDBP (73)

VDBP has 6 main functions:

1. Plays a vital role in the transport of 25OHD and 1,25$(OH)_2$D in the circulation thereby regulating the bioavailability of vitamin D.
2. Acts as a reservoir of 25OHD and prolongs the half-life of vitamin D metabolites.
3. Offers degree of protection against short term, dietary induced vitamin D deficiency.
4. Binds with megalin for the uptake and activation of 25OHD to its active 1,25(OH)2D form in the kidneys.
5. Binds to actin and prevents the actin mediated vascular damage.
6. Acts as a macrophage-activating factor.

1.6.3 Protection of short-term vitamin D deficiency

White and his colleagues in 2000 reported that VDBP null mice have low circulating levels of serum 25OHD when compared to the VDBP wild mice. Despite the low serum 25OHD levels, the VDBP null mice did not exhibit any evidence of biochemical or functional vitamin D deficiency indicating normal circulating levels of active 1,25$(OH)_2$D. When these mice were fed on a vitamin D deficient diet for a period of 4-6 weeks, a rise in serum PTH, alkaline phosphatase levels along with rachitic changes were observed in the VDBP null mice indicating true intracellular paucity of 1,25$(OH)_2$D. However no biochemical changes were noted in wild mice proving the short term protective effect of VDBP in dietary vitamin D deficiency (51).

1.6.4 VDBP action on megalin mediated endocrine synthesis of $1,25(OH)_2D$

Megalin also known as multifunctional clearance receptor is a member of the LDL receptor family. It is expressed on the brush border of the proximal tubular epithelium of the kidneys. While VDBP and 25OHD when filtered through the glomerulus enter the renal tubular epithelial cell directly, VDBP bound 25OHD complex enters the cell either directly or mediated by megalin. The VDBP bound 25OHD complex associated with megalin is broken down inside the lysosome followed by the release of VDBP and 25OHD into cytoplasm. 25OHD then gets activated into $1,25(OH)_2D$ by the enzyme 1 alpha hydroxylase within the mitochondria. Both the vitamin D metabolites then get secreted into the interstitial fluid to bind to VDBP and reach the target tissues (51) [Figure 1.7].

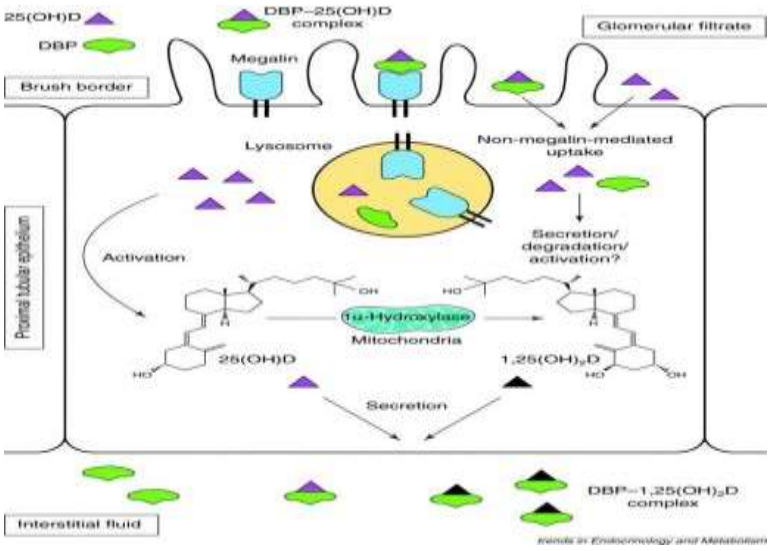

Figure 1.7 Megalin mediated uptake and activation of 25OHD.
Reproduced with permission from White and Cooke, 2000 (51)

Similar to megalin mediated intracellular uptake of VDBP in the proximal renal tubular epithelium, the uptake of albumin (another member of the VDBP family) is mediated by cubulin (74-76).

1.6.5 VDBP action on actin mediated intravascular damage

Another major function of vitamin D is the rapid clearance of actin. Actin is released from the cells damaged by injury or inflammation and polymerised into actin filaments. These polymerised actin filaments cause damage to vascular endothelium leading to thrombo-emboli formation. VDBP binds to actin with a much higher affinity (1x10^9M^{-1}) when compared to 25OHD and 1,25(OH)2D (68) thus preventing its polymerisation and subsequent damage to blood vessels(77).

1.6.6 VDBP acts a macrophage-activating factor

The deglycosylated form of VDBP also known as VDBP-MAF (macrophage activating factor), plays a vital role in chemotaxis, phagocytosis, tumour lysis and destruction intracellular of parasites (51, 68). Gc1f & Gc1s isoforms carry 2 glycosylation sites when compared to Gc2 that has only one glycosylation site. Hence individuals with Gc2 genotype have reduced macrophage-activating capacity with an increased susceptibility to conditions such as osteopetrosis and chronic obstructive pulmonary disease (COPD) (68). VDBP also potentiates the chemotactic effect of C5a during an injury or inflammatory process (78).

1.7 VDBP and VDBP genotype

Two most common single nucleotide polymorphisms (SNP's) in the VDBP (Gc) gene are (i) D432E (formerly known as D416E or rs7041) resulting in the conversion of threonine (A***C***G) to lysine (A***A***G) by ***C*** → ***A*** transversion and (ii) T436K (formerly known as T420K or rs4588) resulting in the conversion of aspartate (GA***T***) to glutamate (GA***G***) by **T→G** transversion, (60). This can give rise to 3 VDBP (Gc) variants (i) Gc1f, (ii) Gc1s and (iii) Gc2 (79, 80) [Figure 1.8]

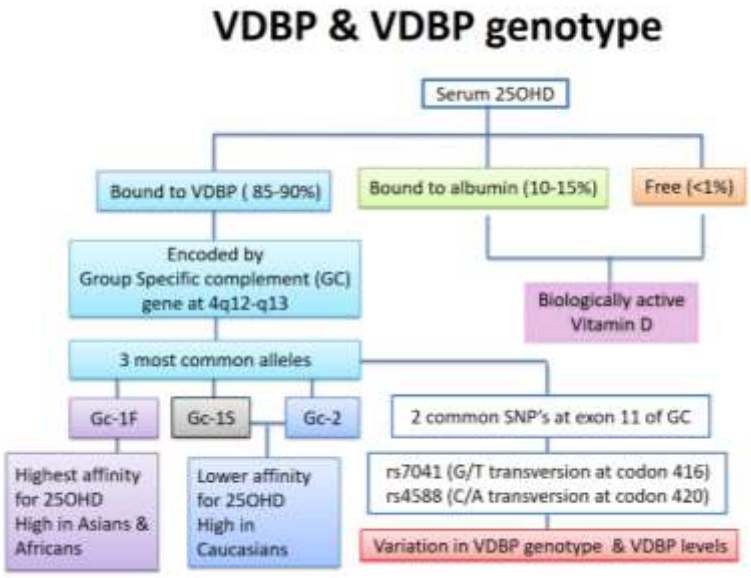

Figure 1.8 VDBP and VDBP genotype – affinity and concentration.
Reproduced with permission from Schwartz et al 2014, (56).

All three variants have similar amino acid sequence except as positions 416 and 420. Gc1f is the ancestral genotype and has aspartate at position 416 and threonine at 420. Gc1s has glutamate instead of aspartate at position 416 and threonine at 420 and Gc2 has aspartate at position 420 and lysine instead of threonine at position 420. The combination of glutamate at position 416 and lysine at position 420 is very rare in humans. The relationship between VDBP genotype, polymorphism allele and amino acid position is illustrated in Table 1.3 (54).

DBP genotype	Allele at polymorphism		Amino acid at position	
	Rs7041	Rs4588	416	420
GC1F	T	C	Asp	Thr
GC1S	G	C	Glu	Thr
GC2	T	A	Asp	Lys

Table 1.3 Relationship between DBP genotype, polymorphism allele and amino acid residue.
Reproduced with permission from Chun et al, 2012 (54)

Gc1f has the highest, Gc1s - intermediate and Gc2 - lowest affinity for serum 25OHD. GC1f, the wild type is found to be commonly associated with Africans and East Asians. Gc1S is highly seen in Europeans, Middle Eastern and South Asian population. Gc2 is found to be generally low in the entire population (81) [Table 1.4].

Population	GC1F (%)	GC1S (%)	GC2 (%)
Australia (Central Desert)	39	43	10
Bolivia (Aymara-Quechua)	23	64	12
Cameroon (Bantu)	82	8	9
China (Beijing)	48	25	26
France (Pyrenean)	8	51	41
India (Delhi)	14	55	31
Mexico (La Manita)	35	50	14
Sweden	14	61	25
USA Alaska (Eskimos)	26	35	33
USA Black (Minnesota)	67	18	10
USA White (Minnesota)	15	56	27

Table 1.4 Prevalence of DBP polymorphic forms in selected populations.
Reproduced with permission from Chun et al, 2012 (54) and Kamboh & Ferrell,1986 (81)

Chun et al. reported that Gc1f is highly associated with Africans, Gc1s in Europeans and intermediate association of both Gc1f and 1s Asians (54). Schwartz et al. reported a higher frequency of GC1f allele in African Americans and Asians and a lower frequency of Gc1s and Gc2 alleles in Caucasians (56). GC1s allele frequency is reported to be high in central Indian population (82). Similarly, Mastana et al. reported a higher frequency of Gc2 allele in Eastern Indians (83).

Few reports have also reported the comparison made between Gc1 and Gc2 isoforms and this means they have studied only the rs4588 SNP, in which, at position 420, Gc1 has threonine and Gc2 has Lysine (82). The differences between the Gc isoforms were derived based on their (i) amino acid position representing its binding affinity and (ii) glycosylation pattern representing its concentration. The presence or absence of trisaccharide glycosylation at position 420 determines the metabolism of Gc isoforms. While Gc1f and Gc1s show trisaccharidic glycosylation Gc2 does not. This leads to a faster metabolism of Gc2 thereby a lower serum concentration. These three VDBP (Gc) variants /isoforms combine to form 6 different diplotypes / haplotypes – Gc1f-1f, Gc1f-1s, Gc1f-2, Gc1s-1s, Gc1s-2, Gc2-2.

There is emerging evidence in the literature that these single nucleotide polymorphisms lead to a significant variation in circulating Vitamin D (25 OH D) levels (84). What is not known is the extent to which such variation results in altered response to standard treatment. Bouillon et al in 1977 has shown that there is no correlation between serum 25OHD and VDBP levels in maternal serum. The same group in 1981 reported a significant positive correlation between serum 1,25$(OH)_2$D and VDBP levels and in addition to the absence of correlation between VDBP and 25OHD similar to their previous report. They also reported a highly significant correlation between the (total and free) serum 25OHD and serum 1,25 $(OH)_2$D in both maternal and cord serum during the last trimester of pregnancy.

Engelman et al. (2008) reported that the higher prevalence of Gc1f in blacks resulted in a similar concentration of bioavailable vitamin D in whites (85). Powe et al has recently shown in their cross-sectional study that black Americans have low levels of total vitamin D and VDBP resulting in similar concentrations of estimated bio-available (free) vitamin D compared to their white counterparts. The black population had a higher BMD and calcium levels, only slightly higher parathyroid hormone levels compared to whites. This observation raises the question of whether standard treatment with vitamin D based on the total 25OHD levels and VDBP is appropriate across all ethnic groups. The authors also

proposed that bio-available 25OHD may be a more appropriate cross-racial marker of vitamin D deficiency (65).

1.7.1 VDBP genotype and VDBP concentration

The VDBP concentration is reported to be higher in individuals with Gc1s-1s when compared to Gc1f-1f, intermediate in Gc1f-1s and low in Gc2-2 genotypes, (50, 63, 86). The higher prevalence of Gc1f genotype that has the highest affinity for 25OHD is found to be associated with low VDBP levels in Black and Asians. Gc2 with a lower affinity for 25OHD is associated with higher DBP levels in white Caucasians and very rarely in Blacks (65, 87) [Table 1.5]

Average human serum levels [23]		**Association constants (Ka) [9,10]**		
DBP (mixed)	5.0 μM	DBP for 25OHD	$7\times10^{8}\ M^{-1}$	
Albumin	650 μM	DBP for $1,25(OH)_2D$	$4\times10^{7}\ M^{-1}$	
25OHD	50 nM	Albumin for 25OHD	$6\times10^{5}\ M^{-1}$	
$1,25(OH)_2D$	0.1 nM	Albumin for $1,25(OH)_2D$	$5.4\times10^{4}\ M^{-1}$	
DBP concentration				
by genotype (average) [12]		**Relative affinity [13]**	**25OHD**	**$1,25(OH)_2D$**
GC1F/1F	5.17 μM	GC1F	1.000	1.000
GC1F/1S	5.15 μM	GC1S	0.536	0.356
GC1S/1S	5.27 μM	GC2	0.321	0.233
GC1F/2	4.77 μM			
GC1S/2	4.79 μM			
GC2/2	4.35 μM			

doi:10.1371/journal.pone.0030773.t001

Table 1.5 Binding protein and ligand biochemical parameters for eSS mathematical model
Table reproduced with permission from Chun et al, 2012 (88)

1.7.2 VDBP genotype and Serum 25OHD concentration

Recently Thongthai et al has genotyped 4,476 individuals from Thailand aged between 14 and 93 years for rs2282679 (rs 4588) polymorphism and have reported that vitamin D deficiency is significantly associated with the presence of c allele in this genotype (Odd's ratio (OR) 1.80, 95%CI 1.57 -2.01) independent of other associated risk factors such as age, sex and obesity (45).

Braithwaite et al. genotyped nearly 3000 Gambian children for 6 different Gc haplotypes [Gc1f- 1f, Gc1f -1s, Gc1-2, Gc1s-1s, Gc1s-2, Gc2-2] and found the highest distribution by Gc1f (0.86), followed by Gc1s (0.11) and Gc2 (0.03). They also noted a higher serum 25OHD concentration in subjects with Gc1f-1f haplotype (89).

Pekkinen et al. in 2014 examined the role of VDBP-Gc [SNP rs4588] in serum 25OHD and bone mineral density (BMD) in 231 Finnish children aged 7 -19 years. Gc1-1 haplotype was found in 68%, Gc1-2 in 26% and Gc2-2 in 6% of their cohort. They reported a significant difference in serum 25OHD (p 0.001) and PTH (0.028) levels between different genotypes with Gc2-2 having the lowest 25OHD and PTH concentrations. The authors also demonstrated the influence of Gc genotypes on BMD in children (90).

Carpenter et al. in 2013 reported the prevalence of Gc1f allele frequency as 72% in African Americans, 13% in Hispanics and 6% in Caucasians. They proposed that a progressive substitution of lysine by threonine at position 420 leads to low circulatory 25OHD levels. They demonstrated that the circulating levels of both VDBP and 25OHD are significantly influenced by the genotypic variation in Gc and that circulating DBP significantly correlates with 25OHD. The Gc genotype can affect the serum 25OHD levels independent to its effect on VDBP (50).

Sinotte and his colleagues in 2009 found a low baseline serum 25OHD concentration in Gc2-2 homozygotes in premenopausal Caucasian women of French descent (91). Engelman and his

colleagues observed a similar finding in African Americans compared to Hispanics, (85) and so as Fu et al. in 2009 with a baseline low 25OHD concentration in subjects with Gc2-2 genotype (59).

Lauridsen and his colleagues studied 595 early postmenopausal Caucasian women from the Danish osteoporosis study cohort. They found the highest levels of serum 25OHD and $1,25(OH)_2D$ in women with Gc1-1 genotype, intermediate levels in GC1-2 and lowest levels in Gc2-2 type. The authors also proposed that Gc phenotype is a significant predictor of serum 25OHD whereas its concentration is an independent predictor of $1,25(OH)_2D$ (92).

1.7.3 Response to vitamin D dosing based on VDBP genotype

Fu and his colleagues in 2009 subjected a total of 98 individuals (42 healthy adults; 56 post-menopausal women) aged 52.2+/-1.0(mean, SE) years to either low dose (600 IU/day) or high dose (4000 IU/day) for a year. They found that subjects with GC2-2 (KK) genotype had the (i) lowest baseline serum 25OHD [Figure 1.9] (ii) highest increment to supplementation [Figure 1.10] and (iii) reduction in VDBP binding affinity in all subjects after a year of vitamin D supplementation with a significant reduction in Gc2-2 (KK) genotype [Figure 1.11]. The authors concluded that the association between genotype and response to 25OHD supplementation was not fully accounted by the differences in VDBP binding capacity.

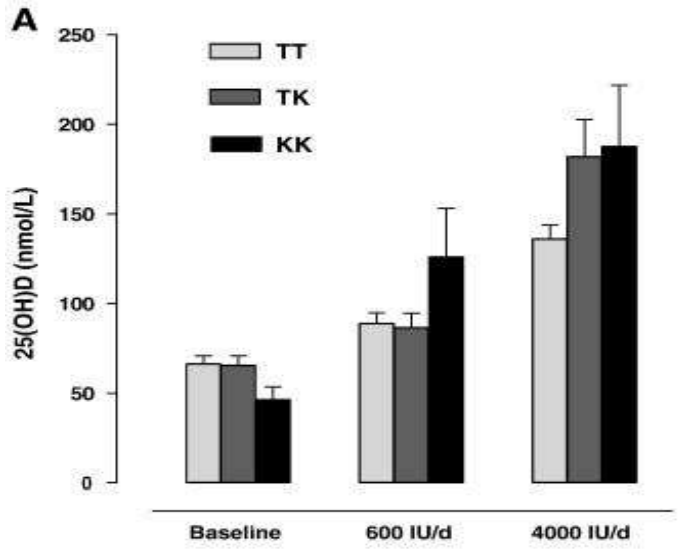

Figure 1.9 Association of serum 25OHD with Gc genotypes at baseline and 1 year post supplementation in low dose and high dose.

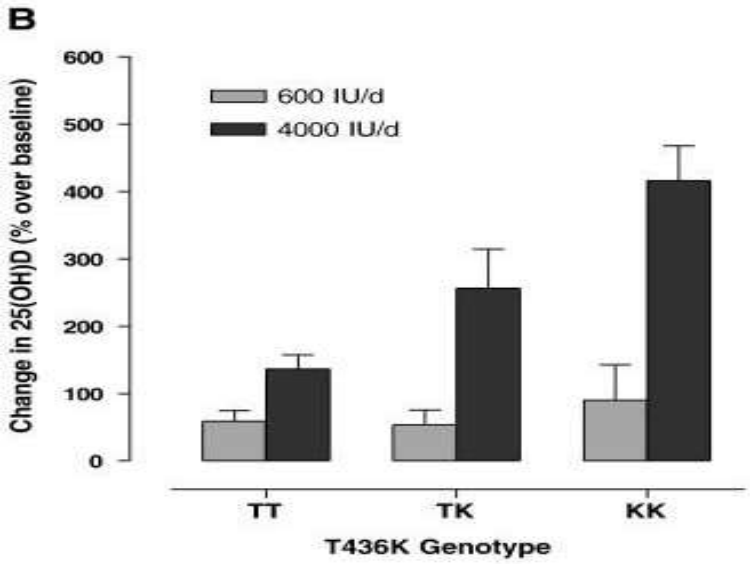

Figure 1.10 Association between genotypes and serum 25OHD increment (%) at one year.

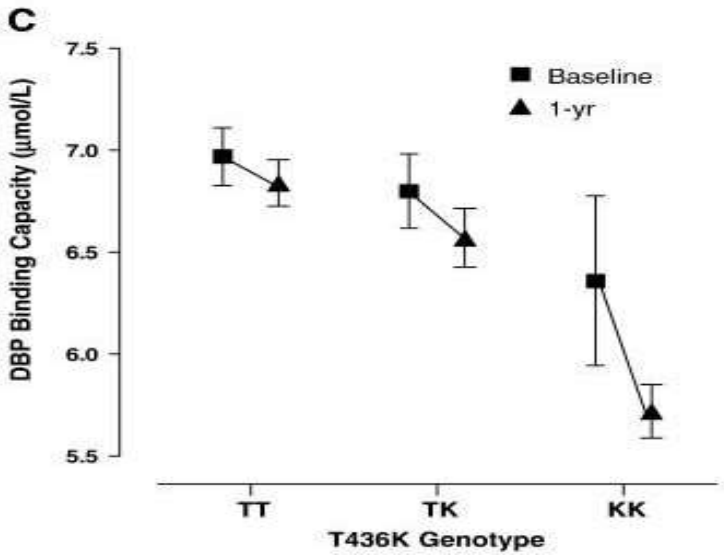

Figure 1.11 Specific VDBP binding capacity at baseline and 1 year post supplementation. Figure(s) 1.9 – 1.11 reproduced with permission from Fu et al, 2009 (59)

1.8 VDBP gene polymorphism in non-skeletal disorders

1.8.1 Chronic obstructive pulmonary disease [COPD]

Hortia et al and Xie et al. have reported in their meta-analysis recently that VDBP GC-1F allele is associated with the risk of COPD (79, 93). Xie et al also found that the GC-1F homozygotes are a risk factor for COPD whereas GC-1S homozygotes are a protective factor against COPD in the Asian population. Jung et al reported an association of GC-1F variant and COPD in the Korean population (94).

1.8.2 VDBP and colorectal cancer

Anic et al. reported a positive association between 25OHD level and colorectal cancer, which is stronger in men who are smokers and with higher VDBP concentrations (95).

1.8.3 VDBP and asthma

The VDBP GC-1s/1s genotype is found to be protective against the development of asthma when compared to GC-1f/1f in the Hispanic population (96).

1.8.4 VDBP and infection

The non-classical action of vitamin D on macrophages and polymorphonuclear leucocytes is proposed to be executed by the intracrine cell specific conversion of 25OHD to $1,25(OH)_2D$. A higher antimicrobial response to vitamin D treatment is more pronounced in subjects with low affinity forms of VDBP such as Gc1s and Gc2 that are associated with higher concentration of 'free' 25OHD (88). In contrast, Liong et al. reported that Gc2 with a single glycosylation site has less macrophage activation capacity thus more susceptible for infections (68).

1.8.5 Vitamin D, VDBP genotype and bone turnover markers (BTM)

There is conflicting evidence regarding the association between 25OHD, VDBP genotype and BTM. Lowe et al. conducted a cross sectional study in 2010, comparing the vitamin D status between 66 South Asian (Pakistani) and 42 Caucasian postmenopausal women in UK. South Asian women had significantly low serum 25OHD, high PTH and bone specific alkaline phosphatase levels when compared to their Caucasian counterparts. However, no significant difference between the 2 groups for BTM (P1NP and CTX) was found. The authors concluded that a low 25OHD status and high PTH concentration was not associated with an increased bone turn over in South Asian women (97).

In contrast, Khan and his colleagues found increased BTM in their study subjects with vitamin D deficiency (VDD) and secondary hyperparathyroidism but not in individuals with a blunted PTH response. They demonstrated that VDD and reduced calcium intake but normal PTH levels is not associated with increased BTM. Thus, the authors concluded that that VDD increases the BTM through PTH (98).

A study done by Saadi et al. has shown that VDBP genotype does not influence bone turnover makers (99). In contrast, Nimitphong and his colleagues have shown a positive correlation between serum 25OHD, BTM and BMD in individuals with Gc1f (AA) genotype and no relationship in others (100).

1.8.6 VDBP vs Parathyroid hormone (PTH)

The inverse relationship between serum PTH level and 25OHD concentrations is well established however the data available on the association between serum PTH and VDBP is very limited. Wang and his colleagues found an inverse correlation between VDBP levels and PTH concentration, suggesting the regulation of VDBP production by PTH (101). Lauridsen and his colleagues studied the Gc genotype of nearly 600 white Caucasian women who entered the postmenopausal phase recently. Nearly 60% women in their cohort had Gc2-2 genotype and associated low serum 25OHD concentrations (<50 nmol/L). However, none of their PTH was elevated above the reference range. The authors concluded that neither the Gc phenotype nor the Gc concentration is a significant predictor of PTH levels (92). The authors also did not find a significant correlation between serum PTH and $1,25(OH)_2D$ levels in that cohort.

On the other hand, Pekkinen and his colleagues demonstrated a significant difference in serum 25OHD and PTH concentration between the genotypes along with a liner trend in Gc2-2 type associated with both lowest serum 25OHD and PTH concentrations. They conducted this study in a group of 231 Finnish children and young adults aged between 7 and 19 years of age (90).

1.9 Free vitamin D

Vitamin D in circulation in blood is bound to proteins such as VDBP, albumin and the levels of the proteins affect the serum 25OHD measurement (19). The free hormone hypothesis suggests that the hormones exert their biologic action when they are unbound from the proteins (102-105). Hence the free fraction (25OHD not bound to VDBP) together with albumin bound fraction are considered to be biologically active and may be a better measure of vitamin D status (106).

Total serum 25OHD = 25OHD~VDBP + 25OHD~Albumin + free 25OHD

Bioavailable 25OHD = 25OHD~Albumin + free 25OHD

Free 25OHD can be calculated based on the law of mass action using serum total 25OHD concentration, VDBP and the VDBP – 25OHD affinity constant (64). The affinity constants measured by Bikle for albumin is 6 x 10^5 and VDBP is 7 x 10^8.

The formula for free 25OHD calculation is (64)

Free 25OHD (pmol/L) = Total 25OHD (mol/L) / 1+ [6 x 10^5 x albumin (mol/L)] +

[7 x 10^8 x VDBP (mol/L)]

Chun et al has proposed that free 25OHD levels are not only determined by the VDBP and the albumin concentration but also by the VDBP binding affinity for specific vitamin D metabolites (106).

Chun et al proposed a mathematical model that designates different affinity constants for the different DBP genotypes to calculate free and bioavailable 25OHD (88). The authors proposed 2 mathematical models (i) extracellular steady state (eSS) and (ii) intracellular steady state (iSS) for serum 'free' 25OHD and 1,25$(OH)_2$D calculation. The eSS model was used to measure the serum 'free' 25OHD and 1,25$(OH)_2$D levels based on an individual's VDBP genotypic affinity and its concentration.

The iSS model was used to measure the impact of VDBP on the biological actions of 25OHD and $1,25(OH)_2D$ in vivo [Figure 1.12].

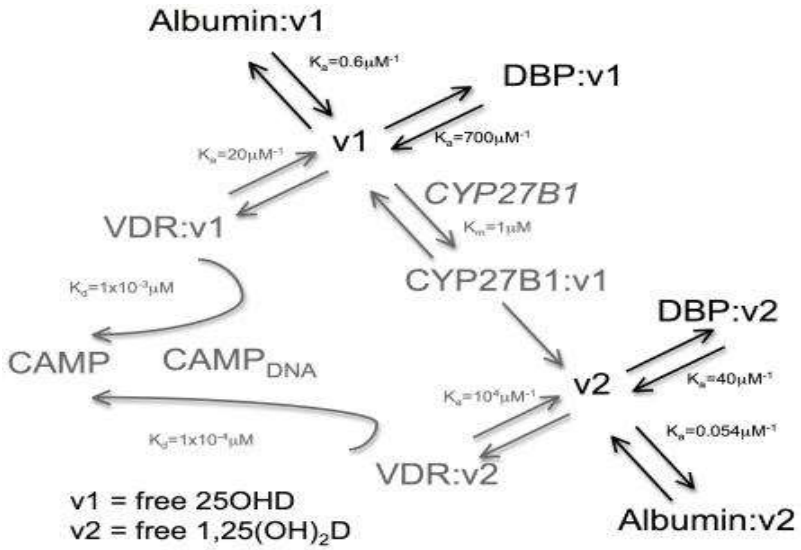

Figure 1.12 Schematic framework of eSS and iSS mathematical models for vitamin D metabolism and function. Black text and arrows indicate the eSS model interaction between free 25OHD & $1,25(OH)_2D$ and VDBP or albumin. Grey text and arrows indicate the iSS model interaction involving the CYP27B1, VDR and CAMP via interaction between VDR and the CAMP – DNA.
Reproduced with permission from Chun et al, 2012 (88)

The eSS model for 'free'25OHD estimation involved the input of the following variables – serum total 25OHD, total $1,25(OH)_2D$, albumin, VDBP concentration and its genotype (affinity). This model predicted the 'free' concentrations of 25OHD as 50nM (<0.1%), $1,25(OH)_2D$ as 100pM (<1.5%) in vivo. The advantage of this model is that it can be used to generate a spectrum of results even if the data on one or more variable is not available. For instance, if the data on VDBP genotype and $1,25(OH)_2D$ are not available, all the six different VDBP haplotypes can be plugged in individually to derive a range of results from low to high affinity VDBP genotypes. Similarly, a constant of 100pM can be used as a stand value for $1,25(OH)_2D$. Thus, an individual who possess a Gc2 genotype (low affinity and low serum concentration) and has low serum 25OHD levels, the calculated free or bio-available 25OHD reaches an optimal level when compared to an individual with a Gc1 genotype (high affinity and high serum concentration). This effect is further accentuated in dark skinned individuals with Gc1 genotype

such as Africans, in whom the cutaneous synthesis of vitamin D is rather low leading to very low levels of 'free' 25OHD.

Powe et al. in 2011 used another method to calculate bioavailable 25OHD (free and albumin bound) is called as modified Vermeulen method. They adapted the formula used by Vermeulen to calculate free testosterone using total testosterone, sex hormone binding globulin (SHBG) and albumin with the known affinity constants of testosterone for SHBG and albumin (107). Both Bikle and Powe used the same affinity binding constants hence the calculated free 25OHD using both the methods highly correlated with each other (Spearman's r =1). But the advantage of using Vermeulen method is to calculate both free 25OHD and bioavailable 25OHD. Powe et al. reported the strong positive correlation between both 'free' and 'bio-available' 25OHD and BMD. They also proposed that 'free' 25OHD could be a better marker for determining the biological actions of vitamin D than total 25OHD (108).

Chun et al. incorporated the calculations used by Bikle in 1985 and 1986 along with BMD data reported by Powe in 2011 to derive 'free' 25OHD concentration. The 'free' 25OHD levels estimated by Chun was comparable to the levels obtained by Bikle (88).

Free 25OHD can also be measured in blood directly, but there are biochemical arguments with regards to its reliability and correlation with calculated free 25OHD levels. Hollis in 2008 and Schwartz et al in 2014 reported that the available methods for direct measurement of free 25OHD in serum are very complex or not very reliable (56, 109). However, Bikle in 1986 reported a strong correlation (0.925) between calculated and directly measured free 25OHD by centrifugal ultrafiltration method (64). An alternative is to measure vitamin D in saliva, where there are no binding proteins.

Fairney et al in 1987 reported the presence of 25OHD in saliva and a huge variation in its levels depending on an individual's ethnic origin, dietary vitamin D intake, timing of sampling. Asian children who are on a predominantly vegetarian based diet have low levels of salivary 25OHD when compared

to their white counterparts, (110). Nuclear uptake and retention of radiolabelled vitamin D in salivary gland is demonstrated in 2007 by Stumpf and his colleagues (111). In 2008, Higashi et al. developed and validated a method for salivary measurement of 25OHD using liquid chromatography – electrospray ionization-tandem mass spectrometric (LC-ESI-MS/MS). They observed a positive correlation (r=0.83) between the serum and salivary 25OHD measurements using this method [Figure 1.13] (112). The authors also demonstrated an increment in salivary 25OHD levels post vitamin D supplementation.

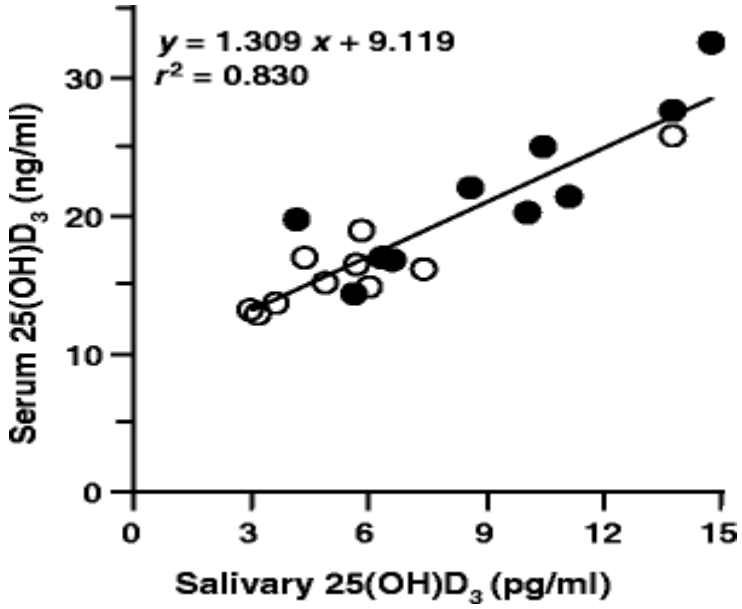

Figure 1.13 Correlation between the salivary 25OHD and serum 25OHD.
Reproduced with permission from Higashi et al, 2008(112)

1.10 Efficacy of Vitamin D2 (Ergocalciferol) and Vitamin D3 (Cholecalciferol)

Despite the presence of a clear biochemical difference between D2 and D3, the biological difference between the two forms remains controversial. Trang et al reported that Vitamin D3 is 1.7 times more efficacious than D2 (113). Armas et al. in 2004 demonstrated that D2 is not only less potent but also

has a shorter half-life (114). This is further supported by the work shown by Houghton in 2006 and Binkley in 2011 (115) (116).

On the contrary, Holick et al. in 2008 reported that 1000 IU of D2 increased the serum 25OHD concentration similar to 1000 IU of D3 hence both the forms are equally effective (117). However, in the current clinical practice the functional outcome of these two forms is considered equal and used interchangeably depending on the availability of the local resources.

1.11 Efficacy and safety of Vitamin D treatment

Vitamin D excess is defined when serum 25OHD levels exceed >250nmol/L and vitamin D intoxication when levels exceed >325nmol/L (24). A single dose of 150,000 units of Vitamin D2 was found to be effective and safe in healthy children aged between 7 and 10 years with no evidence of hypercalciuria or hypercalcaemia at 6 weeks and 5 months post administration. However a higher increment in PTH during winter months was observed in the same population (118).

Fu and his colleagues in 2009 also demonstrated that a high dose vitamin D3 (4000 IU/day) administered over a year was safe with no evidence of hypercalciuria (59). Holick in 2006 reported that neonates and children can tolerate a single dose of 200,000 IU or up to 3000 IU per day of vitamin D2 /D3 without any side effects (119).

A double-blinded placebo controlled randomized clinical trial was conducted by Smith and his colleagues in 2007, involving 9000 men and women over the age of 75 years. A single dose of 300,000 IU of intramuscular vitamin D2 injection was administered annually over a 3-year period. The authors found an increase in fractures but not fall in that cohort (120).

Similarly, Sanders and his colleagues observed a higher incidence of fractures and falls in older women administered with a single annual dose of 500,000 units of vitamin D3 more so within 3 months of supplementation, when compared to placebo in a randomsied clinical trial (121). Dawson-Hughes

postulated the possible mechanism for this as a short term up-regulation of CYP24 enzyme resulting in increased catabolism of $1,25(OH)_2D$. This in turn led to low levels of $1,25(OH)_2D$ in tissues and circulation resulting in increased falls and fractures (122).

Cesur et al. in 2003 compared three different doses of vitamin D administered to infants with nutritional deficiency vitamin D deficient rickets. A total of 56 patients aged between 3 and 36 months along with 20 age matched controls were enrolled into the study. Follow up data were available from 52 children. While the authors did not find an improvement in rickets between the three doses, risk of hypercalcaemia was observed in 2 patients who received 300,000IU of vitamin D and 6 patients who received 600,000IU. The authors concluded that a dose of 150,000 IU or 300,000 IU is adequate to treat vitamin D deficiency in children with no or minimal risk of hypercalcaemia (123).

Markestad and his colleagues in 1987 assessed the risk of hypercalcaemia in 42 infants receiving intermittent high dose (600,000 IU) vitamin D prophylaxis every 3-5 months. They measured the serum concentrations of vitamin D metabolites before and after 2 weeks after each dose. While the serum 25OHD, increased well above normal limits $1,25(OH)_2D$ level was maintained within the normal range without much fluctuation. Despite the lack of cumulative rise in serum 25OHD levels, hypercalcaemia was noted in nearly 35% of the participants prior to dosing indicating vitamin D excess (124).

Zeghoud and his colleagues in 1994 assessed the safety and efficacy of three different doses (100,000 IU, 200,000 IU, 600,000 IU) of vitamin D administered to 60 healthy Algerian neonates born between September and October to mothers who did not receive vitamin D supplementation in pregnancy. They enrolled (i) 30 neonates born between 1984 and 1985 and received a single dose of 600,000 IU (15mg) of vitamin D3 at 2 weeks of age as a usual prophylactic dose (ii) 30 infants born between 1991 and 1992 who were randomised into 2 groups. Group 1 was administered with 100,000 IU (2.5mg) of vitamin D3 at birth, 6 months and 9 months of age. Group 2 was administered with a single dose of

200,000 IU (5mg) of vitamin D3 at birth. Serum 25OHD levels were found to be the highest in the group who received 600,000 IU of vitamin D followed by 200,000 IU and 100,000 IU. In the 600,000 IU group, 50% of the children demonstrated prolonged vitamin D overload up to 6 months. A transient increase in serum calcium 2 weeks following the dosing was observed in children in 600,000 IU group when compared to other groups. The authors concluded that an oral dose of 100,000 IU administered every 3 months is a safe and effective option to protect against vitamin D deficiency.

1.12 Vitamin D and Rickets

In infants younger than 6 months, vitamin D deficiency presents as hypocalcaemic seizures, whereas older infants and toddlers classically present with clinically and radiographically evident rachitic changes alongside biochemical changes, osteopaenia, elevated parathyroid hormone (PTH) and alkaline phosphatase (ALP) levels (125). In addition, the severity of vitamin D deficiency is proportional to the rachitic changes seen on radiographs (126). Rickets is the qualitative deficiency of bone mineralization, most recognizable at the growth plate, which results in radiographically evident abnormalities. Currently, there is little data regarding a child's fracture risk in the case of a mild vitamin D insufficiency versus that in florid clinical and radiographically evident rickets. This is further discussed in Chapter 3 of this thesis. The radiographic changes of rickets [Figure 1.14] are well characterised and a 10-point scoring system proposed by Thacher is widely used to assess the severity of rickets (127).

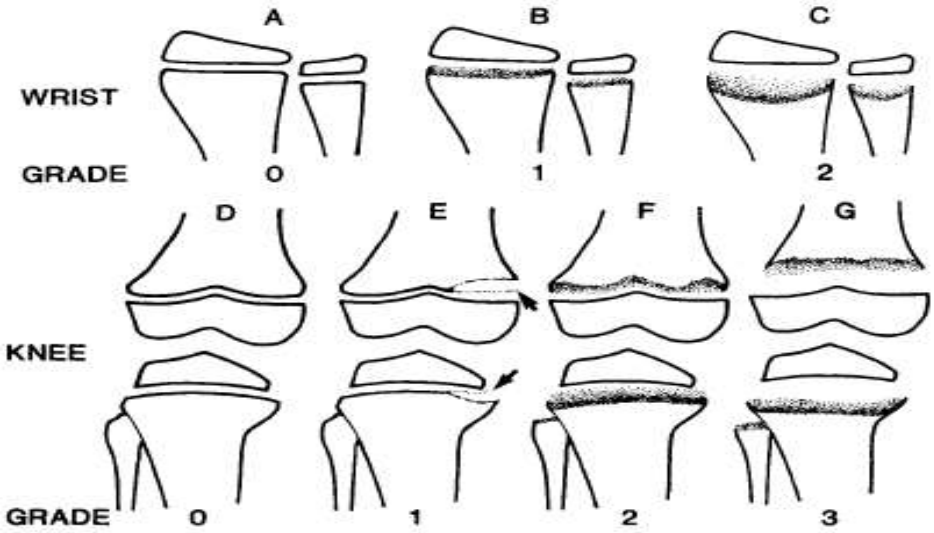

Figure 1.14 Radiological grading of Rickets.
Reproduced with permission from Thacher et al, 2000 (127)

TABLE 1
Ten-point radiographic scoring method for rickets

WRIST[a] — score both radius and ulna separately

Grade	Radiographic features
1	Widened growth plate, irregularity of metaphyseal margin, but without concave cupping
2	Metaphyseal concavity with fraying of margins

2 bones × 2 points = 4 points possible

KNEE[a] — score both femur and tibia separately

Multiply the grade in A by the multiplier in B for each bone, then add femur and tibia scores together

A: Grade	Degree of lucency and widening of zone of provisional calcification
1	Partial lucency, smooth margin of metaphysis visible
2	Partial lucency, smooth margin of metaphysis not visible
3	Complete lucency, epiphysis appears widely separated from distal metaphysis

B: Multiplier	Portion of growth plate affected
0.5	≤ 1 condyle or plateau
1	2 condyles or plateaus

2 bones × 1 point × 3 points = 6 points possible

Total: 10 points possible

[a]Score the worst knee and the worst wrist.

Figure 1.15 Ten-point radiographic scoring system for rickets
Reproduced with permission from Thacher et al, 2000 (127)

1.13 Vitamin D and Fracture

Fractures in children are common and the incidence is increasing (128). Rennie et al in 2000 has documented the epidemiology of fractures in Edinburgh, Scotland and has reported the incidence of fractures in children as 20.2 /1000/year with a higher incidence in males (61%) (128). Fractures are more common in children who have small, narrow and weak bones. Studies have shown that fractures in early childhood are associated with later bone strength. There are several (i) non-modifiable factors such as age, gender, race & genetic make-up and (ii) modifiable factors such as nutrition (vitamin D & calcium intake), lifestyle, body weight and exercise that can contribute to bone strength. Low calcium intake is associated with an increased risk of fracture. Vitamin D plays a pivotal role in bone health by increasing the absorption of calcium from the gut. Severe vitamin D deficiency and rickets may increase the risk of fracture in infants and children. The existing evidence on the link between severe vitamin D deficiency and fractures is quite variable and are discussed in detail in chapter 3.

1.14 Osteoporosis and fracture risk

Osteoporosis is a major public health concern and is caused by the loss of bone mass and architectural deterioration with subsequent predisposition to fracture. It accounts for nearly 300,000 fractures in the UK each year (129). These fractures typically occur at hip, spine and wrist (129). Nearly £1.7 billion of the NHS budget in the UK was spent on osteoporosis annually a decade ago, (130); current estimates are £3.4 billion (131). Peak bone mass, the amount of bone mass acquired by the end of skeletal maturation, predicts the risk of osteoporosis in later life (132) (133). Failure of bone accrual during growth is associated with an increased risk of osteoporosis in later life (134). Hence there is a clear rationale for enhancing bone mass accrual in early life.

Peak bone mass depends on both genetic and environmental factors such as diet, exercise and sex steroid status (135). The University of British Columbia 'Healthy Bones Trial' demonstrated a 5% greater bone accrual ($p < 0.05$) observed at 20 months in both girls and boys that was approximately double that reported at 7 months (136). Studies have also shown a positive effect of weight bearing exercises exerted during childhood on adult bone mass (137). A retrospective cohort study involving 99 female retired dancers mean (SD) = 51 (14) years and 99 controls has reported that classical ballet classes undertaken between the ages of 10 and 12 years are independently and positively associated with a difference in BMD at both the femoral neck site (β= 0.73, p=0.001) and the total hip site (β= 0.55, p<0.01) between dancers and controls (137). Although targeted interventions with Vitamin D in later life might stall or reverse the symptoms of osteoporosis to some extent (138) existing evidence does not support the sole use of vitamin D in fracture prevention (139), (140).

1.15 Bone

1.15.1 Composition and Structure

Bone is a hierarchically structured organ comprised of a complex network of cells and interconnected cell processes (141). It is primarily made up of a protein collagen (20-40%), mainly type 1 collagen and a mineral calcium phosphate (50-70%), mainly hydroxyapatite. Remaining 10% of the bone is made of non-collagenous proteins, lipids and water (142). These components are finely organised to form a complex three-dimensional hierarchical structure [Figure 1.16 and Figure 1.17] and are responsible for the mechanical properties of the organ (143) (144).

Macroscopically, bone is made up of cortical bone, which is the densely packed compact outer layer bone and an inner core of spongy cancellous or trabecular bone. Cortical bone is made of osteons that are comprised of layers of lamellar bone with Haversian canals containing blood vessels and nerves in the centre. Within the lamellar bone are the collagen fibrils and bone crystals (145). Trabecular bone

is formed in plate and rod-like structures with spaces in between. And it is also made up of lamellar bone containing lacunae and osteocytes. The spaces in between are filled with marrow that provides blood supply and nutrients to the bone. And this organization makes the bone strong and light (145).

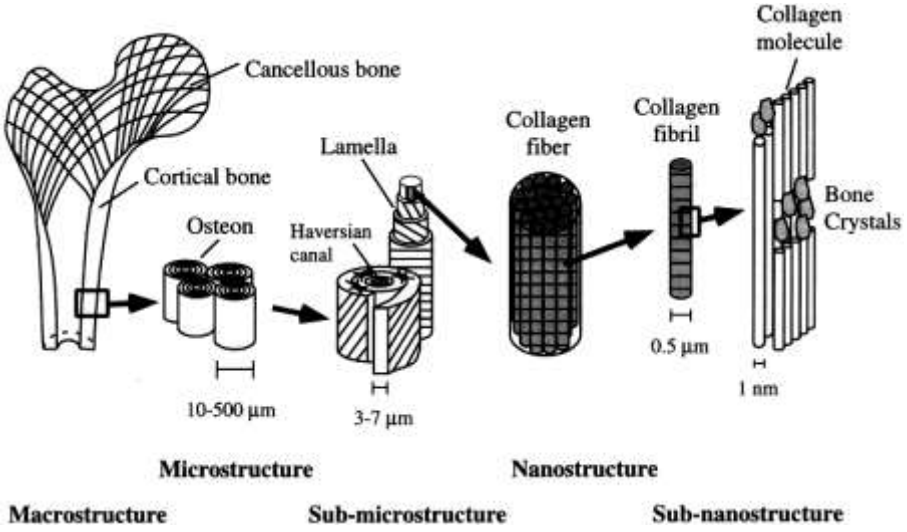

Figure 1.16 Hierarchical structural organization of bone.
Reproduced with permission from Rho et al, 1998 (143).

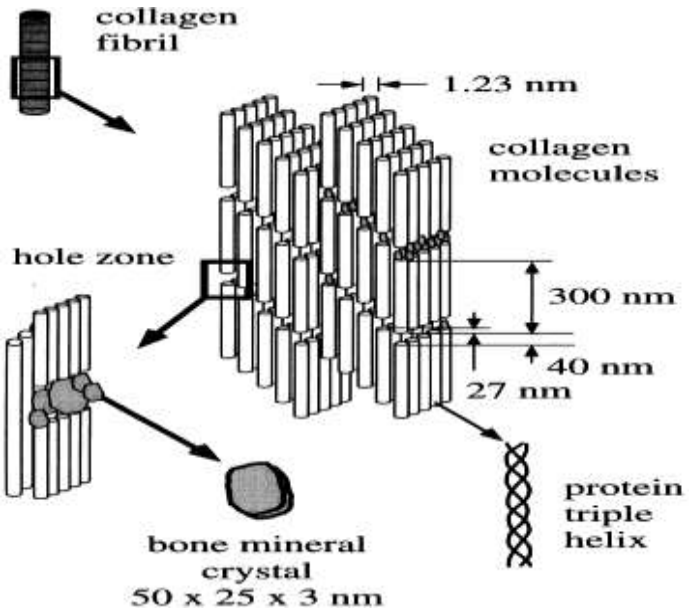

Figure 1.17 Assembly of collagen fibrils and fibres and bone mineral crystals.
Reproduced with permission from Rho et al, 1998 (143).

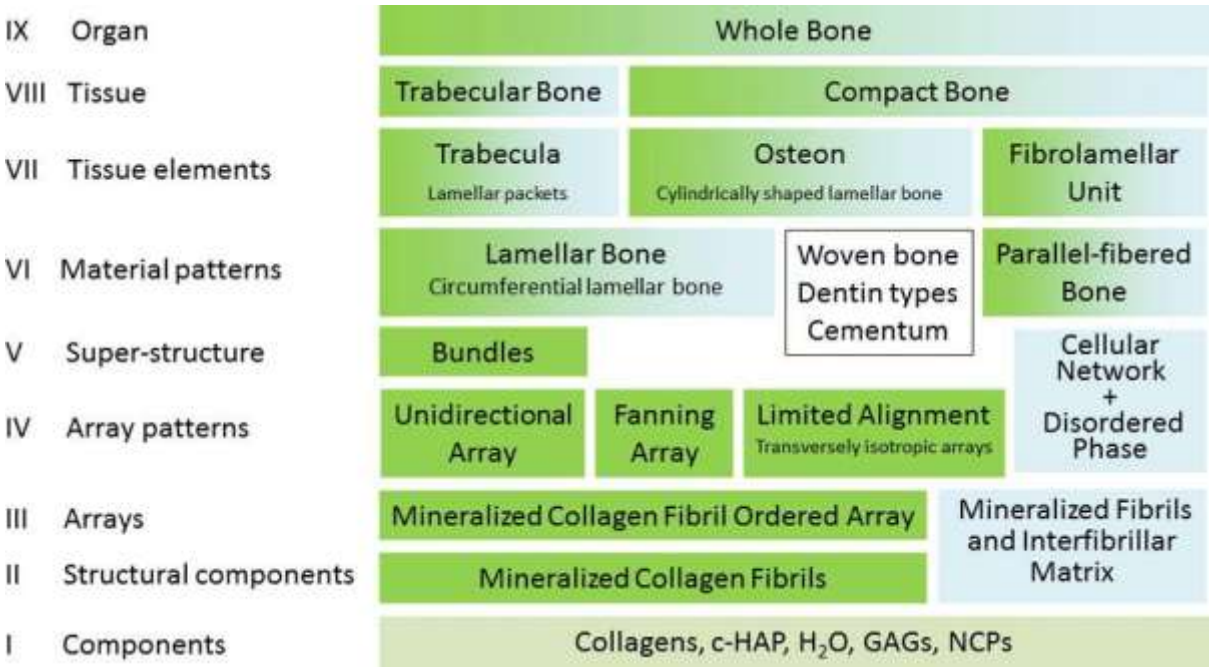

Figure 1.18 Scheme showing the hierarchical organisation of bone
Reproduced with permission from Reznikov et al, 2014 (141).

1.15.2 Bone cells

There are three types of bone cells [Figure 1.19], osteoblasts, osteocytes and osteoclasts (146). Osteoblasts and osteocytes are involved in bone formation and are derived from mesenchymal stem cells. Osteocytes are osteoblasts that are trapped during matrix formation (146). Osteocytes play a key role in the response of bone to mechanical loading (147). Sclerostin, a protein secreted by osteocytes inhibits bone formation through the Wnt signalling pathway (148). Mechanical loading triggers an anabolic response by inhibiting sclerostin secretion (149). Osteoclasts are multinucleated cells like macrophages that are derived from the haematopoietic system and are responsible for bone resorption (146). Osteoblasts also have an indirect influence on bone resorption through RANK-L and OPG (150) as described below.

1.15.3 Physiology and Function

The growth, development and maintenance of the skeletal system is underpinned by two synchronous process, modelling and remodelling. Bone modelling is primarily responsible for bone formation and resorption and remodelling is primarily responsible for the removal and repair of the damaged bone. Osteoblasts, osteoclast and osteocytes are the three types of bone cells that are responsible for this synchronous process of bone modelling and remodelling which takes place at the tissue level. Bone modelling is responsible for gain in bone mass and occurs right from birth to adulthood. It is not coupled i.e. bone formation and resorption occur on separate surfaces (151).

Bone remodelling is responsible for maintaining the bone mass in adults. It is coupled i.e bone formation and resorption takes place on the same surface where old bone is removed and new bone is formed (152). The process of bone remodelling takes place over a period of several weeks in a sequential phase of activation, resorption, reversal, formation and termination as illustrated in Figure 1.19 (151)

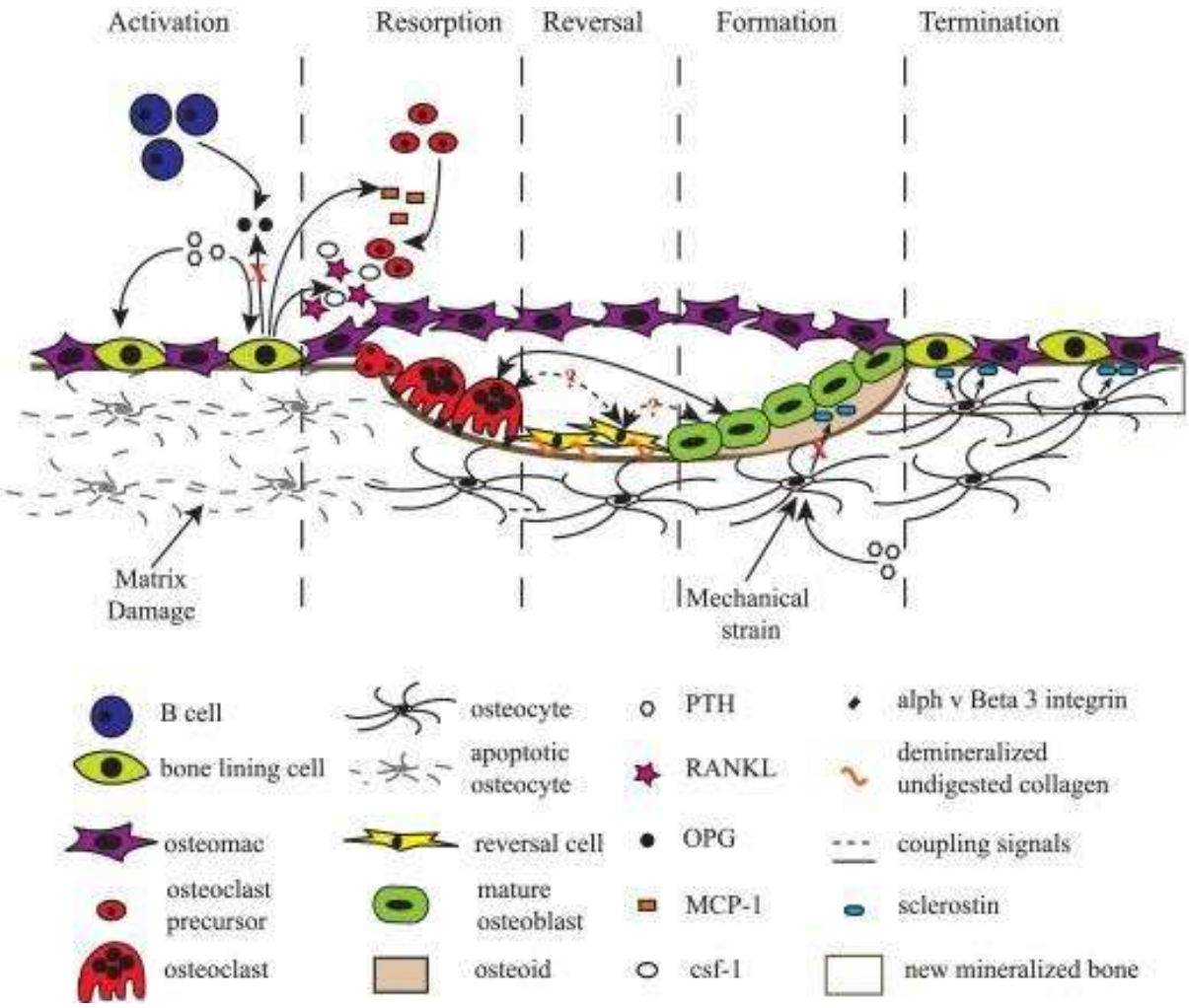

Figure 1.19 Stages of Bone Remodelling
Reproduced with permission from Raggat et al, 2010 (151)

At the pre-activation stage, the resting bone surface is covered with pre-osteoblasts and osteomacs. Osteomacs are tissue macrophages that reside within the bone cells on the endosteal and periosteal surfaces. Osteoprotegerin (OPG) secreted by the B-cells in the marrow suppresses the osteoclastogenesis. During the activation stage, parathyroid hormone (PTH) binds to PTH receptor on pre-osteoblasts which results in localised apoptosis. Subsequently, resorption stage takes place where monocyte chemoattractant protein-1 (MCP-1) is released from osteoblasts and recruits pre-osteoclasts to the bone surface. In addition, the osteoblast expression of OPG is decreased and

production of colony stimulating factor-1 (CSF-1) and receptor activator of NF-kB ligand (RANKL) is increased to promote the proliferation of osteoclast precursors and differentiation of mature osteoclasts. A sealed localised microenvironment is created for the degradation of the mineralised bone matrix through anchoring of the mature osteoclasts to the RGD binding sites. This is followed by the stage of reversal where removal of the demineralised and undigested collagen takes place. During this stage transition signals are generated to stimulate bone formation and halt bone resorption. Next is the formation stage where molecules and signals for bone formation arise from the degraded bone matrix, mature osteoclasts and reversal cells. Expression of sclerostin is reduced by PTH and osteocyte activation resulting in Wnt-medicated bone formation. This is followed by the termination phase when the bone formation terminates. Mineralisation of the newly deposited osteoid occurs and the bone surface returns to a resting with pre-osteoblasts intercalated with osteomacs concluding the remodelling cycle (151)

Bone is highly dynamic and has three most important physiological functions (1) structural support and protection for internal organs of the body (ii) host for haematopoiesis (iii) reservoir for calcium and phosphate (153) (154). Bone homeostasis is crucial for the maintenance of bone mass and strength. Bone strength is determined by a number of intrinsic and extrinsic biomechanical properties (155) as outlined in Figure 1.20. A tight balance between extrinsic and intrinsic factors is crucial for maintaining the bone integrity and preventing fracture.

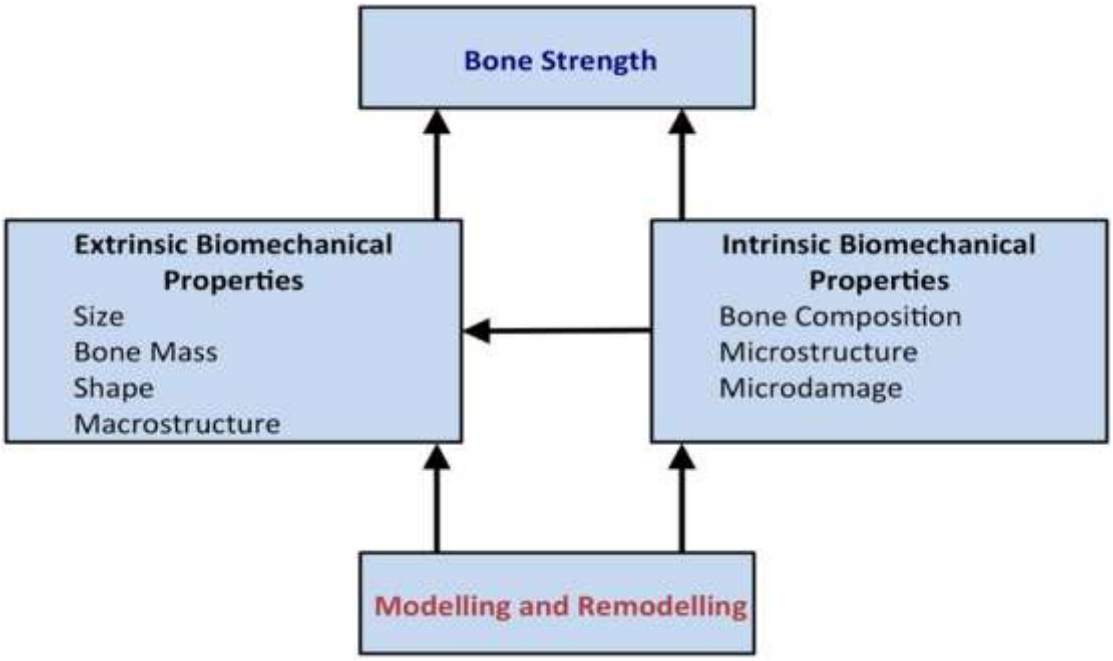

Figure 1.20 Determinants of bone strength.
Reproduced with permission from Forester-Zhang et al, 2016 (155)

1.16 Bone turnover markers

Biochemical markers of bone turnover (156) can be broadly classified into two groups (1) markers of bone formation and (2) markers of bone resorption based on their origin from the bone multicellular unit (BMU) as listed in Table 1.6 and shown in Figure 1.21 (157).

Serum	Bone formation	Bone resorption
	Matrix proteins	Type I collagen degradation products
	N-terminal propeptide of type I procollagen (PINP)	Cross-linked C-terminal telopeptide of type I collagen (CTX) Cross-linked N-terminal telopeptide of type I collagen (NTX) Carboxy-terminal telopeptide of type I collagen (ICTP)
	Osteocalcin (OC)	Pyridinoline (PYD) Deoxypyridinoline (DPD)
	Enzymes	
	Bone specific alkaline phosphatase (BSAP)	Tartrate resistant acid phosphatase (TRACP) 5b
	Regulators	
	Sclerostin, Dkk	Osteoprotegerin (OP), RANKL
Urine	Bone formation	Bone resorption
		Calcium Cross-linked C-terminal telopeptide of type I collagen (CTX) Cross-linked N-terminal telopeptide of type I collagen (NTX) Pyridinoline (PYD) Deoxypyridinoline (DPD) Hydroxyproline Galactosyl-hydroxylysine

Table 1.6 Biochemical Bone Turnover Markers

The most recent consensus statement on the use of biochemical BTM's for osteoporosis has recommended the measurement of serum PINP for bone formation and serum CTX for resorption in the clinical setting due to their bone specificity and low analytical variability (157), as approved by the International Osteoporosis Foundation and the International Federation of Clinical chemistry (158)

The prime clinical utility of BTM is to monitor adherence to oral bisphosphonate therapy. The algorithm also states that BTMs cannot be used to diagnose osteoporosis but to evaluate the causes

for secondary osteoporosis in the face of increased BTMs. Serum BTMs can also be used to predict fracture risk along with bone mineral density and clinical risk factors for fracture (157). Measurement of BTMs are relatively inexpensive and can be measured repeatedly to assess response to treatment. Several controllable and uncontrollable factors influence the levels of BTMS. The controllable sources are diet, diurnal variation and exercise that can be overcome by the measurements of more than one fasting early morning samples. The uncontrollable sources are age, gender, growth, fractures, drugs and disease. To overcome that age and gender specific reference range should be used. (159)

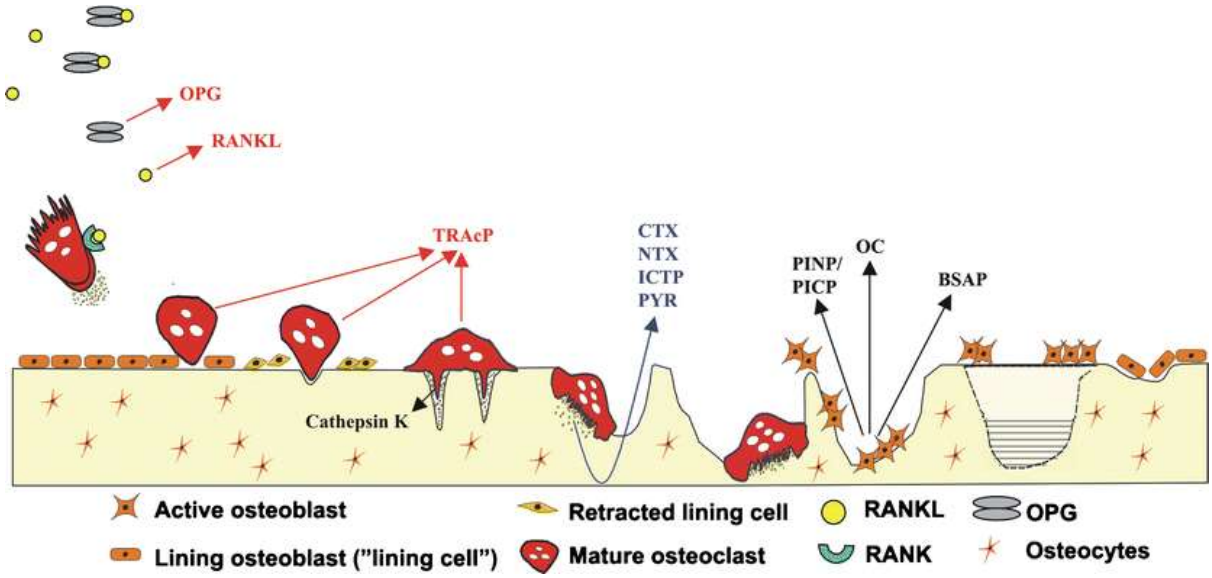

Figure 1.21 Schematic representation of BTMs
Reproduced with permission from Leeming et al, 2006 (157)

1.17 Functional adaptation of bone

The primary mechanical function of the bone is to provide rigid levers for the muscles and to hold the body upright against gravity (160). The skeleton is exposed to thousands of repetitive mechanical loads each day and responds to mechanical stimulation by an increase in bone size and mass and that increment can persist into adult life (161).

Adaptation of bone to these mechanical loads occurs from birth to adulthood through a complex multistep process called cellular mechanotransduction (162). Mechanotransduction is a process through which mechanical signals are converted to biochemical signals within the bone cells to cause bone formation or resorption (163). The process of mechanotransduction includes (1) mechanocoupling, sensation of the mechanical loads and conversion to mechanical signals (2) biochemical coupling, transduction of the mechanical signals to a biochemical response (3) cell to cell signalling, sensor cells (bone lining cells and osteocytes) to effector cells (osteoblasts or osteoclasts) using prostaglandins and nitric oxide as signalling molecules and finally (4) effector response, formation or resorption of bone (164).

1.18 Basic rules of bone adaptation

The three fundamental rules for the adaptation of bone to a mechanical stimulus (160) are (1) Dynamic strain stimulus - adaptation is driven by dynamic but not static loading, (2) Case of diminishing returns – a short duration of mechanical loading is sufficient to initiate an adaptive response. Longer the duration, diminished the response (165), and (3) Bone adaptation is 'error driven' - cells accommodate to a customary loading environment that makes them less responsive to routine loading signals (166).

Wolff's law states that bone will adapt to the load under which it is placed (167). This understanding is further enhanced by Harold Frost who proposed the mechanostat theory (168) (169) (170), which suggests that bone responds to the mechanical forces acting upon it by increasing or reducing mass until a state of equilibrium is reached in which bone mass and architecture are appropriate to the continuing demands placed upon it. As part of the proposed system there is a sensing mechanism that has a set point – akin to a thermostat in a heating system – that switches the system on and off.

The relationship between load and deformation of the bone is shown in a stress-strain curve [Figure 1.22] (171). Stress is the load placed on the bone divided by its cross-sectional area (155) and the

strain is the deformation of the bone divided by its length (172). The curve is divided into 2 regions at the yield point (i) elastic region, in which the bone can withstand the strain(deformation) of the stress(load) and return to normal upon removal of stress, and (ii) and plastic region in which the bone cannot withstand the strain and permanent bone loss occurs. Stress is represented by the height of the curve (b), strain by the entire length of the curve along the x-axis (c) and Young's modulus of elasticity by linear part of the curve (a). Area under the curve (d) is calculated as the modulus of toughness (155). Vitamin D is associated with greater failure displacement, greater post-yield displacement and greater work-to-failure (155).

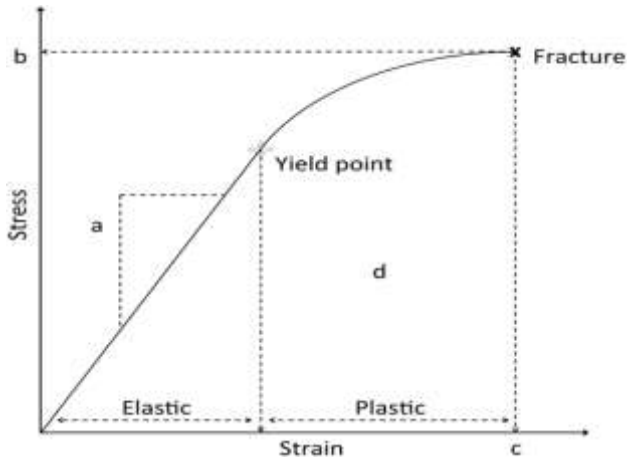

Figure 1.22 Stress Strain Curve.
Reproduced with permission from Forestier-Zhang et al, 2016 (155)

Bone growth can be achieved by loading of bone during childhood in the form of regular sport activities such as gymnastics and exercise programmes. Equally it can be achieved by using whole body vibration (WBV). WBV is the application of vibratory stimulus to the body in a synchronous fashion by which the bones are made much stronger reducing the risk of fracture in later life. Thus, WBV can be used as a means to assess bone responsiveness to mechanical stimulation. This thesis focuses on whole body vibration as a means of mechanical loading and is detailed as below.

1.19 Whole Body Vibration

1.19.1 Introduction

Whole Body Vibration (WBV) is defined as the exposure of the body of the patient to mechanical stimulus via the use of vibrating plates. WBV as a form of an exercise intervention is increasingly becoming popular in medical research over the last decades due to its combined effects on neuro muscular and neuroendocrine systems (173). WBV has been used to deliver mechanical accelerations to both axial and appendicular skeleton (174) to increase bone mass.

Prisby et al. has proposed that an interplay between several systems such as bone, muscle, nerves, blood vessels and hormones may play a role on the effects of WBV on physiological systems (175) as illustrated in figure 1.23.

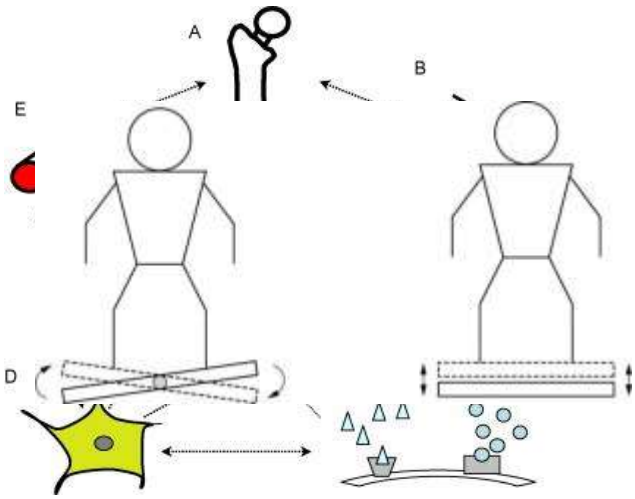

Figure 1.23 The potential effects of WBV on physiological systems and the potential interplay among systems. WBV modulates the (A) Bone (B) muscular (C) endocrine (D) nervous and (E) vascular systems that may elicit secondary responses through interaction among the systems of which the figure presents only the most obvious.
Reproduced with permission from Prisby et al, 2019 (175)

There are two ways these vibrations are transmitted [Figure 1.24] either by vertical planar (up and down) or oscillating axial fashion (side to side) (176). The amount of vibration transmitted to each individual body part varies significantly as energy is lost the further away from the plate the vibration has to travel (177).

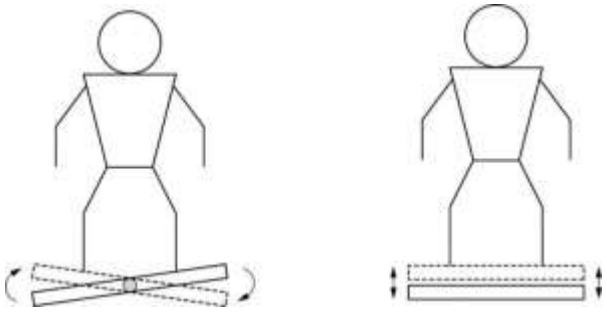

Figure 1.24 Designs of whole body vibration plates
Reproduced with permission from Cardinale et l, 2005 (173)

The side-to-side alternating platform is designed to deliver high frequency high magnitude vibration which can either be delivered in a synchronous vertical motion or side-to-side motion. This type of vibration mimics activities like walking which induce muscle fatigue and stimulate bone loading (178). The mechanism of action has been attributed to (1) reflex muscle activation (179), (2) muscle twitch potentiation (180) and neuromuscular modulation (181). The muscles attached to the bone provide them with the greatest stress. Mechanical stimulation in the form of WBV acts on the muscles to initiate tonic vibration reflex (182).

The synchronous vertical up and down platform is designed to deliver low magnitude high frequent vibration (183). This type of vibration mimics mild activities like standing that form a dominant component of the skeleton's 24 hours strain (183) and has been shown to have a direct anabolic effect on the bone. The vast majority of animal studies have been undertaken using this system (184) (185) and have demonstrated an increase in bone mass, strength and content.

The numerous devices to deliver vibration that are available on the market vary greatly; some of those available are compared in Table 1.7. The terms most frequently used to describe the performance of vibration plates are vibration frequency (Hz, number of complete up and down movement cycles per second) and amplitude (mm, oscillation size) (186).

Name	Manufacturer	Vibration frequency (Hz)	Amplitude (mm)
Galileo Advanced	Novotec GmbH	1-30	2, 4, 6
Juvent MDT 1000	Marodyne	32- 37	0.085
AV-001A	Body Green	8- 40	0.4- 2.0
NEXTgeneration	Power Plate	25- 50	2- 4
LivMD	Marodyne	30-90	0.4*g (magnitude)*

Table 1.7 Comparison of example vibrating plates currently available

The public within the gym setting can use these devices as part of training to improve muscle strength, power and flexibility. As a result, researchers have sought to test its use and efficacy in a wide range of common childhood conditions / treatment associated with poor muscle tone and strength (187).

Prisby et al. (175) in their recent review article on WBV has reported the summary of skeletal effects of whole body vibration in animals and humans, as listed in Table 1.8 [Reproduced with permission]

Target population	Wave type	Vibration protocol	Duration	Intensity (accelerations (g); amplitudes (mm); force (N))	Frequency (Hz)	Outcome	Reference
Animal studies							
OVX Wistar rats (12 wks)	NR	30 min/d, 5 d/wk	12 wks	2.0 g	50	Vibration: ↓ OVX-induced bone loss ↑ BMD	Flieger et al. (1998)
Female Sprague Dawley rats (6–8 mon)	NR	10 min/d	4 wks	0.25 g	90	↑ BFR and MS/BS with normalized BFR in vibration + HU group similar to CON	Rubin et al. (2001b)
C57BL, BALB/c, and C3H mice (age NR)	SIN	10 min/d	NR	0.25 g	45	↑ BV/TV in C57BL only (85%); ↑ BFR/BS in BALBc (32%), ↔ in C3H	Judex et al. (2002)
OVX Wistar rats (1 yr)	NR	30 min/d	17 wks	0.5 g	17	@ 45 Hz: ↑ periosteal BFR and ↓ OVX-induced endocortical resorption, ↓ declines in biomechanical properties	Oxlund et al. (2003)
				1.5 g	30		
				3.0 g	45		
Male mice (7 mon)	SIN	15 min/d	5 wks	0.1 g	45	↑ trabecular bone BV/TV in 0.1 and 1.0 g groups	Christiansen and Silva (2006)
Female mice (2 mon)	SIN	15 min/d	3 wks	0.3 g	45	↓ osteoclastic activity; ↑ BFR	Xie et al. (2006)
C57BL/6 mice (12 wks)	SIN carrier waveform	3 d/wk	4 wks	2 N	?0–50	No effect of vibration on periosteal bone formation in the ulna	Castillo et al. (2006)
OVX Sprague Dawley rats (6–8 mon)	NR	10 min/d 5 d/wk	4 wks	0.15 g	45 90	90 Hz: ↑ BFR, BV/TV and TbTh	Judex et al. (2007)
HU female mice (4 mon)	SIN	20 min/d, 5 d/wk	3 wks	0.6 g	45	↓ unloaded-induced bone loss and ↓ deterioration of microarchitecture	Ozcivici et al. (2007)
Adult female C57BL/6J mice (19 wks)	SIN	10 min/d	3 wks	0.3 g, 0.6 g	45	0.3 g: 88% ↑ trabecular BFR/BS, ↑ MS/BS (64%); 0.6 g: 66% ↑ BFR/BS, 22% ↑ MS/BS, 8% ↑ epiphyseal cortical area and 8% ↑ thickness	Garman et al. (2007)
BALB/cByJ mice (8 wks)	NR	15 min/d, 5 d/wk	6 wks	0.3 g	45	Proximal tibial metaphysis: ↑ MS/BS; cortical bone: ↑ BV, periosteal bone area, bone marrow area, cortical area and moment of inertia; ↑ soleus cross-sectional area	Xie et al. (2008)
OVX Sprague rats (3 mon)	SIN	20 min/d, 5 d/wk	8 wks	0.6 g, 3.0 g	30	Longitudinal analysis: no differences in cortical or trabecular BMD by pQCT; cross-sectional analysis: no differences in histomorphometric analysis; *in vitro* analysis → vibration at 3 g increased total cortical and medullary areas and periosteal and endosteal perimeter in OVX rats	Rubinacci et al. (2008)
Human studies							
PMP (58–74 yrs)	SIN	30 min, 3 times/wk	24 wks	2.28–5.09 g, 1.7–2.5 mm	35–40	↑ hip BMD (0.93%); ↔ markers of bone remodeling; ↑ isometric (15%) and dynamic (16%) muscle strength	Verschueren et al. (2004)
Pre- or postpubertal disabled, children (4–19 yrs)	SIN	10 min/d, 5 times/wk	6 mon	0.3 g	90	↑ vTBMD proximal tibia (6%); vTBMD in control group ↓ 12% ↔ cortical BMD, ↔ muscle parameters	Ward et al. (2004)
PMP (57 yrs)	SIN	2 × 10 min/d	12 mon	0.2 g	30	↔ BMD hip, lumbar spine, ↑ spine BMD in compliant subjects	Rubin et al., 2004
Osteoporotic PMP (55–88 yrs)	NR	1 time/wk, 4 min	12 mon	0.7–4.2 mm	20	↔ BMD lumbar spine, hip; ↔ markers of bone remodeling; ↓ chronic back pain	Iwamoto et al. (2005)
Low-BMD females w/ fractures (15–20 yrs)	SIN	10 min/d	12 mon	0.3 g	30	2.1% ↑ cancellous vertebral BV/TV, 3.4% ↑ femoral cortical bone, 5% ↑ cross-sectional area of paraspinous muscle	Gilsanz et al. (2006)
PMP (66 yrs)	NR	3 times/wk WBV vs. walking	8 mon	3 mm	12.5	↑ BMD femoral neck (4.3%) ↔ BMD lumbar spine ↑ balance (29%)	Gusi et al. (2006)

Table 1.8 Summary of skeletal effects of whole body vibrations in animals and humans

Here I present the literature review on WBV studies undertaken in children. The rationale for the use of WBV to either increase bone mass, or mitigate possible bone loss is common to most of the studies described in this thesis.

1.19.2 Healthy Children

The effect of intervention in healthy children was examined in which 36 healthy boys aged between 9 and 12 years were asked to stand on a low (<1g, frequency 32-37 Hz, amplitude 0.085 mm) or high (>2g, frequency 5 to 30Hz, amplitude 0+/-4.5mm) magnitude vibrating plate for either 1, 3 or 5 successive days (178). A control group of 15 boys stood on an inactive platform. For all participants, blood samples were taken before and after intervention to assess markers of bone formation (P1NP and osteocalcin) and resorption collagen type I C-telopeptide (CTX). The focus of the study was to measure the change in bone turnover markers and not to measure the functional outcome. This study showed a significant rise in both P1NP 25.1% (p=0.005) and CTX10.9% (p=0.009) from baseline to day 8, in the group exposed to 5 days of WBV. These platforms were very well tolerated with the boys only experiencing a mild tickling sensation in the feet (low magnitude WBV) or a strange or itching feeling in the calves (high magnitude WBV). These symptoms resolved shortly after completion of the intervention (178).

1.19.3 Cerebral Palsy

A total of 12 studies were reviewed and one area of improvement for study participants was a reduction in spasticity. This positive outcome was revealed in five (188-192) of the twelve studies identified (14-24) with the participants in these studies all measuring improvement on the Modified Ashworth Scale (MAS). The scale is measured 0 with no increase in muscle tone, to 4 with affected part(s) rigid in flexion or extension.

The intervention in these studies ranged from 20 minutes of WBV (8 to 40 Hz and 0.4 to 2.0 mm) on 2 separate days 1 week apart (188) to 12 weeks of 3 times weekly sessions of 40 minutes of physical therapy and WBV (191) (40Hz frequency). The reduction in spasticity was significant in all of these studies; one study reported MAS reducing by 1.43 (p=0.036) (189). These effects continued for three days after the intervention with children able to more actively engage with therapeutic interventions and daily activities (188, 189). Improvements in gait, posture and walking speed were also found in many of the studies with walking speed and step length of the less affected side having increased, and step width of the affected side having decreased (188, 189, 193-196).

Muscle strength and bone density also significantly improved following WBV therapy (190, 197, 198) along with range of movement but it is unclear how much of this effect is due to additional physical therapy, which was often used alongside the WBV therapy, such as, *"stretching exercises for Achilles tendon, hamstrings, hip flexors and adductors of both lower limbs, upper abdominal and pectoralis muscles"* (190). Within this study, the control group of children without cerebral palsy, did not show any improvement following the 12-week program of *"9 minutes 3 times per week for 3 months of WBV along with other strength building exercises"* (190).

These studies reported no side effects from the use of WBV within children with CP. However, one of these studies limitations is that most of the children involved were less severely disabled as they were required to stand on the platform during the WBV, meaning that those at greatest risk to fracture weren't exposed to the intervention.

1.19.4 Children with motor difficulties

Ward et al. (199) examined the osteogenic potential of short durations of low-level mechanical stimuli in children with disabling conditions in 2004. The authors conducted a prospective, double-blind, randomised placebo-controlled pilot trial (RCT) to elucidate the effect of WBV on tibial and spinal

volumetric trabecular BMD (vTBMD) in 20 pre and post pubertal, ambulant and disabled children (mean age, 9.1 +/- 4.3 years). The children were randomised to active or placebo devices for 10 minutes a day for 5 days a week for 6 months. The net benefit of treatment at the end of 6 months for tibial vTBMD was +15.72 mg/ml (17.7%; p= 0.0033). The authors concluded that low magnitude, high frequency mechanical stimulation was anabolic to trabecular bone in children with limited mobility (199).

A final study assessed the efficacy of WBV in severe motor disorders such as Cerebral Palsy, Rett Syndrome and also in children for whom their neuromuscular diagnosis remained unclear despite thorough investigation (200). Nineteen non-ambulatory children between the ages of 5 and 16 years underwent WBV twice per week for 6 months for between 5 and 15 minutes (40–42 Hz frequency, amplitude of 0.2 mm). At the start, 6 and 12 months, bone mass parameters and bone markers were measured including osteocalcin, P1NP and CTX. The markers of bone formation (PINP and bone ALP) remained within the normal reference range, but osteocalcin (another marker of bone formation) did remain 50% lower than the age and gender-adjusted reference intervals throughout. The relatively high CTX levels with low osteocalcin levels in children with disabilities do indicate that they have lower bone formation rate when compared to their healthy counterparts. During the 6-month WBV intervention period the levels of Vitamin D and calcium fell (82 nmol/l to 58 nmol/l, $p < 0.01$ and 2.45 mmol/l to 2.37 mmol/l, $p < 0.05$) respectively). The levels of these markers returned to normal after the intervention (200). Fractures did occur within this study with 5 children receiving one during the study period. However, this is on an increased background risk of their likely immobility induced osteoporosis, with 63% of the participants having a fracture either pre or during the study, high when compared to the 42% risk of a fracture in a healthy 16 year old male. Their total body BMD increased after 6 months of WBV (200).

1.19.5 Osteogenesis Imperfecta (OI)

Semler et al. studied eight children or adolescents with osteogenesis imperfecta type 3, (N=5) and osteogenesis imperfecta type 4 (N=3) who received 2 daily therapy sessions. Subjects laid on a tilting table whilst receiving 3 sets of 3 minutes of WBV through the soles of their feet (10-15 Hz frequency, 1-2mm amplitude). The table began at 10 degrees and was gradually raised towards vertical as the study progressed. Throughout, the patient's ability to put pressure on the vibrating plate was measured and used to assess muscle strength. This study found that a 6-month regime of WBV improved the participants' muscle strength (P=0.002). Participants also had individual, more subjective benefits which were documented within the paper such as being *"independent getting in and out of his wheelchair, walking distance 30-250 steps with posterior walker"* (201). Regarding safety, within this study one child "*developed a localised pain at the end of an intramedullary rod, which was already dislocated before starting whole body vibration*" which required no surgical management. 2 patients acquired fractures unrelated to the WBV intervention. Other than these events musculoskeletal safety wasn't reported amongst the cohort. Some participants experienced itching directly after the vibration. This was felt to be due to increased blood flow to the calves (202).

1.19.6 Duchenne Muscular Dystrophy (DMD) and Spinal Muscular Atrophy (SMA)

Two studies are reported in the literature, the first enrolling boys with DMD alone, the second both DMD and SMA patients.

Soderpalm et al. focused solely on boys with DMD, participants received 2 to 3 sessions of WBV per week for 3 months (16-24Hz and 2-4mm on a side to side alternating platform). Nine month follow-up showed no significant increase in bone mass or bone strength (203). This study found that WBV in this group was well tolerated with no adverse events.

Vry et al. studied patients with DMD and SMA used the Galileo MedM© platform for 3 x 3 mins, twice a day, on 5 days per week (18 to 24 Hz frequency, 4mm amplitude). After one day of training, the DMD

group had increased levels of creatinine kinase (CK). However, at the end of eight weeks these levels had returned to normal. Children with SMA showed no change in CK. In the children with SMA the average six-minute walking test (6MWT) distance increased from 371.3m to 402.8m ($p<0.01$). There were no changes in 6MWT, time to climb 4 stairs, time to walk 10m and the time to rise from supine for boys with DMD (204). However, within this second study many adverse events were reported and some participants experienced more than one. These included leg erythema, muscle pain and talus fracture.

1.19.7 Down syndrome

Three studies in children with Down syndrome have been reported. Gonzalez-Aguero et al. examined a group of 30 children with Down syndrome aged between 12-18 years underwent 30- 60 second periods of WBV totalling 5-10 minutes of WBV, three times per week for 5 months on a vertical vibrating platform (Power Plate Pro5, 25-50Hz, 1-2mm). Body fat and lean body mass were measured with dual energy X-ray absorptiometry (DXA) at baseline and at study end. The study group were found to have reduced body fat in the upper limbs ($p < 0.05$) and a non-significant tendency toward a higher percent increase in lean body mass ($p=0.229$) (205).

Matute-Llorente et al. studied the effects of increasing periods of time and intensity of WBV on BMC, BMD and structure variables, measured by DXA and compared with a control group. This study used the same platform (Power Plate Pro5, 25-50Hz, 1-2mm). The whole body BMC of the study group increased by 2.8% and BMD also increased by 4.8% (both $p<0.05$) when compared to baseline (206).

In the third study, Eid MA. randomised 30 patients to either a control (15) or study group (15) who took part in a physical therapy program of three, one-hour sessions per week for 6 successive months. In addition, the study group also received 3 sessions per week of WBV of increasing duration (Galileo Basic, 25- 30Hz frequency, 2mm amplitude). The balance of the children, which was measured using the Overall Stability Index (OSI), significantly improved from baseline (1.4-1.19) in the study group

when compared to the control group (1.42-1.37) (p=0.004). Along with this the study participants experienced improved muscle strength of the knee flexors and extensors (13.71 - 15.65lbs, P=0.04 and 14.04 -16.04lbs, P=0.01 respectively) (207).

These three studies identified no complications or side effects of using the platforms as part of physiotherapy for children with Down syndrome.

1.19.8 Spina Bifida

Sixty children with Spina Bifida aged between 4 and 15 years received side-alternating WBV platform (System Galileo®, Novotec Medical, and Pforzheim, Germany) over a six-month period in a study conducted by Stark et al (208). The intervention led to the participants receiving 3×3 min of 10-15 Hz frequency, 1-2mm amplitude vibration 10 times per week during the home-based WBV training periods. The children's walking velocity, assessed by measuring ground reaction force, significantly improved, meaning they sped up by 0.11m/s (p=0.0026). Their mobility also significantly improved by 2.54 points according to the Gross Motor Function Measurement (GMFM-66) (p=0.001. Within the 60 patients, 12 children's parents reported (subjective) increased contractures. When analysed these were shown to have decreased during the active training period and increased during the active follow up period of 6 months each respectively. Other events were reported by participants such as spontaneous bladder emptying (3 patients), a femoral fracture (1, not study related), a symptomatic tethered cord that required surgery (1, unclear if study related) and one patient requiring a shunt revision during the study period (again unclear if related to the trial).

1.19.9 Idiopathic Toe Walking (ITW)

ITW is one of the uncommon gait disorders of childhood and is defined as toe walking where the cause can't be identified as being related to another condition. The exact prevalence of this condition is unknown but one study has suggested that around 2% of children persist in toe walking until 5 and a half years (209). One study analysed the effect of using a single dose of WBV for children with idiopathic toe walking. In the study conducted by Fanchiang et al. 15 children diagnosed with ITW and 15 apparently healthy children aged 4 to 10 years received 1 minute of 30 Hz WBV. No change in gait was observed when analysed using HR32 (an algorithm designed to highlight aspects of the toe-walking pattern by analysing at what point in the cycle the ankle height rises). For example, early heel rise (starting before 32% of the cycle is complete) means that the patient has a toe walking pattern. It was also found that the "healthy controls" experienced no benefit on receiving this brief intervention (210). No participants experienced any adverse events or discomfort during the interventions.

1.19.10 Obesity

Twenty obese Latino boys were assigned by Erceg et al. either to a no intervention control group (n=9) or a study group (n=11) who received WBV for increasing lengths of time three times a week using a NEXT generation vibration platform© (30–40 Hz frequency, 2–4 mm amplitude). Bone formation (serum osteocalcin), and bone resorption (CTX) were measured at baseline and after a ten week WBV intervention. Oral Glucose Tolerance Tests (OGTT) and a total body dual-energy x-ray absorptiometry (DXA) scan were also undertaken. Within the vibration group, the results showed statistically significant increases in BMC and aBMD when compared to baseline (4.5±3.2%, p=0.01 and 1.3±1.3%, p<0.01 respectively). In the control group, BMC increased significantly with no change observed in aBMD (2.0±2.2%; p=0.02 and 0.8±1.3%; p=0.11 respectively). There were no significant changes in osteocalcin or CTX in the study group; the control group showed a significant increase in CTX (p<0.03) (211). There was no effect of the intervention on insulin resistance as measured by the homeostatic

model assessment of insulin resistance (HOMA-IR) (211). No adverse events were reported amongst the study participants.

1.19.11 Burns affecting more than 30% of the total body surface area

Within the one study identified, nineteen children were randomly allocated to receive 12 minutes of WBV with a standardised exercise regime or exercise alone five times per week (212). The 6-week program consisted of progressive resistance exercise and aerobic conditioning that lasted from 30 min to 1 h with the intervention group also receiving WBV (30–40 Hz frequency, 2–4 mm amplitude). Total body BMC, BMD and Lean Mass (LM) were measured by DXA. Muscle strength was measured using isokinetic testing. The WBV intervention had no effect on any parameter outlined above, but both groups improved from baseline when examining absolute knee extension peak torque (Δ23.8 +/- 2.7, $p < 0.001$ and Δ15.1 +/- 2.3, $p < 0.001$ respectively) (212). No adverse outcomes occurred as a result of WBV; the intervention was reportedly acceptable to all participants.

A table of all the WBV studies conducted in children based on the conditions and platform types are listed in Table 1.9.

Name of Trial	Trial Type	Study Pop.	Platform Type	Vibration Type	Frequency (Hz) Amplitude (mm)	Vibration Regime	No. of patients	Gender M: F	Age (range /mean)	Primary outcomes of study
Harrison, R. et al (2015)	Intervemtion	Healthy children UK	Galileo Advanced platform (Novotec Medical GmbH, Pforzheim, Germany) Juvent MDT 1000 platform (Marodyne, Lakeland, Florida, USA)	Side to Side oscillating platform Vertical plate displacement	5 - 30Hz 4.5mm 32-37 Hz 0.085mm	**18 Low magnitude/ 18 High magnitude** Vibration daily for 10 minutes with blood taken immediately pre and post, 60 mionutes post, 3 days (pre and post) and 5 days (pre and post). **15 Control**	51	51:0	9-12	Increased bone turnover markers in the blood. In 1avour of bone formation. (increased the bone formation marker P1NP by 25.1% and the resorption marker CTx by 10.9%)
Side to Side										
Eid, M. et al. (2015)	Randomised controlled trial	Down Syndrome, Eygpt	Vibraflx Home Edition II; Orthometrix Inc, USA (. Side to side movement)	Side to Side oscillating platform	25- 30 Hz 2mm	**15 Control** **15 Study** 3 sessions per week with increasing duration of vibration therapy and total training time. Alongside vibration, balance and muscle contraction exercise performed	30	17:13	(8.93)	Statistically significant improved balance and muscle strength in the study group
El- Shamy, S. et al. (2013)	Randomised controlled trial	Diplegic cerebral palsy Eygpt	Vibraflx Home Edition II; Orthometrix Inc, USA	Side to Side oscillating platform	25- 30 Hz 2-6mm	**15 Control** **15 Study** 1 hour per day, 5 days per week. Each session consisted of 9 minutes of exposure to WBV	30	23:7	(9.79)	Significantly improved speed of quadriceps movement in the study group.
Gonzalez-Aguero, A, et al (2013)	Randomized controlled trial	Down Syndrome Spain	Power Plate1 Pro5; PowerPlate, Amsterdam, The Netherlands	Side to Side oscillating platform	25-30 Hz 2mm	**13 Control** **11 Study** 3 days per week WHOLE BODY VIBRATION for 20 weeks	30	19:11	12-18	Slightly reduced whole body fat (not sig)
Kilebrant, S. et al (2015)	Intervention	Severe motor disabilities Sweden	Hoppolek, Jump & Joy AB, Förmansvägen 19 SE-117 59 Stockholm Sweden		40–42 Hz 0.2 mm	**All Study** At least twice a week, 5–15 min/ treatment, at	19	6:13	5.1-16.3 (12.5)	Increased bone turnover resulting in increased Bone Mass Density

Lee, B. et al (2013)	Randomised controlled trial	Cerebral Palsy Korea	Galileo system (Novotec Medical GmbH, Pforzheim, Germany)	Side to Side oscillating platform	5-25Hz 1-9mm	**15 Control** **15 Study** Conventional physical therapy (30 minutes of muscle stretching, balance training and gentle massage) +/- Whole Body Vibration (1 hour per day, 3 days per week for 8 weeks)	30	15:15	(9.83)	
Matute-Llorente, A. et al (2015)	Randomized controlled trial	Down syndrome Spain	Power Plate® Pro5; PowerPlate, Amsterdam, The Netherlands	Side to Side oscillating platform	25-30 Hz 2mm	**14 Control** Continued with their daily life **11 Study** Whole Body Vibration group received training for 20 weeks	25	17:8	15.7	Improved DXA in hip area No changes on peripheral quantitative computed tomography (pQCT) when scanning the tibia.
Myung-Sook, K. et al (2015)	Intervention	Cerebral Palsy Korea	Galileo system (Novotec Medical GmbH, Pforzheim, Germany). S	Side to Side oscillating platform	20-24Hz 1-2mm	**12 Control** **12 Study** 3 minutes of vibration stimulation and 3 minutes of rest, followed by 3 minutes of vibration and 3 minutes of rest, and another 3 minutes of vibration. Therefore, the total duration of vibration was 9 minutes	24	10:14	(9.45)	Improved walking speed, stride length in study groups.
Olama, K. et al (2010)	Intervention	Cerebral Palsy Eygpt	Galileo Basic (Vibraflex Home Edition II. Orthometrix Inc, White Plains, NY.)	Side to Side oscillating platform	12Hz 2-4mm	**15 control** **15 study** 6 times per week for 6 weeks. Either just physiotherapy or physio plus WBV (9 minutes. WBV session consisted of the following schedule: (3 min of WBV) – (3 min rest) – (3 min of WBV) – (3 min rest) – (3 min of WBV).)	30	Not disclosed	8-10 (8.92)	Significant improvement in stability for patients undergoing whole body vibration
Semler, O. et al (2008)	Intervention	Osteogenesis Imperfecta Germany	Galileo system (Novotec Medical GmbH, Pforzheim, Germany).	Side to Side oscillating platform	15-25Hz 1-2mm	**8 study** 10-degree angle of tilt table. 3 3 minute sessions of WBV with 3 minute breaks in between. 6 months of training	8	3:5	4.9-14.9	Increased muscle force and mobility

Soderpalm, A. et al (2013)	Intervention	Duchenne Muscular dystrophy Sweden	Galileo Delta (Novotec Medical GmbH, Pforzheim, Germany)	Side to Side oscillating platform	16-24Hz and 2-4mm	**6 study** 2-3 times per week, for two minutes per session in the first 2 weeks. Following this the duration increased to 6 minutes per session in the remaining 10 weeks.	6	Not disclose d	5.7-12.5 (6.8)	No significant change in bine mass density, muscular density or muscle strength. No change in Creatinine Kinase activity.
Stark, C. et al (2013)	Intervention	Spina Bifida United Kingdom	Galileo system (Novotec Medical GmbH, Pforzheim, Germany).	Side to Side oscillating platform	15-25Hz 1-2mm	**60 study** 6 months of home based Whole Body Vibration with interval blocks at the rehabilitation center: 13 days of intensive therapy at the beginning and 6 days after 3 months.	60	28:32	8.71±4.7	Walking velocity improved significantly by 0.11 m/s (p=0.0026) Mobility (GMFM-66) by 2.54 points (p= 0.001)
Vry, J. et al (2013)	Intervention	Duchenne muscular dystrophy and spinal muscular atrophy	Galileo®MedM platform without rails (Novotec Medical GmbH, Pforzheim, Germany).	Side to Side oscillating platform	18- 24Hz 4mm	8 week vibration training program 3x3minute sessions twice a day 5 days a week	22	17:5	5.7-16.2 (9.4)	Improved 6-minute walking, walking speed and also time to climb 4 steps was reduced
Wren, T et al (2010)	Intervention	Cerebral Palsy USA	Juvent Medical Inc., Somerset, NJ		30Hz	**31 study** 10 minutes per day for 6 months, with an additional 6-month control (vibration free) period	31	18:13	6-12	Increased bone deposition during the vibration study period om the midshaft of the tibia
Vertical										
Name of Trial	**Trial Type**	**Study Pop.**	**Platform Type**	**Vibration Type**	**Frequency (Hz) Amplitude (mm)**	**Vibration Regime**	**No. of patients**	**Gender M: F**	**Age (range /mean)**	**Primary outcomes of study**
Cheng, H. et al (2015)	Crossover	Cerebral Palsy Taiwan	AV-001A, Body Green, Taipei, Taiwan	Vertical plate displacement	8- 40 Hz 0.4- 2.0 mm	**8 received** 20 minutes of WHOLE BODY VIBRATION on 2 separate days 1 week apart **8 received** 20 minutes stood on platform (not turned on)	16	9:7	7.5- 12.1 (9.8)	Increased active range of movement in ankles and knees, reduction in spasticity and improvement in ambulatory function

Edionwe, J. et al (2015)	Randomized controlled trial	Burns covering around 30% of their total body surface USA	Power Plate Next Generation Vibration Platform; Power Plate North America, Chicago, IL, USA	Vertical plate displacement	30–40 Hz 2–4 mm	6-week program consisted of progressive resistance exercise and aerobic conditioning that lasted from 30 min to 1 h. Exercise durations ranging from 12 to 15 min.	9	5:4	11.7 +/- 3.7	In children recovering from burns, use of exercise in conjunction with WBV is well tolerated, improves strength, and may have had a small protective effect on bone loss in the leg and trunk.
Erceg, D. et al (2015)	Randomized controlled trial	Obesity Los Angeles, USA Latino	Power Plate Next Generation Vibration Platform; Power Plate North America, Chicago, IL, USA	Vertical plate displacement	30–40 Hz 2–4 mm	**9 Control** **11 Study** 3 days per week WHOLE BODY VIBRATION exercise (VIB) for 10 weeks	20	20:0	8- 10	Improved bone metabolism (increased bone mineral content and bone mineral density)
Fanchiang , H, et al (2014)	Intervention	Idiopathic toe walking (ITP) Taiwan	Soloflex, Hillsboro, OR		30 Hz	**15 Control (without ITP)** **15 ITP** Gait assessment, vibration session then further gait assessment	30	15:15	4-10	No change in gait post vibrattion
Ibrahim, M. et al (2014)	Randomized controlled trial	Cerebral Palsy Eygpt	Power Plate; Northbrook, IL	Vertical plate displacement	12- 18 Hz 2 – 6 mm	**15 Control** **15 Study** 9 minutes 3 times per week for 3 months along with other strength building exercises	30	N/A	8-12	Increased muscle strength Reduction in spasticity of knee extensors Improved walking speed Improved gross motor performance
Tupimai, T. et al (2016)	Intervention	Cerebral Palsy Thailand	AIKO vibrator, ETF-001CG, Thailand	Vertical plate displacement	20Hz	**6 Control** **6 Study** Two-period cross-over trial. Control group received passive muscle stretching was performed while the subjects stood on a tilt table for 40 minutes per session. Study group, a combination of passive muscle stretching and whole body vibration was conducted	12	Not disclose d	6–18 (age, 10.58)	Decreased spasticity and increased muscle strength and spasticity in study group.
Ward et al (2004)	prospective, double-blind, randomised placebo-controlled pilot trial	Disabling motor and muscle conditions United Kingdom	Fritton et al, 1997 (213)	Vertical ground based vibration	0.3*g*, 90 Hz	**10 active** **10 placebo** The children were randomised to active or placebo devices for 10 minutes a day for 5 days a week for 6 months.	20	14:6	4-19(age,9.1)	Increased tibial and spinal volumetric trabecular BMD (vTBMD) in active group

Vibro-acoustic bedpad										
Katusic, A. et al (2013)	Randomised controlled trials	Cerebral Palsy Croatia	ISIC bedpad-VSM 10, Acouve Laboratory Inc, Japan	Vibro-acoustic bedpad	40Hz	**44 Control** **45 Study** 12 weeks. 3 sessions of 40 minutes per week. In addition study group received vibration in one on one sessions.	89	52:37	4.0- 5.7 (4.75)	Improved gait speed, stride length, cycle time and ankle ankle in the study group
Not Disclosed										
Unger, M. et al (2013)	Randomised controlled trial	Cerebral palsy South Africa	Not disclosed			**27 study** 8-week exercise programme consisting of abdominal and back exercises, weight training. 4 week study period with participants acting as their own control	27	17:10	6-13	Improved posture and gait

Table 1.9 WBV studies conducted based on the conditions and platform in children

1.20 Summary

This review found positive effects of WBV in children across a range of conditions affecting the neuromuscular and skeletal systems. There was also research in healthy boys that shows the potential for WBV to be used as a bone stimulation test as one study therefore supports the idea that a *"growing skeleton can respond quickly to vibration of either high or low magnitude*" (178).

With high prevalence of childhood overweight and obesity in the United Kingdom (14% and 15% respectively) the developmental physiology of children is also changing. Physical inactivity in these children places them at risk of poor skeletal development and developing insulin insensitivity and diabetes (211). Insulin sensitivity has been shown to improve using dietary and exercise interventions, but the authors of one study hypothesized that exercise may stimulate osteocalcin production in bone, which might have a positive impact on insulin secretion and sensitivity. One study included in this review found that bone metabolism in obese children remained stable when exposed to WBV alongside their normal activity with overall weight loss in children who continued their daily activity alone. Therefore, this may mean that WBV is advantageous for overweight children by improving/maintaining their bone mineral content and body mass density and thus improving their overall skeletal development (211).

WBV was found to be effective in increasing function e.g. walking distance, range of movement and reducing spasticity in children diagnosed with severe burns, spinal bifida, OI, DMD, SMA, Down syndrome and CP. It was also found to increase rates of bone formation and to a lesser extent increase bone resorption in healthy and obese children and those diagnosed with Down syndrome or those having severe motor difficulties. These potentially positive effects were measured qualitatively by the use of functional questionnaires assessing spasticity and function, the use of objective six-minute walking tests and quantitatively by the assessment of the serum bone turnover markers.

We found very little in the way of safety data reported in the majority of studies. Although the lack of safety data might appear to suggest that the intervention is without complication, the accelerations entrained by the use of the side-to-side alternating platforms have been estimated by some to exceed 6G. No studies in children have assessed the possible consequences of such acceleration for retinal or cerebral vasculature as far as we can determine.

In summary, the current literature has shown some evidence of effectiveness of WBV in enhancing bone mass in individuals with low BMD, adolescents and elderly. The focus of this thesis is to demonstrate WBV as a surrogate marker for bone stimulation through which bone formation is increased and bone resorption is decreased rather than as prescription to enhance bone mass or strength.

1.21 Recommendations

Further research assessing the efficacy and safety of WBV in a greater range of medical conditions as well as healthy children is warranted. The use of short periods of WBV as a mechanical stimulation test needs to be validated across a broader age range. It has potential as a method to assess the effects of both diseases and interventions on an important aspect of skeletal function – the appropriate response to mechanical stimulation. Short period of vibration mimics mild activities like standing that form a dominant component of the skeleton's 24 hours strain (183) and has been shown to have a direct anabolic effect on the bone.

In addition, with the wide range of platforms and exercise regimes found in the literature, larger studies to assess the effectiveness of the different "doses" of WBV would be useful to look at the disease-specific consensus-derived outcome parameters.

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Summary

Despite an exponential growth in our knowledge over the last decade on vitamin D metabolism and its effects on skeletal health; several knowledge gaps are being identified with regards to assessing its status, response to its treatment and its functional outcome.

The composition of this thesis has been chosen to ensure that role of vitamin D in skeletal health is considered from a holistic perspective across the life course ranging from antenatal period to adulthood. Whilst each chapter has its own detailed aim and objective, the overall aims and objectives for the thesis are:

Aim

To identify and bridge the existing knowledge gaps on 'The Role of Vitamin D in Skeletal Health' across the life course with regards to assessing its status, effect on postnatal bone development and response to mechanical stimulation, relationship between fractures and rickets in infants and response to its treatment in young adults.

Key Objectives

1. Does antenatal vitamin D supplementation influence the postnatal response of bone to mechanical stimulation?
2. What is the relationship between Vitamin D, Rachitic Radiographic Changes and Fractures in Infants?
3. Whether standard treatment with vitamin D based on the total 25OHD levels is appropriate across all ethnic groups? Alternatively, should we adopt a personalised approach for vitamin D supplementation and treatment based on an individual's skin colour, race and vitamin D binding protein genotype?

Chapter 2: Vitamin D and Vibration (VIVID) Study

Title

Maternal pregnancy vitamin D supplementation increases offspring bone formation in response to mechanical loading: Findings from a MAVIDOS Trial sub-study

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2.1 Abstract

Objectives:

The Maternal Vitamin D Osteoporosis (MAVIDOS) trial (1) reported higher total body bone mineral content in winter-born infants of mothers receiving vitamin D supplementation compared with placebo during pregnancy. This sub-study aimed to determine whether antenatal vitamin D supplementation altered postnatal bone formation in response to mechanical stimulation.

Methods:

Thirty-one children born to MAVIDOS participants randomised to either placebo (n=19) or cholecalciferol (n=12) were recruited at age 4-5 years. Children received whole body vibration (WBV) for 10 minutes on 5 consecutive days. Fasting blood samples for bone homeostasis, 25 hydroxyvitamin D (25OHD), parathyroid hormone (PTH), and bone turnover markers (Pro-collagen Type 1 N-terminal propeptide, P1NP; Cross-linked C-telopeptide of Type I Collagen, CTX) were collected pre-WBV and on day 8 (D8).

Results:

Mean changes (Δ) in P1NP (ng/ml) between baseline and D8 in the vitamin-D intervention and placebo groups were 40.6 and -92.6 respectively and mean changes (Δ) in CTX (ng/ml) were 0.034 (intervention) and -0.084 (placebo) respectively. Between-group ΔP1NP difference was 133.2ng/ml [95% CI 0.4, 266.0; p=0.049] and ΔCTX 0.05ng/ml (95% CI -0.159, 0.26ng/mL; p=0.62).

Conclusion:

Antenatal vitamin-D supplementation resulted in increased P1NP in response to WBV, suggesting early life vitamin D supplementation increases the anabolic response of bone to mechanical loading in children.

2.2 Introduction

Vitamin D is widely recognised as essential for normal skeletal metabolism. In childhood, maintenance of sufficient serum 25-hydroxyvitamin D (25OHD) levels is critical to prevent adverse outcomes, including hypocalcaemic seizures, cardiomyopathy, rickets and growth failure.

There is also increasing recognition that maternal vitamin D status might be an important determinant of offspring bone development (2) (3) (4). Lower bone area in relation to body size is predictive of increased fracture risk in children aged 10 years (5). Observational studies have demonstrated that lower maternal serum 25OHD during pregnancy and at birth are associated with reduced bone width and mass in the offspring at 8-9 years age (6). The Southampton Women's Survey also reported a positive correlation between maternal 25OHD status and offspring bone mineral accrual (7) at age 6-7 years (8). The study from the Australian Raine Cohort demonstrated a positive relationship between maternal vitamin D status and offspring bone mass at 20 years (9).

Equally there are observational data that do not support these findings (10). The ALSPAC study demonstrated no association between maternal vitamin D status and offspring bone mass at 9 years (10) (11) . Similarly, Garcia et al. reported no association between maternal 25OHD concentrations in mid-pregnancy and offspring bone mineral density (12). There could be a number of reasons for the inconsistencies in establishing a positive relationship between maternal vitamin D status and offspring bone accrual in these studies. Some of these studies are underpowered to detect a small difference, have used different reference range for vitamin D status during pregnancy, different methodological approaches. Examples of the latter are some studies categorised vitamin D status and looked at difference between groups, others used continuous data and regression/correlation approaches, for instance in the ALSPAC study they looked at correlation between age and season at DXA measurements (11).

The Maternal Vitamin D Osteoporosis Study (MAVIDOS), a multicentre, randomised, double-blind, placebo controlled trial of 1000 IU/day cholecalciferol vs. placebo from 14 weeks gestation to birth, was conducted at three UK centres (Southampton, Oxford and Sheffield) (4). MAVIDOS study participants included women older than 18 years, who had a singleton pregnancy, had gestation of less than 17 weeks and a serum total 25OHD concentration between 25-100 nmol/L.

Women in both intervention and placebo groups had similar age, parity, educational qualification (A level or higher), smoking status, exercise status (strenuous activity at least once per week), ethnic status (80% White British). Although height was similar between both groups, median weight, BMI, and mean sum of skinfold thickness were nominally greater in the placebo group than the intervention group (1).

The primary outcome was neonatal whole body bone mineral content (BMC) measured within 2 weeks of birth (4). Although no significant differences in neonatal bone indices (bone area, bone mineral content (BMC), bone mineral density (BMD)) were detected between randomization groups overall, in a pre-specified analysis, an interaction between treatment group and offspring season of birth was detected. Whole body BMC and BMD were shown to be approximately 9% and 5% higher, respectively, in children born in winter (December to February) to mothers randomised to cholecalciferol compared to those randomised to placebo, leading to a 0.5 SD (63.0±10.8g vs 57.5±10.9g, p=0.004) increase in neonatal whole body BMC in the intervention cohort (1). Follow-up DXA data from the Southampton cohort at 4 years of age has demonstrated an increase in WBLH aBMD in the intervention group children compared to placebo [mean (95%CI): supplemented group: 0.477 (0.472, 0.481) g/cm^2; placebo group: 0.470 (0.466, 0.475)g/cm^2, p=0.048]. These findings suggest a sustained beneficial effect of maternal vitamin D supplementation in pregnancy on offspring bone health.

The growing skeleton responds to mechanical stimulation with an increase in bone size and mass and that can persist into adult life (13), (14). In contrast, without mechanical stimulation, peak bone mass may not be fully achieved (15) (16). Data from our preclinical murine model study looking at the effect of reduced vitamin D intake during pregnancy and early life on the skeleton's response to mechanical loading demonstrated that antenatal complete vitamin D depletion substantially reduces the loading-dependent increase in both cortical and trabecular bone mass of offspring mice (17). We have also demonstrated in a cohort of healthy pre-pubertal boys aged between 9 and 11 years, that brief exposure to whole body vibration - 10 minutes daily for five days - increases the bone formation marker N-terminal propeptide of type I procollagen (PINP) by 25% and the bone resorption marker C-terminal telopeptide of type I collagen (CTX) by 11% (18)

The main aim of our study was to investigate whether antenatal vitamin D supplementation altered postnatal bone formation in response to mechanical stimulation by WBV. Our hypothesis was that children born to mothers who received vitamin D supplementation during pregnancy would have a greater increase in the bone formation marker P1NP in response to WBV than children whose mothers had received placebo.

2.3 Materials and Methods

2.3.1 Study Design

This was a prospective single centre interventional study in which each subject participated in the trial for a total of 8 days. The study sample consisted of children born to mothers who had participated in the MAVIDOS study. Details of the MAVIDOS study have been published previously (4), but briefly, women with a baseline 25(OH)D between 25 and 100 nmol/l at 11-14 weeks' gestation were randomized to either placebo or 1000 IU/day cholecalciferol from 14 weeks gestation until delivery (n=1134). Women were recruited into the MAVIDOS study from three UK centres: Southampton, Oxford and Sheffield. Only children of women who had been recruited in Sheffield (n=56) were invited to participate in this sub-study.

All potential participants were contacted by methods discussed and agreed by the patient and public involvement (PPI) focus group of this study. Information sheets detailing the study procedures with our contact details were sent out to the families of potential participants who had expressed an interest in the study prior to their attendance. Parents and families were clearly informed that this study was an additional study to MAVIDOS, so they were free to decline without their participation in the core trial protocol being affected.

The Yorkshire and Humber South Yorkshire Research Ethics Committee approved the study. Written informed consent was obtained from the parents and guardians of all study participants. The ClinicalTrials.gov registration number is NCT02743559.

2.3.2 Study Participants

There were a total of 56 potential participants. The children were aged between 4 and 5 years at the time of recruitment and they formed 2 groups, (i) an intervention group whose mothers had received antenatal vitamin D (cholecalciferol 1000 IU/day) supplements (n=29) and (ii) a placebo group (n=27) [Figure 2.1].

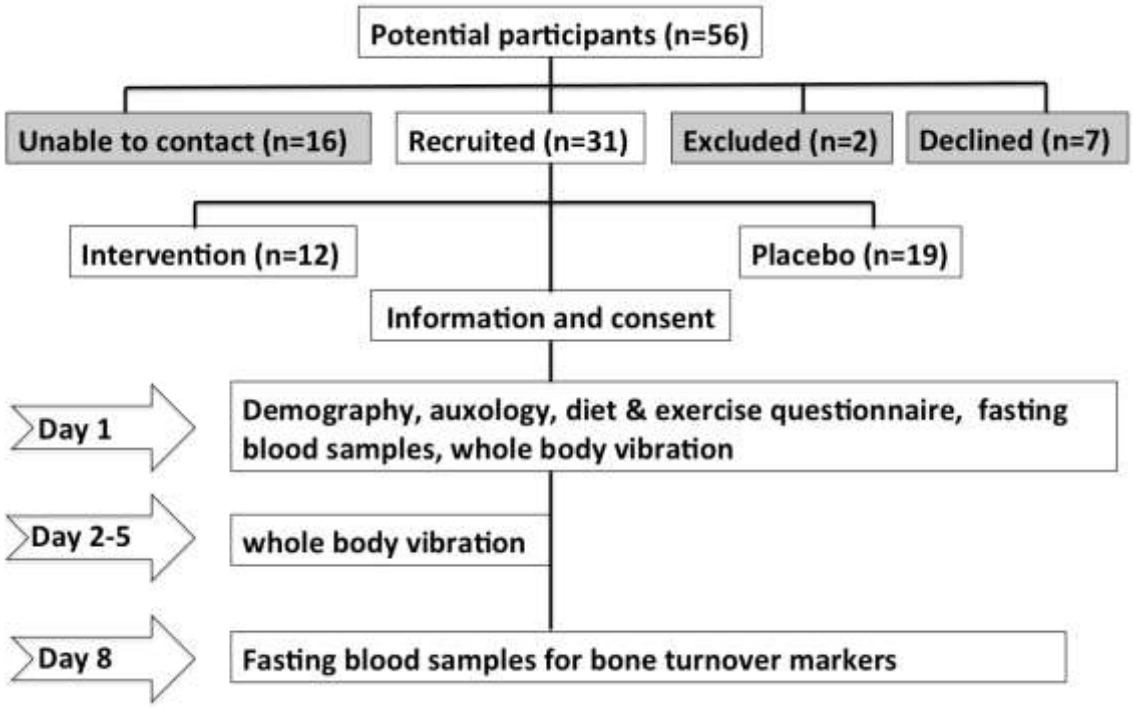

Figure 2.1 Study Design

Children with balance problems, existing or healing fractures, any chronic illness involving bone, liver or renal systems, with known disorders of growth or glucose metabolism, or with existing long-term use of steroids, anticonvulsants or any medication that might affect calcium and vitamin D metabolism were excluded.

Enrolment into the study and first day procedures took place in the Clinical Research Facility at Sheffield Children's Hospital; other study procedures took place at the participant's home.

2.3.3 Anthropometry, Activity and Diet

Anthropometry was undertaken with the participants wearing light clothing (vest and pants). Height (without shoes, to next succeeding 1 mm) by wall-mounted stadiometer (Holtain, Crymych), and weight (to nearest 0.1 kg) by electronic balance scales (Seca GmbH & Co, Hamburg, Germany) were measured. Body mass index (BMI) was derived as [weight (kg) / height (m) ^{2}]. A validated questionnaire regarding exercise to derive MET's score (19) was used to ascertain the amount of physical activity undertaken over the 7 days prior to standing on the platform and then again on day 8 to determine whether undergoing WBV had had an effect on participant's levels of physical activity. METs = metabolic equivalents and one MET is the amount of energy we use when we are resting. Physical activities are rated using METs to indicate their intensity and can be translated into light, moderate and vigorous intensities of exercise. The frequency and intensity of the types of exercise given in answer to these questions were multiplied by the anticipated metabolic equivalents (METs) of nine, five and three for strenuous, moderate, and mild exercise respectively, to provide an activity score for comparison. The participants came from a mixture of areas from leafy green suburbs to the city. The "Rapid assessment of dietary calcium intake" questionnaire was given to all participants (20).

2.3.4 Laboratory methods

Fasting blood samples for bone homeostasis: calcium, phosphorus, alkaline phosphatase, magnesium, PTH, serum 25OHD; and for bone turnover markers (P1NP, CTX); were collected immediately pre-vibration on day 1 and again on day 8.

Serum calcium, phosphate, albumin, and alkaline phosphatase were measured using Micro Slide Technology Colorimetric/Rate by Reflectance Spectrophotometry in the Vitros 5, 1 FS System (Ortho

Clinical Diagnostics; Raritan, New Jersey) analyser. The interassay CVs were: calcium (1.4%), phosphate (1.6%), albumin (2.9%), and alkaline phosphatase (2.4%). Intact parathyroid hormone (PTH) was measured using Immunoassay (Chemiluminescent Microparticle Immunoassay) in the Architect *i* 1000 System (Abbott; Abbott Park, Illinois) (PTH analytical sensitivity ≤1 ng/L). Serum total 25-hydroxyvitamin D (25OHD) levels were determined using an UPLC/Mass Spectrometer Semi-automated hexane extraction in the Acquity Ultra Performance LC/Quattro MS (Waters; Milford, Massachusetts) analyser. Lower limit of detection for $25OHD_2$ was 6 nmol/L and for $25OHD_3$ 3.5 nmol/L. The interassay coefficient of variation (CV) for $25OHD_2$ and $25OHD_3$ were 5.7% and 5.4% respectively. Blood samples for bone formation marker P1NP was measured using automated immunoassay (Elecsys, Cobas E11, Roche Diagnostics, UK; intraassay % CV <1.7%) and bone resorption marker CTX (Elecsys β-CrossLaps/serum kit, Cobas E411, Roche Diagnostics, UK; intraassay % CV 2.8 - 8.4%). All samples were collected in the mornings approximately between 0800 and 0900 hours following an overnight fast. Samples were centrifuged, separated and stored at -80°c within 2 hours of collection.

2.3.5 Whole body vibration intervention

Figure 2.2 LivMD vibrating platform.

Study volunteer having a demo at the platform, picture produced with parental permission in figure 2.2.

The participants were asked to stand barefoot and facing forwards on a portable Marodyne LivMD low magnitude (0.4*g*), high frequency (30-90 Hz) vibrating platform (Marodyne Medical, Inc., Lakeland, Florida) to receive WBV [Figure 2.2]. From our previous research experience (18) we knew that children could tolerate this well. We also encouraged them to hold the back of a chair or their parent for support.

The period of vibration was delivered in 4 cycles of 2 minutes 30 seconds each, separated by 30 seconds off the platform, providing 10 minutes of vibration every day for 5 consecutive days around the same time between 7 and 8 am. Both participants and their carers were taught to use the vibrating plate at home. Delivering vibration in this pattern will allow the participants to become accustomed to the platform in a more comfortable manner. Additionally it has been demonstrated that insertion of rest periods enhances the anabolic effect of loading on bone (21) (22). Compliance to the intervention with WBV was monitored by parental reporting from daily phone calls made by the researcher.

2.3.6 Statistical analysis

The study was powered to detect a difference of 50% in the change in PINP between children of mothers who were supplemented with high dose vitamin D (1000 IU) versus placebo based on our previous WBV study in healthy pre-pubertal boys (18) with 90% power at the 5% significance level (two-tailed). Sample size was determined to achieve a minimum of 20 observations (10 per group).

We performed all our analyses using Statistical Package for the Social Sciences version 22 (SPSS by IBM), Data Desk™ v6.2.1, and Stata v15 (23). Groups were compared at baseline using either t-test (continuously distributed data) or proportions test (categorical data). Statistical tests for normality

(eg. Shapiro-Wilk) were not carried out. These tests are known to be too sensitive for large samples and the reverse for small samples. And normality was therefore based on eyeball.

Baseline data are summarized by median (25^{th}/75^{th} centiles) for continuously distributed data or n (%) for categorical data. Graphical presentation of bone turnover markers (P1NP and CTX) was illustrated using box and whisker plots, which have the following interpretation.

Box and whisker plots represent a visual representation of P1NP, and CTX over the different days. The median (50th centile) of each bone marker is indicated by a horizontal line in the middle of the rectangular box. The two ends of the box are named the lower and upper quartiles (or 25th and 75th centiles) respectively. The difference between the upper and lower quartiles is called the interquartile range (IQR). The 'whiskers' on either side of the box are calculated according to a formula. The upper whisker is calculated as upper quartile + (1.5 x IQR); the lower whisker by lower quartile – (1.5 x IQR). Oddly this formula is different in the USA, reasons for which are unclear. Whiskers may or may not be capped by a small horizontal line. The dots beyond the whiskers are extreme values of the distribution. Note, not all data will have extreme values or even whiskers.

Differences (post vibration - baseline) between randomised groups (intervention i.e vitamin D supplementation in pregnancy vs placebo) for each bone turnover marker were analysed by an independent t-test using an arbitrary level of 5% statistical significance (two-tailed). Ninety five percent (95%) confidence intervals (CIs) were estimated.

Maternal characteristics were not adjusted in the analysis owing to (1) small sample size of our study (2) ongoing nature of the MAVIDOS study and (3) restricted access to maternal records due to blinding.

2.4 Results

31 children (placebo group n=19; cholecalciferol group n=11) participated in the study. Baseline characteristics of the study subjects are shown in Table 2.1.

Variable	Intervention	Placebo	p value
Age (years)	4.9(4.4,5.2)	5.1(4.5,5.4)	0.318
Gender (female)	7(58%)	8(42%)	0.295
Ethnicity (Caucasian)	12(100%)	14(74%)	0.21
Height (cms)	107.5(102.5, 113.4)	108.1 (103.8, 114)	0.43
Weight (kg)	18.3 (16.9, 21.7)	17.8 (16.2, 18.8)	0.68
BMI (kg/m^2)	16.1 (15.7, 17.0)	15.0 (14.1, 16.2)	0.16
Serum 25OHD (nmol/l)	71(58,98)	79(61,81)	0.537
Serum calcium (mmol/l)	2.4(2.37,2.42)	2.35(2.31,2.42)	0.615
Serum phosphate (mmol/l)	1.52(1.5,1.62)	1.61(1.55,1.69)	0.058
Serum alkaline Phosphatase (u/l)	204.5(180,215)	200(186,213)	0.864
Serum albumin (g/l)	42(41,44)	42(41.5,43.5)	0.634
Serum magnesium (mmol/l)	0.85(0.85,0.89)	0.84(0.82,0.88)	0.379
Serum parathyroid hormone (ng/l)	27.9(24.0,34.1)	36.5(22.3,40.0)	0.685
Serum P1NP (ng/ml)	552.2(512.3,678.6)	587.1(518.2,801.7)	0.558
Serum CTX (ng/ml)	1.52(1.3,1.81)	1.59(1.3,1.81)	0.618
Dietary calcium intake (gms/day)	783(577,842)	521(430,535)	**0.040**
Activity score (METS units)	66 (44,83)	104 (68,120)	**0.040**

Table 2.1 Baseline characteristics. Data are median (25th/75th centiles) or n (%).

Age, gender, ethnicity, height, weight, and BMI were similar between the groups. Bone profile including serum 25OHD and PTH were normal in the entire cohort and there were no significant differences between the groups. Although median serum 25OHD levels were comparatively lower in the intervention group, levels were well within the normal range in both groups (50 nmol/L → vitamin D sufficiency). 94% of the MAVIDOS participants were White British on both arms. This could reflect the vitamin D sufficient status in the children participated in our study. Similar argument applies to

serum phosphate and PTH levels. This is a pre-selected cohort of children and we did not have any control on this recruitment.

Data acquisition on diet and exercise shown in Table 2.1 were limited due to non-filling of questionnaires or failed recall of requested information in 30% of cases (similar in both groups). Dietary calcium intake (grams/day) was significantly higher in the intervention group (p= 0.04). The activity score (METs units) was significantly higher in the placebo group (median 104) when compared with the intervention group (median 66) (p= 0.04).

Median (25^{th}/75^{th} centiles) P1NP (ng/ml) at baseline was 552.2 (512.3, 678.6) for the intervention group and 587.1 (518.2, 801.7) for the placebo group. Comparison of P1NP at baseline and post vibration by randomisation group is shown in Figure 2.3.

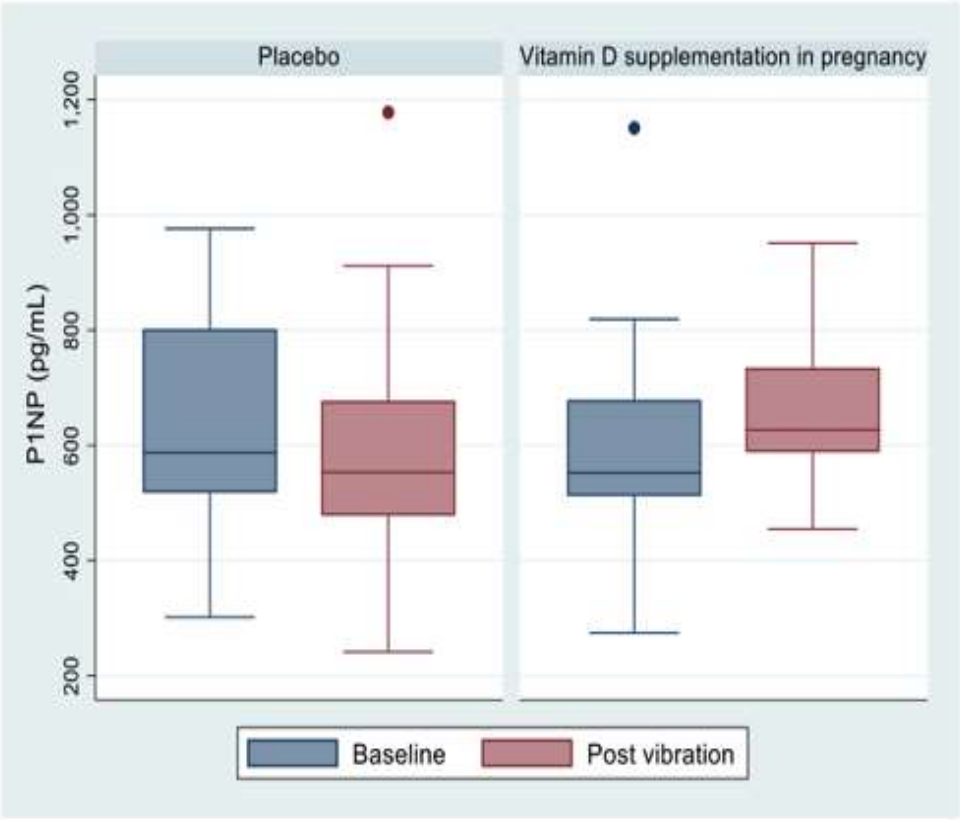

Figure 2.3 P1NP at baseline and post vibration

The mean change (Δ) in P1NP (ng/ml) between baseline and D8 in the intervention and placebo groups was 40.6 and -92.6, respectively. The between-group difference in ΔP1NP was 133.2ng/mL (95% CI 0.4, 266.0; p=0.049). Mean (%) change in P1NP within the intervention group was 11% (pre vs post vibration by patient) and within the placebo group was -13.3%; difference between groups 24% [Table 2.2].

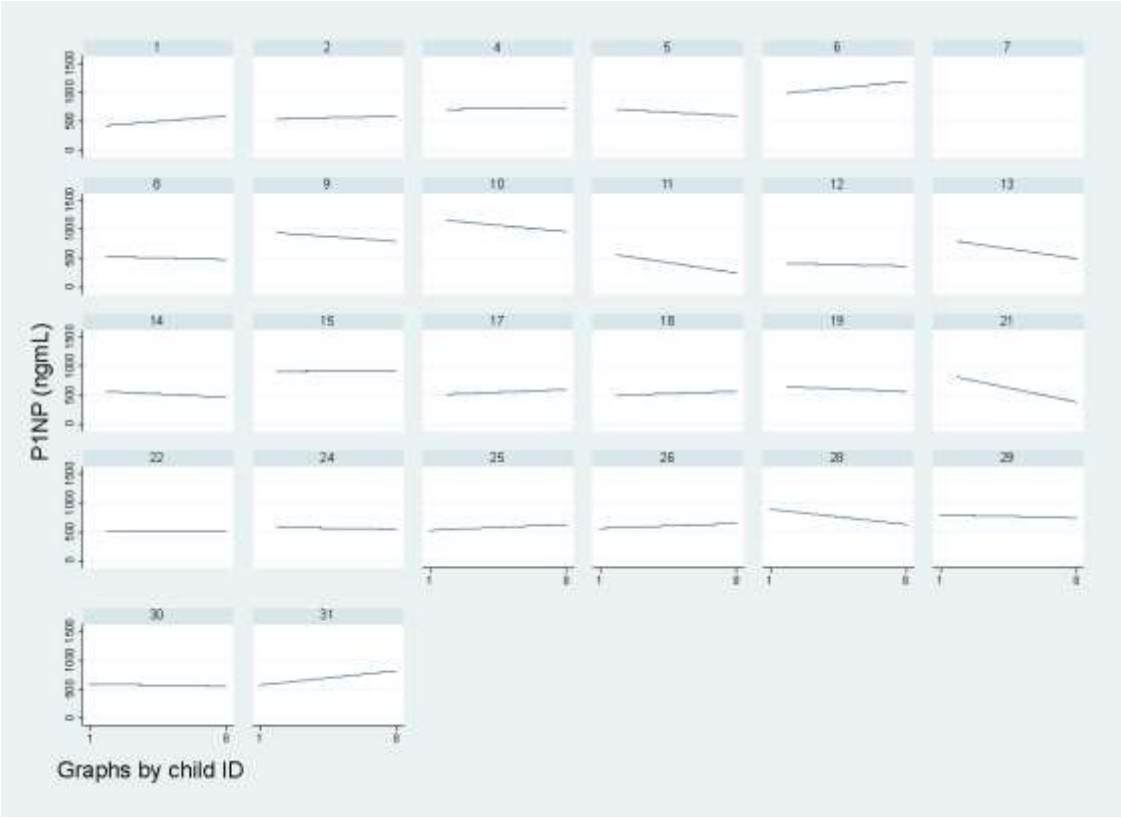

Figure 2.4 PINP individual line plots

Individual child plots for PINP were drawn as shown in figure 2.4. And there was little or no evidence of regression towards the mean indicating that no single child skewed the results.

Variable	Intervention	Placebo	Between group difference (95% confidence interval)	P value
Median serum P1NP (ng/ml) at baseline	552.2(512.3,678.6)	587.1(518.2,801.7)	-	0.558
Mean change in P1NP (ng/ml) between baseline and D8	40.6	-92.6	133.2 (0.4, 266.0)	**0.049**
Mean % change in P1NP between baseline and D8	11	-13.3	24	-
Median serum CTX (ng/ml) at baseline	1.52(1.3,1.8)	1.59(1.3,1.8)	-	0.618
Mean change in CTX (ng/ml) between baseline and D8	-0.034	-0.084	0.05 (-0.159,0.26)	0.620
Mean % change in CTX between baseline and D8	-0.5	-4.9	4.4	-

Table 2.2 Comparison of bone turnover markers

Comparison of bone turnover markers at baseline and post vibration by randomisation group. Data are median (25^{th}/75^{th} centiles) or n (%). Bold type indicates significant p value. We have summarised the data by median but presented mean % change. We have not adjusted the differences according to previous arguments.

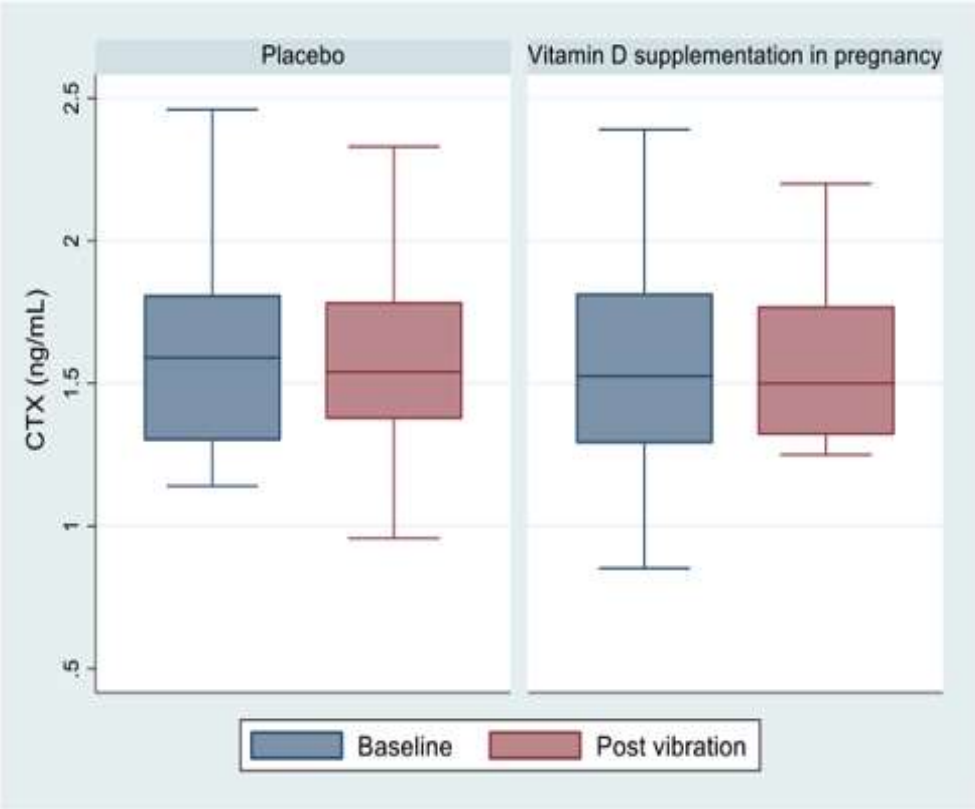

Figure 2.5 CTX at baseline and post vibration

Median (25^{th}/75^{th} centiles) for CTX (ng/mL) at baseline was 1.52 (1.3, 1.8) for the intervention group and 1.59 (1.3, 1.8) and for the placebo group. Comparison of CTX at baseline and post vibration by randomisation group is shown in figure 2.5.

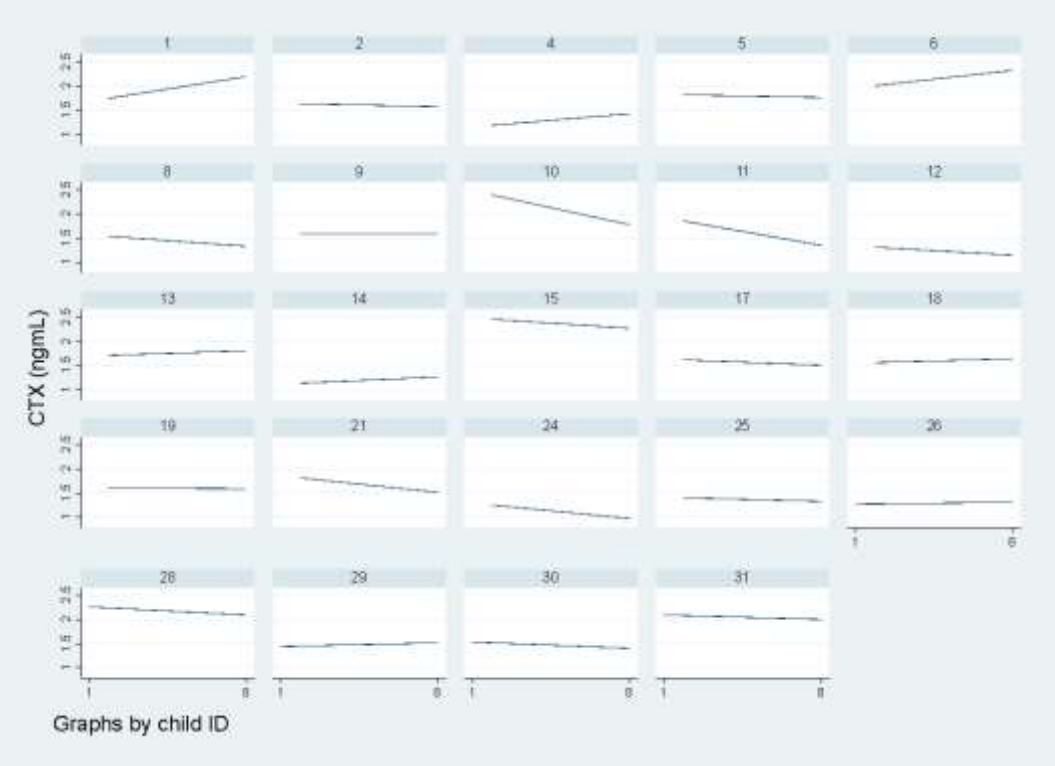

Figure 2.6 CTX individual line plots.

Individual child plots for CTX were drawn as shown in figure 2.6. Regression towards the mean is illustrated with child #10. The largest difference (0.62 ng/mL) was found from the highest starting point (2.39 ng/mL).

2.5 Discussion

In this study we have shown that antenatal vitamin D supplementation increases the anabolic response of growing bone to mechanical stimulation. We found that five consecutive days of low magnitude high frequency WBV increased the bone formation marker PINP by 11 % between the pre-vibration baseline and the day 8 measurement in children whose mothers had received 1000 IU vitamin D supplementation during pregnancy. By contrast the P1NP decreased by 13% in the placebo group, showing a net benefit of 24% in the intervention group. We did not find a change in the bone resorption marker CTX in either the intervention or the placebo group. Bone turnover markers in children are high during the first 2 years of life, which then gradually decrease during childhood before peaking up during puberty followed by a fall during adulthood (24). Serum concentrations of bone turnover markers are affected by several physiological factors in children such as age, gender, ethnicity, growth velocity, diet, pubertal growth spurt and pathological factors such as malnutrition, vitamin D deficiency, prematurity, growth hormone deficiency respectively due to rapid skeletal growth and bone turnover (25). There are few controllable factors that could account for the variation in serum bone turnover markers such as the timing of the sample, fasting and feeding, day to day variation, seasons and menstrual cycles. Feeding acutely decreases bone turnover markers by around 30% in CTX and 0-10% in P1NP (26). However, we collected fasting samples in our cohort hence it is unlikely for feeding to be accountable for this variation. Equally PINP is more stable and not affected by the circadian rhythm than CTX (27). And in our cohort we found a fall in PINP in the placebo group when compared to the intervention group and this may well be attributed to the day to day variation since we only measured the bone turnover markers as a single measurement on day 0 and day 8.

The central results are in contrast to the findings of our previous study in which the effect of WBV in 36 healthy pre-pubertal boys was examined. Participants in that study received either low (<1g, frequency 32-37 Hz, amplitude 0.085 mm) or high (>2g, frequency 5 to 30Hz, amplitude 0+/-4.5mm)

magnitude vibration for 1, 3 or 5 consecutive days. A significant rise in both P1NP 25.1% (p=0.005) and CTX 10.9% (p=0.009) from baseline to day 8 was observed in the group exposed to 5 days of WBV, irrespective of the mode of vibration (18). However, subjects in that study were older (9-11 years) and had higher baseline P1NP and CTX levels. It is possible some of those participants, despite being Tanner stage 1, had some early hormonal changes of puberty resulting in a higher P1NP and CTX response to vibration. It may also be the case that there were simply insufficient numbers in this study to detect significant changes in CTX.

The increase in the bone formation marker but not the bone resorption marker in our current study suggests that there is an uncoupling of bone turnover favouring formation more than the resorption in response to mechanical stimulation. While this may be true for adults, but for children, the bone turnover markers reflect growth and modelling as well as remodelling, so the effect on PINP may also reflect changes in these processes. We did not measure osteocalcin or sclerostin in our study based on the results from our previous study on WBV in healthy pre-pubertal boys where we did not find a significant change in them after 5 days of WBV. Similarly, in the previously mentioned study of pre-pubertal boys a greater increase in bone formation than resorption marker was also observed (18).

Studies have reported increased expression of osteoprotegerin [a factor affecting bone resorption] in serum in association with reduced bone resorption through the activation of the canonical wnt-signalling pathway through LRP5/6 (28); however we did not measure OPG in this study, as we had not previously demonstrated a significant difference in OPG levels between baseline and day 8 following WBV in pre-pubertal boys (18).

Soderpalm et al. studied the tolerability and effects of WBV (16 -24 Hz and 2-4 mm on a side to side alternating platform) on muscle and bone in six ambulatory children with Duchenne Muscular Dystrophy (DMD) aged between 5-12 years (29). Ward et al. has demonstrated that WBV is osteogenic in children with disabling conditions such as DMD (30). The authors did not find a significant change

in bone mass or bone turnover markers such as osteocalcin, osteoprotegerin, or sclerostin. But observed a non-significant trend towards an increase in bone specific alkaline phosphatase after 3 months of WBV, which returned to baseline, 3 months post discontinuation of WBV. Their study population differed to ours, having a significant muscle disease and also being treated with the steroid prednisolone and we did not measure alkaline phosphatase post vibration in our study to compare with Soderpalm's results. However these results do suggest that the response to WBV of bone turnover markers may be transient and not sustained over long periods of time (29). Kilebrant et al. studied the effects of WBV (40-42 Hz, oscillation amplitude 0.2 mm) on bone mass, bone turnover markers and body composition in 19 children aged between 5 and 16 with severe motor disabilities who used wheelchairs for mobility, but also spent time each day in a standing frame. WBV was delivered 5-15 minutes per treatment twice a week for 6 months. All measurements were undertaken at baseline, 6 and 12 months. They found a significant increase in TBLH BMD and BMC after 6 months of WBV. There were no clear changes in any of the bone formation or resorption markers measured between baseline and 6 months; however, no "early" measurements i.e. within 1-2 weeks of commencing WBV, were made (31). These results do suggest that the response in bone turnover markers or mass to WBV may be transient and not sustained over long periods of time. There are no published studies of the acute response to vibration in children with bone diseases.

The results obtained in our study are consistent with those from our studies of antenatal vitamin D depletion in a preclinical mouse model system. There we demonstrated that despite post-natal vitamin D repletion, the offspring of dams who had been made completely deficient in vitamin D accrued substantially less bone in response to mechanical loading than the offspring of dams who were replete in vitamin D. These effects were apparent during growth affecting both cortical and trabecular bone and after growth had ceased affecting cortical bone accrual at skeletal maturity. It seems possible that this "programming" effect may also be operating in the human situation, given

the results demonstrated here, and the observational data from other studies that suggest a positive association of maternal and early infant vitamin D status with later bone size and mass (17) .There are no similar previous experiments of this nature (i.e combining vitamin D depletion with mechanical loading) with which to compare our data.

2.6 Strengths and limitations

This is the first study to report the results of mechanical loading of bone in children exposed to high dose (1000 IU daily) vitamin D supplementation in pregnancy. The key strength of our study is the unique study cohort that has been randomised to vitamin D or placebo antenatally, combined with the novel technique of stimulating bone in a consistent way and measuring the response using serum markers of bone formation and resorption.

We conducted this study in only 1 out of 3 MAVIDOS centres, thus sample size was relatively small with a limited number of subjects especially in the intervention group. 94% of the MAVIDOS study participants (mothers) are of White British in origin. And the baseline serum total 25OHD level was well within the normal range (> 50nmol/L) in our study cohort. According to most recent NDNS data, there is evidence of low vitamin D status across all age groups and 2% of children aged between 4 and 10 years had low vitamin D status (32). Our study cohort was not a true reflection of the general population in terms of ethnic distribution and vitamin D status in the UK.

We did not adjust the analysis owing to (1) small sample size of our study and (2) ongoing nature MAVIDOS study. We were also not granted access to maternal characteristics from the MAVIDOS research group due to blinding.

The primary focus of our study was only to measure the change in two specific bone turnover markers (PINP and CTX) in response to a very short period of WBV (5 days). Osteocalcin (another marker of bone formation) when measured after 5 days of WBV in our previous study on a pre-pubertal cohort

did not show an increase. This may well be due to osteocalcin being produced later in the process of endochondral ossification during the time of bone mineralisation. Whereas PINP is produced early during the bone formation process at the time of formation and deposition of bone matrix (18). Hence we did not measure osteocalcin and also other factors affecting bone formation such as sclerostin and bone resorption such as osteoprotegerin. Therefore we were unable to determine any mechanism that might link antenatal vitamin D supplementation with this response.

We also did not measure muscle strength, a determinant of bone mass. . We could have considered measuring handgrip strength, as a simple and reliable tool for assessing voluntary muscle strength in our cohort (33).

We found a significantly higher dietary calcium intake (grams/day) in the intervention group (p= 0.04) and significantly higher activity score (METs units) in the placebo group (p= 0.04). The increased METS in the placebo group with a lower, though not statistically significant BMI might suggest a higher exercise exposure in this group that might have modified the response. These could potentially have interacted with the effect of any prenatal programming on bone formation and resorption markers. However, the quality of the data for diet and exercise was very limited. Thereby it was very difficult to draw meaningful conclusions about potential interactions of such factors with the response to vibration.

2.7 Conclusion

Our study demonstrates that children born to mothers who received vitamin D supplementation during pregnancy have a greater bone formation in response to mechanical stimulation. This implies early life vitamin D supplementation increases the anabolic response of bone to mechanical loading in children. We suggest that supplementation of pregnant women with a higher dose of vitamin D may improve bone health in early childhood, which in turn may increase lifetime bone accrual and reduce

the risk of developing osteoporosis and fragility fractures in later life as adults. Given the limited sample size, confirmation in a larger cohort is needed, along with prospective data collection on fractures may shed more light on our study findings

2.8 Acknowledgements

UK Experimental Arthritis Treatment Centre (EATC) for Children supported this work and we thank Professor Michael W Beresford for his support for this study on behalf of the EATC for children. We thank study volunteers and their families for their participation and acknowledge support by the staff at the Sheffield Children's Hospital Clinical Research Facility. We thank Emilia Bettell, Sharon Colyer (Department of Clinical Biochemistry, Sheffield Children's hospital) and Tim Thorpe (Department of Clinical Biochemistry, Bristol Royal Infirmary) for undertaking biochemical assays and Fatma Gossiel (Metabolic Bone Unit, University of Sheffield) for measuring bone turnover markers.

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Chapter 3: Vitamin D and Fracture Study

Title

Unexplained Fractures in Infants & Young Children: (Ir)relevance of Serum Vitamin D.

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3.1 Abstract

Objective To test the null hypothesis that in the absence of radiographic rickets, low total 25 hydroxyvitamin D (25OHD) does not predispose children aged below two years to fracture.

Material and Methods

Design Five-year retrospective cross-sectional observational study.

Setting A single specialist children's hospital in England.

Patients Children aged below 24 months who had skeletal imaging performed within two weeks before or after a serum 25OHD measurement.

Main outcome measure:

Fracture frequency and its relationship to serum total 25OHD levels

Secondary outcome measure:

Interobserver reliability on the radiographic evidence of (1) fracture(s), (2) osteopenia (radiographic low bone density) and (3) rickets based on the independent reporting of two observers, with differences refereed by a third observer.

Results A total of 381 children had skeletal imaging within 2 weeks of a serum total 25OHD measurement, of whom 153 (40%) had a full skeletal survey. Median (range) 25OHD was 60.7 (6 - 301.8) nmol/L; 73 of 381 (19%) children were deficient (≤ 30 nmol/L); 70 of 381 (18 %) insufficient (30.1- 50 nmol/L). 61 children out of 381 (16%) had at least one fracture; total number of fractures was 140 [median = 1 (25^{th} – 75^{th} centile 1-2 respectively)]; 36 of 381 (9%) osteopaenia (radiographic low bone density); and 20 of 381 (5%) had rickets.

Interobserver variability measured by kappa was moderate for Thacher score (0.586), substantial for radiographic low bone density (0.667), and almost perfect for fracture (0.917). In univariable Poisson regression analysis there was weak evidence of an association between fracture frequency and serum total 25OHD level. Incident rate ratio (IRR) = 1.0092 (95% CI 0.999 -1.0193) (p=0.07). This relationship was maintained after correction of age in months, radiographic bone density, and seasons [IRR = 1.0106 (CI 1.0002 – 1.0211) (p=0.044)]. Sensitivity analysis yielded similar conclusions.

Conclusion In children under two years old with unexplained fracture(s), isolated low serum 25OHD is not associated with increased fracture prevalence.

3.2 Introduction

In this study we have used the terms “vitamin D” to describe serum total 25-hydroxyvitamin D (25OHD) levels and “osteopaenia” for radiographic low bone density.

The significance of vitamin D status in children with suspected physical abuse is contentious. Vitamin D status is categorised as deficiency (VDD) when serum total 25-hydroxyvitamin D (25OHD) concentration falls below 30nmol/L (12ng/mL), insufficiency (VDI) between 30.1 and 50nmol/L and sufficiency when levels are above 50nmol/L for prevention of nutritional rickets (1).

There is no objective evidence to show that fractures highly specific for physical abuse occur with VDD or VDI (2) and no association has been reported between low serum total 25OHD and increased fracture risk in infancy (2) .Nevertheless, a minority of researchers have controversially labelled sub-optimal vitamin D status as a cause of fractures in children with otherwise unexplained injury (3) (4) and VDD in the absence of radiological rickets is used as a defence in court cases of suspected child abuse, as exemplified by the high-profile case of Islington vs Al Alas and Wray [2012] EWHC 865(Fam) (5).

If suboptimal vitamin D status increases propensity to fracture, there is a potential risk of misdiagnosis of otherwise unexplained fractures as abuse. Conversely, abused children may be missed if we are to believe those who link suboptimal vitamin D status to a predisposition to fracture (3) (4) (6). The significance of early and accurate diagnosis of child abuse and the emotional, psychological and financial burden of false accusations cannot be overstated.

The objective of this study was, therefore, to test the currently accepted mainstream null hypothesis that isolated VDD (i.e. in the absence of radiological evidence of rickets) is not associated with fractures in < 24-month-old infants and toddlers.

3.3 Materials and methods

3.3.1 Study design

We conducted a retrospective, single centre cohort study in a large teaching hospital for children between 1st January 2010 and 31st December 2014. We interrogated the hospital database and identified infants and young children below 24 months of age and irrespective of sex, who had a serum total 25hydroxyvitamin D (25OHD) i.e vitamin D level measured during the study period, and who had at least one skeletal radiograph performed within two weeks before or after vitamin D measurement.

Vitamin D status was categorised as deficiency (VDD) when serum total 25OHD concentration was < 30nmol/L (12ng/mL), insufficiency (VDI) between 30.1 and 50nmol/L and sufficiency when levels were >50nmol/L (1). The radiographs consisted of either full skeletal surveys or individual radiographs, including at least one knee or wrist joint, to allow the diagnosis of rickets to be made. Radiographs were excluded if there was clear radiological evidence of underlying disease other than rickets.

Blinded to serum 25OHD levels two independent observers scored the anonymised radiographs for (1) fracture(s) [number/site], (2) radiographic bone density [normal/ reduced], and (3) rickets severity

using the Thacher Rickets Severity Score (RSS) for nutritional rickets (7). The Thacher RSS is a validated 10-point scale (0 representing absent to 10 most extreme degree of rickets) to assess the severity of rickets in the radiographs of wrists and knees based on the degree of metaphyseal cupping, fraying and splaying (7).

Thacher scores were translated to rickets severity as follows:

Skeletal surveys: 1-3 = mild, 4-6 = moderate, 7-10 = severe

Knee radiographs: 1-2 = mild, 3-4 = moderate, 5-6 = severe

Wrist radiographs: 1 = mild, 2 = moderate, 3-4 = severe

Radiographic low bone density ("osteopaenia") was scored subjectively, following a consensus training session, by comparison with radiographs of children with known normal and reduced radiographic bone density performed at the same institution, using the same age and size-based radiographic parameters. Rachitic changes were considered absent when the Thacher score (7) was zero and present when the score was equal to or greater than one. One observer (ACO) was a paediatric musculoskeletal radiologist with 15 years of experience and the other (JSG-K), a doctoral student in-training in bone metabolism. A third observer (AS) with 40 years of experience in paediatric radiology arbitrated in a final consensus read of discrepant images.

3.3.2 Demographic and Laboratory Data

Demographic (age, sex, ethnicity) and biochemical (serum 25OHD, plasma PTH, corrected calcium, phosphate, and alkaline phosphatase) data were collated from hospital records. Patients were excluded if they were on drugs that might affect bone strength (e.g. bisphosphonates) but not over the counter multivitamins as this is not always ascertained or recorded. Serum total 25OHD levels were determined using a UPLC/Mass Spectrometer Semi-automated hexane extraction in the Acquity

Ultra Performance LC/Quattro MS (Waters) analyzer. Lower limit of detection for $25OHD_2$ was 2.4ng/ml (6nmol/L) and for 25OHD3 was 1.4ng/mL (3.5nmol/L). The inter-assay coefficient of variation (CV) for 25OHD2 and 25OHD3 were 5.7% and 5.4% respectively.

3.3.3 Statistical Methods

This study was designed as a single centre study to allow a power calculation for a definitive multicentre study; the five-year recruitment period was arbitrarily selected.

Continuously distributed data were summarized by the median (25^{th} /75^{th} centiles) and categorical data by n (%). Missing values were tabulated but not otherwise considered in our analyses. Imputation of missing values is complex statistically and it is outside the scope of this thesis (8).

The number of fractures and the relationship between fracture frequency and serum 25OHD levels was analysed using Poisson regression, from which incident rate ratios (IRRs) were generated and 95% confidence intervals were generated.

Poisson regression is used for modelling occurrences or counts of data (eg. number of fractures, episodes of hypoglycaemia). The assumption of Poisson data is that the mean and variance of the outcome measure should be equal. Rarely is this satisfied with the variance>mean. This is known as 'overdispersion' and caused by an excess of zeros. Robust standard errors were used to control for the violation of strict Poisson assumption (9). Another approach which allows for overdispersion is negative binomial regression; this is considered later.

Linear and non-linear relationships were explored between fracture frequency and vitamin D levels. We split vitamin D by quartiles (Q) of its distribution. Q1 (5.9-34.3 nmol/L n=96), Q2 (34.6-60.7nmol/L n=96), Q3 (61-85.3nmol/L n=94), Q4 (85.5-301.8 nmol/L n=95). Secondary covariates of less interest were age, osteopaenia (radiographic low bone density) and meteorological season.

An arbitrary level of 5% statistical significance (2-tailed) was assumed. In addition p-values were considered using Professor Martin Bland's 'evidence' approach which moves away from an arbitrary cut-point of 5% statistical significance (10). Under his rules a p-value lying between 0.05 and 0.1 is classified as 'weak evidence' of an effect rather than the rigid 'not statistically significant'.

Graphical presentation by histogram, box and whisker or mean lines drawn as appropriate to the data analysis.

Interobserver agreement was estimated by the Kappa statistic (11) and graded according to the scale of Landis and Koch (12). The Stata statistical computer package was used to analyse the data (13).

3.3.4 Ethics Approval

Local Research Ethics Committee approval was waived for this retrospective review of anonymised images. The study received Service Evaluation and Health Research Authority and local Research and Innovation approval and registration, including waiving the need to obtain parental/guardian consent.

3.3.5 Funding

Original research article for which no external funding was received

3.4 Results

Demographic, clinical and laboratory summaries are given in Table 3.1

	Entire Study Cohort: n = 381		Children with Rickets (Thacher ≥ 1): n= 20
Variable	Missing n (~%)	Median (25^{th}/75^{th} centile) or n (%)	Median (25^{th}/75^{th} centile) or n (%)
Age (months)		8 (3,15)	8·5 (6,16.5)
Sex (female)		167 (44)	5 (25)
Pregnancy (singleton)	76 (20)	238 (62)	17 (85)
Gestation (weeks)	89 (23)	39 (37,40)	40(37,40)
Mode of feeding	110 (29)		
Breast		89 (23)	5 (25)
Bottle		151 (40)	8 (40)
Mixed		31 (8)	0
Missing (only children with rickets)			7 (35)
Ethnicity	68 (18)		
White Caucasian		152 (40)	8 (40)
Asian (subcontinent)		75 (20)	6 (30)
Afro-Caribbean		17 (4)	2 (10)
Other		69 (18)	4 (20)
Vitamin D (serum Total 25OHD) status			
Deficient (< 30nmol/L, 12ng/mL)		87 (24)	8(40)
Insufficient (30.1-50 nmol/L)		57(15)	2(10)
Optimal (>50nmol/L, 20ng/mL))		237 (62)	10 (50)
Serum Corrected Calcium (mmol/L)*	114 (30)	2.51(2.42,2.58)	2.41 (1.93,2.51)
Serum Phosphate (mmol/L)*	108 (28)	1.86 (1.67,2.02)	1.93 (1.54,2.13)
Serum Alkaline Phosphatase (U/L)*	96 (25)	233 (169,302)	420 (199,489)
Serum Parathyroid Hormone (ng/L)*	272 (71)	34 (23,73)	29.4 (23.5,210.7)

Table 3.1 Demographic, clinical and laboratory data of the entire cohort and children with rickets Normal reference ranges; corrected calcium = 2.1-2.56 mmol/L; phosphate = 0.8-1.9 mmol/L; alkaline phosphatase = 76-308 U/L; PTH (parathyroid hormone) = no paediatric reference range available for a child under 2 years old given in our laboratory.

3.4.1 General

A total of 402 infants and young children between 0 and 24 months of age were potentially eligible, of which 21 were excluded due to clear radiographic features of osteogenesis imperfecta (n=11), osteopathy of prematurity (n=2), or because they were on medications that affect bone strength - oral glucocorticoids, anticonvulsants, and bisphosphonates (n=8). Data were collected and analysed from the remaining 381 children, including skeletal surveys in 153 (40%) and one or more individual skeletal radiographs in 228 (60%) of the 381 children. Median (range) age was 8 (3, 15) months; median gestational age was 39 weeks. Of the 381 children, 167 (44%) were female; 40% were Caucasian; 20%

Asian; 4% Afro-Caribbean; 18% were of "other" origin; and ethnicity was not recorded in 18%. Median (range) serum 25OHD level was 60.7 (6, 301.8) nmol/L (24.3 (2.4, 120.7) ng/mL). Vitamin D deficiency (VDD) was noted in 19% (73 of 381), insufficiency in 18% (70 of 381), and sufficiency in 63% (238 of 381). Sixty-one children out of 381 (16%) had at least one fracture. Thirty-six children (9%) had osteopaenia (radiographic low bone density). Twenty children (5%) had rickets. The number of children by metrological season was: Spring 98 (26%), Summer 91 (24%), Autumn 98 (26%), and Winter 94 (25%). Radiographic rickets and VDD were not always associated [Figure 3.1 – Figure 3.2].

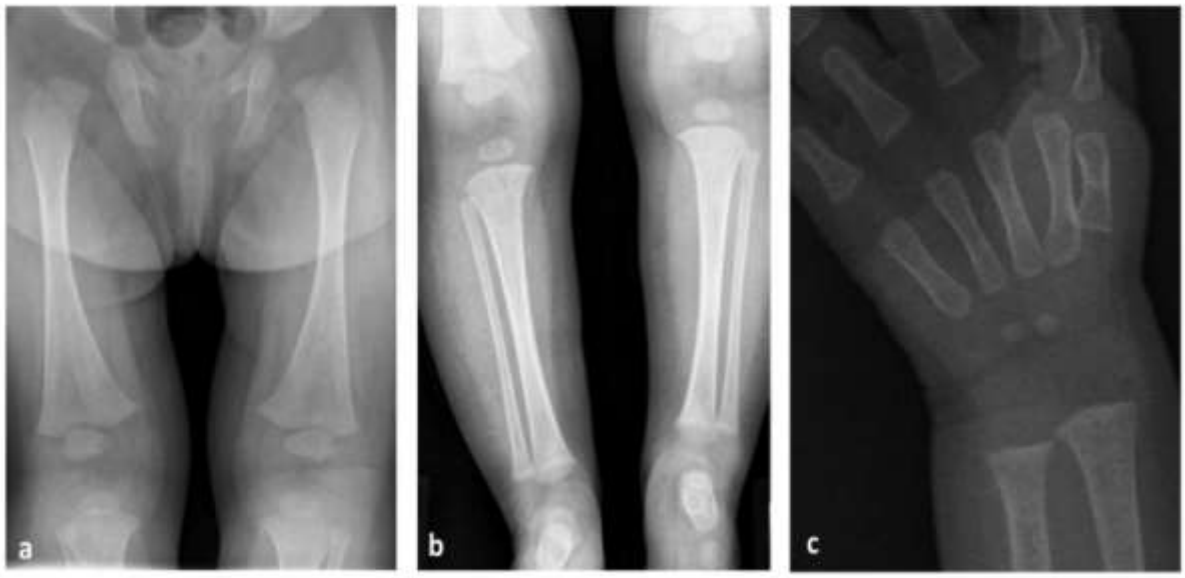

Figure 3.1 Radiographs of a child with severe VDD and normal radiographs with no evidence of rickets. AP both femora (a), AP both tibia and fibulae (b), DP left hand (c)

Figure 3.1 shows images obtained as part of a full skeletal survey in a 4-month old female investigated for sudden unexpected death. Her serum total vitamin D was <6nmol/L (< 2.4ng/mL). Both observers scored the radiographs as "Grade A" (no radiographic rickets). The radiographs are normal.

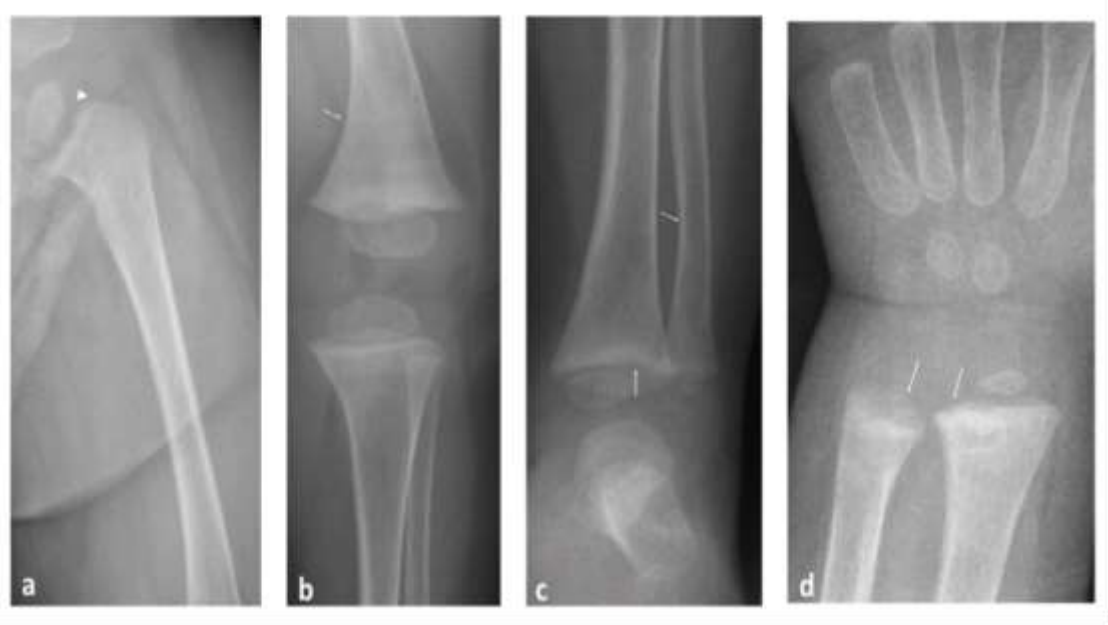

Figure 3.2 Images of a child with normal vitamin D levels, showing signs of rickets, osteopaenia (radiographic low bone density) but no fractures. AP left hip (a), AP left knee (b), AP left ankle (c), and DP left hand (d)

Figure 3.2 shows Images obtained as part of a full skeletal survey in a 15-month old African boy, for initial investigation of suspected rickets. His baseline serum total vitamin D was 159nmol/L (64ng/mL). His serum PTH 31.9 ng/L (ref range 0-0), corrected calcium 2.45 mmol/L (2.1 - 2.56), phosphate 1.98 mmol/L (0.8 - 1.9), alkaline phosphatase 249 U/L (76 - 308). Observer A scored the radiographs as "Grade C" (moderate radiographic rickets), while Observer B and the arbitrator both scored them as "Grade B" (mild radiographic rickets). Note osteopenia with widening of the proximal femoral growth plate (arrowhead), mild cupping of the growth plates (straight arrows) and bowing of the distal femur and fibula (dotted arrows). The skeletal survey was otherwise normal; in particular there were no fractures.

3.4.2 Serum total 25OHD level (vitamin D) and fracture risk

Variable	Fracture (n=61)	Non-fracture (n=320)
	(Median 25/75th) or n (%)	
Age (in months)	5.5 (2, 13.5)	9 (315)
Gender (female)	28 (46)	137 (43)
Pregnancy (singleton)	54 (88)	242 (75)
Gestation (weeks)	38 (38, 40)	39 (37,40)
Mode of feeding		
Breast	16	71
Bottle	15	136
Mixed	4	27
Missing	31	86
Ethnicity		
White Caucasian	37	115
Asian (sub-continent)	13	64
Afro-Caribbean	1	17
Other	3	48
Missing	7	76
Vitamin D status		
Deficient (< 30 nmol/L)	5	68
Insufficient (30.1 – 50 nmol/L)	10	60
Optimal	46	192
Serum total vitamin D (nmol/L)	72.7 (51.5, 91.6)	60.0 (28.9, 83.8)
Serum Corrected Calcium (mmol/L)	2.55 (2.44, 2.64)	2.51 (2.42, 2.57)
Serum Phosphate (mmol/L)	1.80 (1.56, 2.0)	1.87 (1.68, 2.02)
Serum Alkaline Phosphatase (U/L)	233 (169, 402)	222 (167, 287)
Serum Parathyroid Hormone (ng/L)	52.9 (27.6, 87.3)	35.9 (23.02, 60.2)
Rickets	4 (6)	16 (5)

Table 3.2 Demographic, clinical and laboratory data of children with and without fractures

A total of 61 children out of 381 (16%) sustained at least one fracture. Median (range) number of fractures was 1(1-10). Total number of fractures was 140. Median serum vitamin D level was 72.7nmol/L in the fracture group and 60nmol/L in the non-fracture group. Demographic, clinical and laboratory differences between the fracture and the non-fracture group are given in Table 3.2.

A total of 61 children presented with 140 fractures, median (IQR) =1(1, 2). Three children had 10 fractures (Figure 3.3).

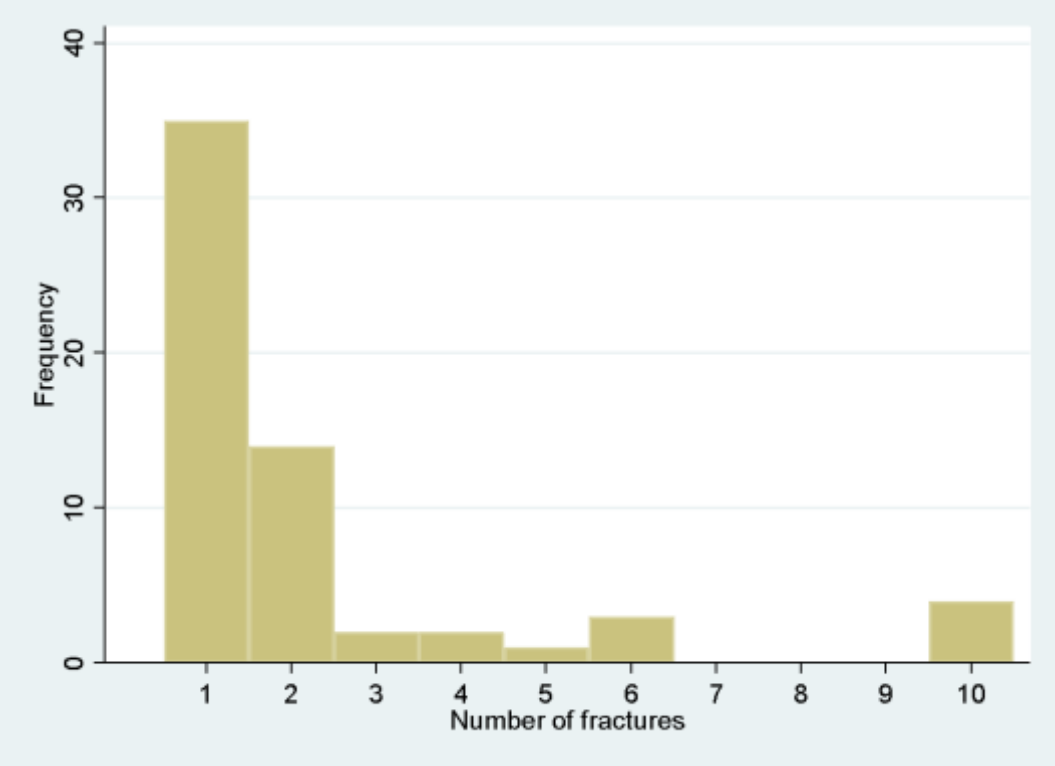

Figure 3.3 Fracture frequency in children with at least one fracture. 35 children had 1 fracture, 14 had two fractures, 3 had 2 etc.

The distribution of serum vitamin D levels is shown in Figure 3.4 Median serum vitamin D level was 60.7 (34.3.85.5 IQR) nmol/L. 73 (19%) were vitamin D deficient, 70 (18%) insufficient and 238 (62%) had optimal vitamin D status.

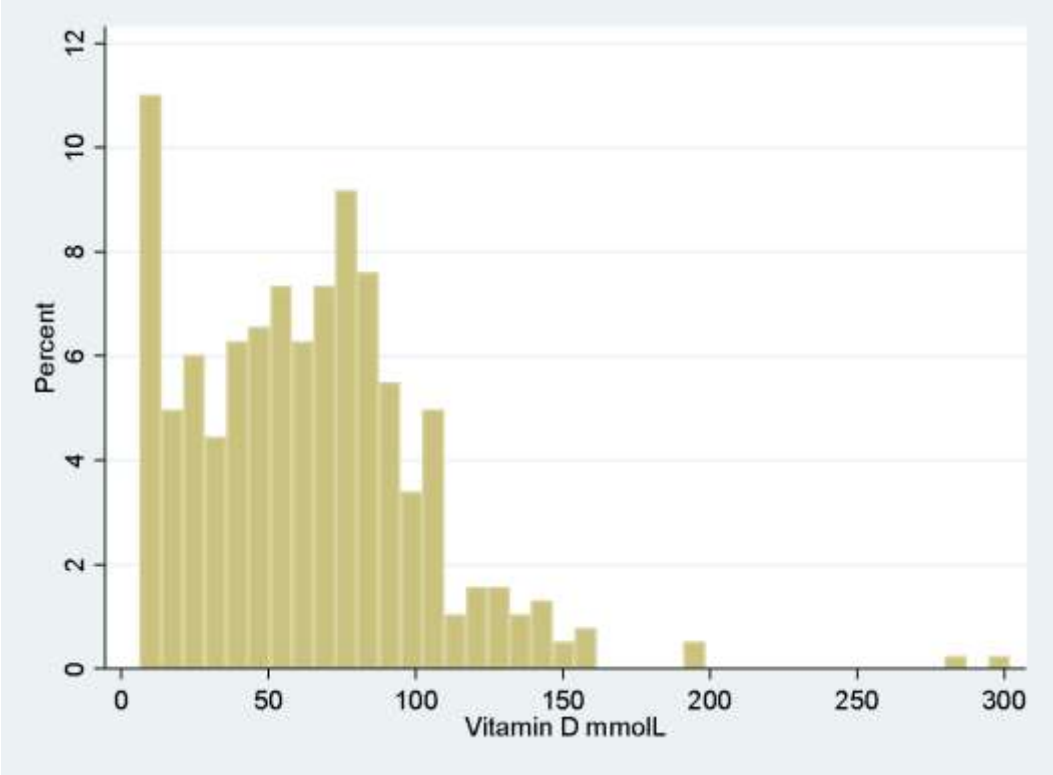

Figure 3.4 Histogram of serum total 25OHD (vitamin D) levels. It is skewed to the right-hand side.

3.4.3 Poisson regression (overdispersion corrected)

The primary outcome measure was fracture frequency. The mean was 0.376, and the variance 1.713 demonstrating evidence of overdispersion.

Univariable analysis

There was evidence of increasing serum vitamin D levels with fracture frequency (Table 3.3) (p=0.031). Osteopaenia (radiographic low bone density) was weakly associated with an increase in fracture frequency (p=0.079). Pairwise comparisons were generated to explore the relationship between fracture frequency and meteorological season. There was weak evidence of higher fracture frequency in winter compared to autumn (p=0.074), all other pairwise comparisons indicated little or no evidence of a difference. The number of fractures by spring, summer, autumn and winter was 38, 26, 22 and 54 respectively.

Variable	Incident Rate Ratio (IRR) (95% CI)	p-value	Evidence of a difference
Serum vitamin D (nmol/L)	1.0092(0.999,1.0193)	0.07	Weak evidence
Age (months)	0.964 (0.926,1.003)	0.07	Weak
Osteopaenia	1.982 (0.695,5.650)	0.2	Little or none
Spring **Summer** **Autumn** **Winter**	1 (reference) 0.736 (0.293,1.851) 0.578(0.229,1.458) 1.481(0.618,3.547)	 0.51 0.24 0.47	 Little or none Little or none Little or none
Multivariable serum vitamin D (nmol/L)	1.0106(1.0002,1.0211)	0.044	Weak evidence

Table 3.3 Poisson regression (overdispersion corrected). Serum vitamin D continuously distributed.

Multivariable analysis

The last row of the table 3.3 shows the IRR for vitamin D and fracture frequency adjusting (statistically) for age, osteopaenia and meteorological season. The IRR (95% CIs) hardly changed on adjustment (little or no confounding). The p-value 0.044 indicating evidence of increasing vitamin D with increasing fracture frequency. Figure 3.5 illustrates this relationship graphically.

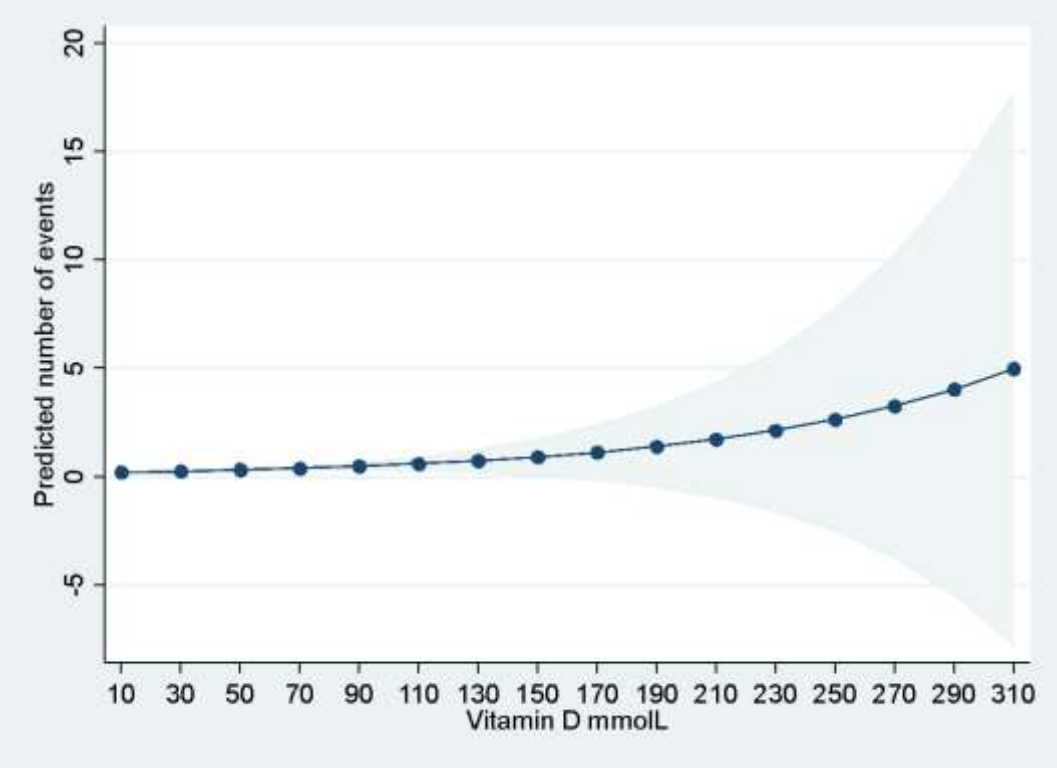

Figure 3.5 Predicted fracture frequency. Vitamin D continuously distributed (adjusted for age, osteopaenia, and metrological season). 95% CI shaded area. Wide CIs reflect few events.

3.4.4 Quartiles of serum vitamin D levels

Univariable analysis

The number of fractures by serum vitamin D levels from lowest to highest quartile was 14, 42, 39 and 45 respectively. There was a non-linear relationship described as a 'plateau' effect between fracture frequency and vitamin D quartiles. The 2nd, 3rd, and 4thquartiles had approximately the same IRRs (Table 3.4). Pairwise comparisons found no other statistical differences between the quartiles.

Variable	IRR(95% CI)	p-value	Evidence of a difference
Univariable vitamin D level			
Q1	1 (reference)		
Q2	3 (1.136,7.922)	0.027	Evidence
Q3	2.844(1.070, 7.563)	0.036	Evidence
Q4	3.248(1.231, 8.563)	0.017	Evidence
Multivariable serum vitamin D			
Q1	1(reference)		
Q2	3.350 (1.263,8.886)	0.015	Evidence
Q3	2.870 (1.038,7.930)	0.042	Evidence
Q4	3.776 (1.372,10.394)	0.010	Evidence

Table 3.4 Poisson regression (overdispersion corrected). Quartiles of vitamin D level. Children in Q2, Q3, and Q4 are 3 times as likely to fracture compared to those in Q1.

Multivariable analysis

Statistical relationships were maintained on adjustment for age, osteopaenia and meteorological season. However, the IRRs for 2nd, 3rd and 4th quartiles were less precise (wider 95% CIs) than on univariable analysis. Pairwise differences as for univariable Poisson. Figure 3.6 illustrates the relationship graphically. Age, osteopenia and meteorological season are not germane to this analysis and their IRRs are not quoted again.

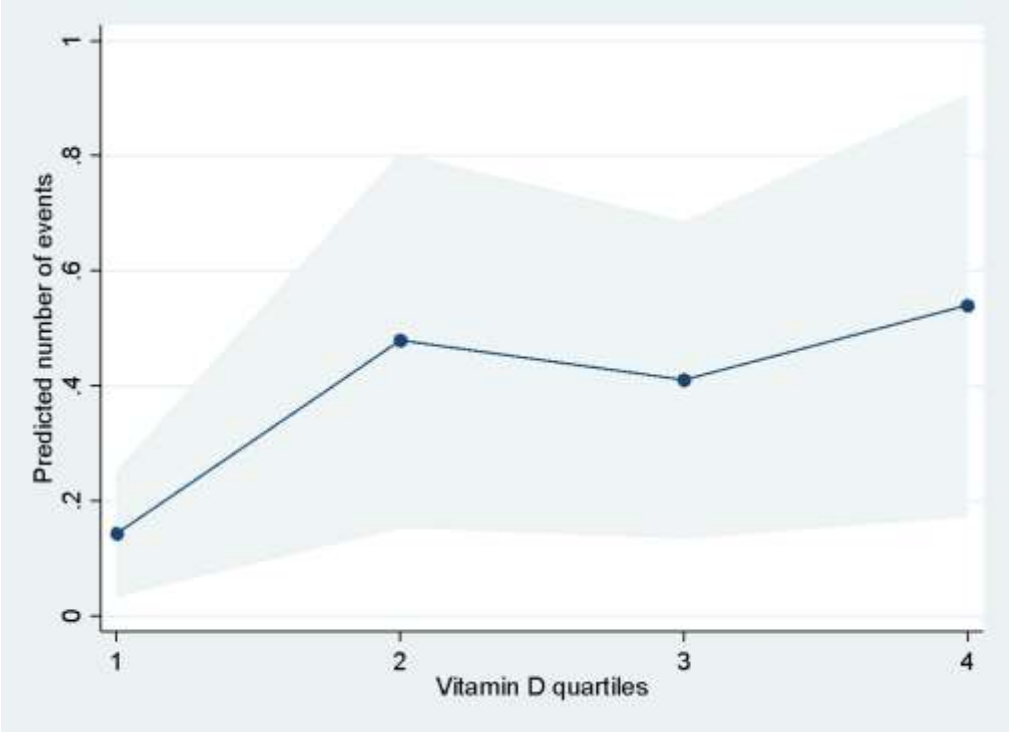

Figure 3.6 Predicted fracture frequency by quartiles of Vitamin D. (Adjusted for age, osteopaenia, meteorological season). 95% CI shaded area.

Sensitivity analysis

A sensitivity analysis was undertaken on quartiles of serum vitamin D (Table 3.5, Figure 3.6) by comparing it to a model based on negative binomial regression. P-values for negative binomial regression for the 2nd, 3rd and 4th serum vitamin D quartiles were 0.013. 0.049, and 0.009 respectively. The conclusions remain unchanged.

Variable	Negative binomial regression IRR(SE)
Vitamin D quartiles	
1st	1 (reference)
2nd	3. 580 (1.112)
3rd	3.169 (0.992)
4th	3.729 (1.154)

Table 3.5 Sensitivity analysis on vitamin D quartiles based on negative binomial regression.

Under negative binomial regression children in 2nd, 3rd and 4th quartiles are 3 times more likely to fracture compared to those in the lowest quartile.

3.4.5 Interobserver agreement

Interobserver agreement according to Landis and Koch (9) for presence of rickets was 'fair' (Kappa = 0.393); for Thacher grading of rickets 'moderate' (Kappa=0.586); for bone density 'substantial' (Kappa=0.667); and for presence of fracture 'almost perfect' (Kappa=0.917).

3.2 a. Presence or Absence of Rickets:

Kappa = 0.393 (fair agreement)

	Observer 1	
Observer 2	Absent	Present
Absent	359	12
Present	4	6

3.2 b. Thacher Grading Score for Rickets:

Kappa = 0.586 (moderate agreement)

	Observer 1			
Observer 2	A	B	C	D
A	359	2	0	0
B	1	4	1	0
C	0	2	1	8
D	1	0	1	1

3.2 c. Radiographic Bone Density:

Kappa = 0.829 (substantial agreement)

	Observer 1	
Observer 2	Normal	Reduced
Normal	334	14
Reduced	8	25

3.2 d. Presence or Absence of Fracture:

Kappa = 0.917 (Almost perfect agreement)

	Observer 1	
Observer 2	Absent	Present
Absent	320	3
Present	5	53

Table 3.6 Interobserver agreement for radiographic parameters. Interobserver agreement for grading of rickets based on Thacher score (2a); presence or absence of rickets (2b); radiographic bone density (2c) and presence or absence of fracture (2d) between Observers 1 and 2. Rickets was considered to be absent if the Thacher score was 0 and present if the score was ≥1.

3.5 Discussion

By confirming the absence of increased fracture frequency in infants and young children with isolated VDD compared to those with VDI/normal vitamin D, our results add to the body of evidence supporting mainstream thinking that low vitamin D on its own is not causative of fractures in this group (1) (14) (15) (16) (17) (18).

Schilling et al studied 118 children younger than two years with fractures and concluded that there is little or no association between low vitamin D and increased fracture risk (17). However, all children had at least one fracture and authors did not document the prevalence of radiological rickets.

Subsequently, Perez-Rossello et al conducted a prospective study, identifying 40 children (8 - 24 months) with VDD and performing radiographs of their wrists and knees. They concluded that development of demineralization, rickets and a subsequent increase in fracture risk is rare in infants and toddlers with VDD (18). All children in their study had VDD, therefore comparison with children with VDI or normal VD was not possible.

To account for the limitations in existing studies, we recruited children based on their having a serum 25OHD level recorded and skeletal imaging within two weeks of that measurement. This therefore included children with all ranges of serum 25OHD, potential for normal radiographs, full skeletal surveys, all grades of rickets and presence or absence of fracture. Our results overlap with those of the studies discussed above (17) (18). We found at least one fracture in 61 of 381 (16%) patients. Of the 61 patients with a fracture, 36 (59%) had radiographic evidence of reduced radiographic bone density. There was no increased prevalence of fracture in children with low serum 25OHD concentration (including in those with full skeletal survey). In fact, the tendency was for children with normal serum 25OHD concentration to have an increased number of fractures, presumably because

of the increased numbers of those children and not because raised serum 25OHD concentration predisposes to fracture.

VDD and VDI are not uncommon and there has been a huge resurgence of VDD and consequent rickets in children globally as well as in the UK over the last 15 years (19). This is in particular the case in ethnic minorities and dark-skinned individuals (20). Yang et al. conducted a multicentre, cross-sectional observational study looking at the prevalence of VDD and VDI among 460,537 children in mainland China. They reported deficiency (<30nmol/L) in 6.69% and insufficiency (30 - 50nmol/L) in 15.92% (21). Bodnar and colleagues reported VDD in 29.2% and VDI in 54.1% of the Afro-Caribbean mothers at the time of delivery and 45.6% and 46.8% in their new-born babies respectively. They reported VDD (< 37.5nmol/L) in 5% and VDI (37.5 – 80nmol/L) in 42.1% of White British women and VDD in 9.7% and VDI in 56.4% in their new-born babies respectively (20). Cohen et al reported an incidence of VDD in 24.5% (< 25 nmol/L) and VDI in 39 % (25 – 50 nmol/L) in a cohort study looking at the association between VDD and sudden unexpected death in infancy and childhood where they measured serum total 25OHD levels in 41 post-mortem cases (19). We could see a huge variation in the definition of vitamin D status used in these studies in particular for the VDI status. And the prevalence of VDD (<30nmol/L) (19%) and VDI (30.1-50nmol/L) (18%) in our cohort of 381 children is not dissimilar to those reported elsewhere (19) (20) (22).

There is increasing interest in the skeletal and extra-skeletal effects of VDD (23). In infants and young children, the debate centres around the extent to which isolated VDD (i.e. in the absence of other biochemical and radiological features of rickets) predisposes to otherwise unexplained fractures (1) (2) (3) (4) (6). Gordon et al studied the vitamin D status in 380 healthy infants and toddlers and concluded that rachitic changes are rare and subtle in healthy children with VDD (24). Recruiting only a cohort of healthy children, they were not able to correlate VDD with fracture prevalence. Perez-

Rossello et al reported an incidence of rickets in only two of 40 otherwise healthy children. They did not assess for rachitic changes in children with normal VD or VDI (18). Ponnapakkam et al reported that none of their 25 infants (0-6 months) with low serum total 25OHD showed evidence of rickets (25).

"Rickets" is a radiological diagnosis (it may be confirmed by histology). Low-grade rickets may be difficult to differentiate from physiological change, particularly of the distal ulna (26), but is never limited to this site. We found rickets (Thacher score ≥ 1) in only 20 (5%) of our 381 population, despite 87(24%) having VDD. Our results support those of others that biochemical VDD is more common in the general population than radiological rickets. It should be noted that 50% of those in our cohort who had radiographic rickets had normal vitamin D. We used a low threshold to diagnose rickets (Thacher score ≥ 1), so that we do not miss any such case and so it could be that these patients didn't have rickets at all. The effect this will have had on our results would be to increase any association between rickets and fracture; nevertheless, we were not able to demonstrate any such relationship. This reflects local practice, where a full skeletal survey is not necessarily performed for known or suspected rickets. To explain further when a full skeletal survey was undertaken in a 15-month-old African boy as part of initial investigations for suspected rickets (as shown in figure 3.2) he was found to have "Grade B" (mild radiographic rickets) and radiographic low bone density. His skeletal survey was otherwise normal; in particular there were no fractures. This reiterates that a full skeletal survey is not indicated for the diagnosis of rickets.

Equally in those who had a full skeletal survey for suspected abuse we were able to identify all fractures. This also reflects national policy, which is to perform a full skeletal survey is if an inflicted injury is suspected to identify all fractures and not to miss any. Equally we found more fractures in the vitamin D sufficient group which reinforces that low serum vitamin D levels in isolation is not attributed to fractures.

We also did not find a significant difference in prevalence of osteopaenia between the individual radiographs and the full skeletal survey groups. Equally DXA whole body bone mineral content (BMC) measurements for children <3 years of age are of limited clinical utility due to the lack of normative data and feasibility. Areal bone mineral density (BMD) should not be used routinely due to the difficulty in appropriate positioning. And should not be used in the context of physical abuse.

3.6 Limitations

Our study has some limitations. Observer 2 was a paediatrician with relatively limited experience in radiology; however, despite this and following a period of training on non-study radiographs, the interobserver reliability was fair (presence of rickets) to almost perfect (presence of fracture). All other statistical analyses were based on the final arbitration read of a radiologist with 40 years' experience in paediatric radiology, including providing a radiographic report in over 1,700 cases of suspected inflicted injury.

We used the radiographs to describe osteopenia in this study. Osteopenia in children is caused by several conditions including vitamin D and /or calcium deficiency rickets, immobility, secondary to excessive use of steroids, chronic inflammation, delayed or arrested puberty. Osteopenia refers to reduced bone mineral density (BMD) and is defined by both a history of pathological fractures and a low BMD score between -1 and -2.5 SD for age measured by DXA as per the International Society for Clinical Densitometry (27). However, the term osteopaenia is often used by the radiologists in a generic and qualitative manner (28) to describe decreased bone density on bone radiographs when ideally it should not be used.

Rickets is defective mineralisation of the growth plate and bone matrix in growing children which is equivalent to osteomalacia in adults where there is defective mineralisation of the bone matrix that

occurs even after the fusion of the growth plate (29). In rickets there are radiographic changes above and beyond osteopenia such as metaphyseal cupping, splaying and fraying. Radiographs of the wrists and knees and skeletal survey remain the gold standard for the accurate evaluation and diagnosis of rickets and fractures in non-accidental injuries respectively (29).

Only 153 (40%) of the study population had a full skeletal survey performed. For the remainder, while rickets (being a systemic condition) could be excluded, it is possible that clinically silent fractures were missed. However, it should be noted that in the skeletal survey group, children with normal 25OHD were more likely to have fractures. We included children who had at least one skeletal radiograph performed within two weeks before or after serum vitamin D measurement. It can be argued that the timing of serum vitamin D measurement and radiological imaging is important firstly because treatment with vitamin D may have commenced in those children who had blood tests prior to imaging, thus altering radiographic features of rickets. However, although radiographic changes may be evident within the first week of treatment, it takes three to six months for the radiographs to return to normal after the commencement of vitamin D treatment. Furthermore, we had a low threshold for defining rickets (Thacher ≥ 1), so that we were unlikely to miss any such case. The second reason why timing is of potential significance is that vitamin D treatment may have been commenced after imaging on the basis of radiographic rickets but prior to a baseline serum 25OHD measurement. In this unlikely clinical scenario, standard age appropriate dose of vitamin D treatment or supplementation as per the national guidelines (30) would be given in oral form and serum 25OHD would not be expected to return to normal levels within the two-week time frame; NICE recommends treatment for three to six months before rechecking serum 25OHD levels (31). Finally, blood tests and radiographs were not necessarily performed on the same day; we limited the time frame between biochemical tests and radiographs to two weeks so that any fractures present will not have healed.

Being a retrospective study there was a high rate of "missing values" in regards to bone biochemistry in our retrospective study. Of the 73 patients with VDD, 43 (59%) had serum corrected calcium measured, of whom 35 (81%) had normal levels; 57 (78%) had serum phosphate and serum alkaline phosphatase levels measured of whom 56 (98%) and 38 (67%) respectively had normal levels and only 28 (38%) had serum PTH measured of whom 9 (32%) had elevated levels. There are no normative data for PTH in infants. There is published data from prospective RCTs of vitamin D that suggest values around 25pg/ml are typical (32), but the manufacturer's reference ranges only apply to older children and adults. In those who had low serum 25OHD and elevated PTH levels and fracture(s), one can argue that raised PTH can very well be the cause for fracture rather than the result of the fracture(s) since chronic vitamin D deficiency leads to secondary hyperparathyroidism which in turn leads to increased bone turnover and fracture risk. Although both PTH and serum 25OHD play a crucial role in bone homeostasis via a tightly controlled feedback mechanism (33), PTH is the primary modulator of bone turnover and it exerts its action via PTH related peptide receptors that are expressed in osteocytes (and kidney) and not 25OHD since there are no receptors on the osteocytes for the latter. This may explain our findings of a higher prevalence of fractures in children with elevated PTH; i.e. a rise in PTH is a response to fractures. Our findings call for further prospective trials to further fill the gap of knowledge as well as cementing the understanding on rachitic changes on radiographs and biochemical markers of bone metabolism.

3.7 Conclusion

This study provides objective evidence to support the view that in the absence of radiological evidence of rickets, vitamin D deficiency is extremely unlikely to be the cause of otherwise unexplained fracture(s) in children less than two years of age. These results should inform both clinical management and legal deliberations in cases of suspected inflicted injury in children.

3.8 Acknowledgement

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3.9 Authors' contribution

Jaya Sujatha Gopal-Kothandapani contributed to scoring of the radiographs as Observer 2, statistical analysis, drafted the manuscript and designed the tables and figures.

Alan S Rigby contributed to the statistical analysis and to the writing of the manuscript.

Elaine Pang contributed to designing the spreadsheet and preliminary data collection.

Alan Sprigg contributed as Observer 3 in arbitrating the discrepancies in radiographic scoring between Observers 1 and 2 and to the writing of the manuscript.

Amaka C Offiah contributed to the design and implementation of the project, scoring of the radiographs as Observer 1, analysis of results and to the writing of the manuscript.

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Chapter 4: Vitamin D Dosing Study

Title

Effect of Vitamin D Supplementation on Free and Total Vitamin D: A Comparison of Asians Vs Caucasians

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4.1 Abstract

Objectives:

It is well established that UK Asians typically have lower vitamin D levels than Caucasians. It is also known that vitamin D binding protein (DBP) is lower in some races than Caucasians. To investigate how ethnicity, skin colour and genetic variation affect the response to vitamin D (150,000 IU) administered to young Asian and Caucasian men.

Design:

Prospective, single centre clinical trial

Participants:

Sixty young men (18-25yr) of Asian (n=30) and Caucasian (n =30) origin

Measurements:

We measured serum calcium, phosphate, magnesium, alkaline phosphatase, albumin, parathyroid hormone; total 25 hydroxyvitamin D (25OHD); calculated and directly measured free 25OHD; DBP at baseline and 4 weeks; DBP genotype, skin colour (Fitzpatrick scale), dietary vitamin D and calcium intake at baseline; and urine calcium:creatinine ratio at baseline, 1 and 4 weeks.

Results:

At baseline, Asians compared to Caucasians had lower serum total 25OHD (26.4 [13.7] vs 34.1 [12.3] nmol/l p=0.0272) and DBP (6.7 [3.4] vs 9.6 [4.4] nmol/l; p=0.0065) but similar free 25OHD (16.7 [10.4] vs 17.8 [7.5] pmol/l p=0.6530). After dosing, total 25OHD rose similarly in each group (≈56 nmol/l), but measured free 25OHD rose more in Asians (18.1 [9.4] vs 12.2 [13.3] pmol/L p=0.0464). Lower DBP at baseline, possibly reflecting genotype differences, was associated with a greater change in measured free 25OHD in Caucasians, but not in Asians.

Conclusions:

Asian compared with Caucasian males had a larger increment in measured free 25OHD following 150,000 units vitamin D3, possibly reflecting differences in DBP affinity for 25OHD. Ethnicity should be considered when devising guidelines for the treatment of vitamin D deficiency.

Key words:

Bio-available 25 hydroxy vitamin D, Ethnicity, Serum Measured Free 25 hydroxy vitamin D, Vitamin D binding protein, Vitamin D binding protein genotype, Vitamin D3

4.2 Introduction

Vitamin D deficiency is a term widely used but rarely defined in terms of tissue specific effects on bone, muscle and vital organs such as gut and kidney. . Based on synthesised evidence, thresholds for low dose supplementation or higher dose treatment have been suggested. The Scientific Advisory Committee on Nutrition Department (SACN) recommends a threshold of 25 nmol/L for serum 25OHD for all individuals at any time of the year to protect the musculoskeletal health (1). The Institute of Medicine defines the threshold for vitamin D deficiency as ≤30 nmol/L (2). The Endocrine Society defines the threshold for vitamin D deficiency as <50nmol/L, which would likely include more than half the population of the UK during winter months (3)

What remains unclear is the extent to which “one size fits all” in providing vitamin D supplementation or treatment. In the UK, vitamin D deficiency is widely reported, more so in those with darker skin including those of African or Asian descent. The effect of ethnicity, independently of other factors, has not previously been considered when devising strategies and recommendations for either low dose supplementation or higher dose treatment.

Vitamin D status is currently assessed by measuring circulating 25-hydroxyvitamin D (25OHD), which exists either free in the circulation (<1%), or bound to albumin or the vitamin D-binding protein (DBP). The terms “free” vitamin D and “bioavailable” vitamin D refer respectively to unbound 25OHD, or unbound plus 25OHD bound to albumin. The extent to which the total measurement adequately reflects either free or bioavailable vitamin D and whether any relationship between “total” and “free” varies following supplementation, or with other factors such as ethnicity, is unclear. Reports of higher free and bioavailable vitamin D for the same total serum 25OHD in African Americans have been challenged because of concerns regarding DBP measurement accuracy (4) (5) (6) (7) (8).

In light of this, we sought to investigate whether there were differences between serum total and directly measured free 25OHD concentrations between Asians and white Caucasians and the changes in these parameters following administration of a single dose of 150,000 IU of vitamin D3. In addition, we aimed to determine the effect of covariates including DBP concentration and genotype, concurrent diet and skin colour on both the free and total 25OHD responses, and the extent of parathyroid hormone (PTH) suppression following dosing.

4.3 Materials and methods

4.3.1 Study design

This was an exploratory study to determine the size of the effect on serum free and total 25OHD of a 150,000 unit dose of vitamin D, given to young adult males from different ethnic groups [Figure 4.1].

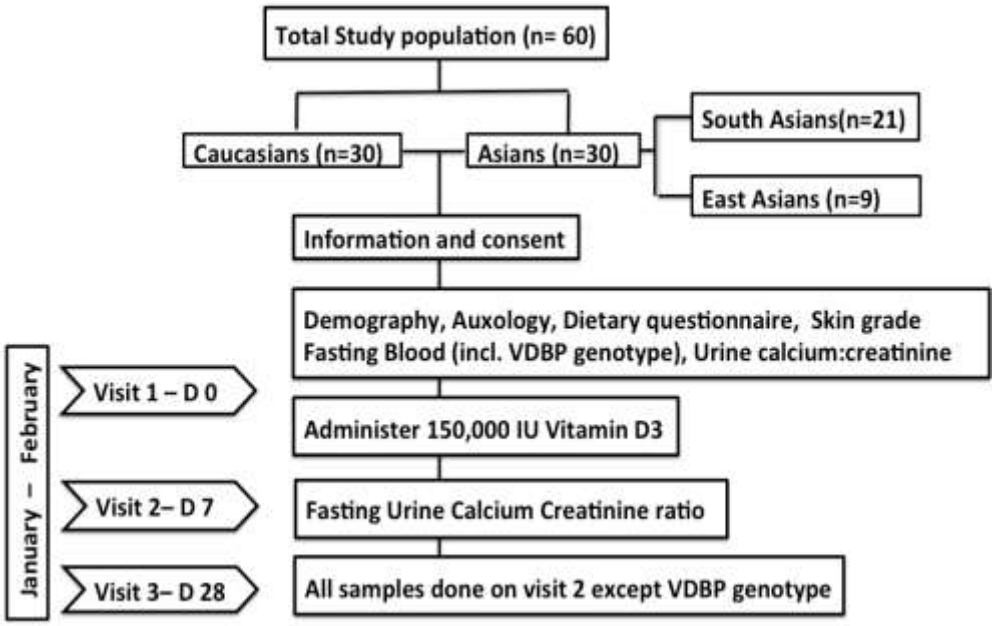

Figure 4.1 Study Design.
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4.3.2 Study participants

We recruited a cohort of sixty apparently healthy young adult men from two ethnic groups - White Caucasian (n=30) or South/East Asian (n=30) origin. Eligibility criteria included men aged between 18 and 25 years who were free from any condition affecting bone health, general nutrition, growth and glucose metabolism. Subjects with any chronic illness involving the liver and kidney, causing malabsorption, who used steroids, anticonvulsants, or vitamin D treatment as well supplementation or any medication that might affect calcium and vitamin D metabolism, were excluded. Recruitment took place within the University of Sheffield using a mixture of email, posters/leaflets and pre-lecture 2-minute talks. Subjects were students of the University of Sheffield, mostly medical and dental students. None of the study participants travelled to destinations where cutaneous vitamin D synthesis could have taken place during the study period. Whilst it was anticipated that gender should not impact on the outcome, the administration of a large dose of vitamin D might have had unexpected effects on an early stage pregnancy, thus we excluded young women.

4.3.3 Demography and Auxology

At baseline, height (without shoes to next succeeding 1mm by wall-mounted stadiometer [Holtain, Crymych]), weight (wearing vest and pants to nearest 0.1 kg by Marsden portable weighing scales, body mass index [BMI (kg/m^2), and waist:hip circumference ratio (paper tape measure) were recorded in all subjects.

4.3.4 Dietary calcium and vitamin D intake

A validated 101-item food frequency questionnaire (DIET-Q) was used to ascertain the dietary intake of calcium and vitamin D at baseline. Mean daily calcium (grams/day) and vitamin D (IU/day) intake were analysed using Q Builder (V4.0) nutritional software (Tinuviel software, Llanfechell, Anglesey UK).

4.3.5 Skin type, season and sun exposure

Skin type was assessed using a 6-point Fitzpatrick scale in all subjects at baseline [4]. Fitzpatrick 1 skin type is fair and freckled; type 6 is very dark/black. The study was conducted in the UK during January and February to avoid sun exposure.

4.3.6 Samples

4.3.6.1 Baseline

Fasting blood samples were collected for serum total 25OHD, free 25OHD, calcium, phosphate, magnesium, albumin, alkaline phosphatase, PTH, DBP and DBP genotype.

4.3.6.2 Four weeks

All blood investigations (except DBP genotype) were repeated.

4.3.6.3 Urinary calcium:creatinine ratio

A fasting second void urine sample for calcium:creatinine ratio was collected at baseline, 1 and 4 weeks after vitamin D3 administration.

4.3.7 Vitamin D3 dosing

A single dose of 150,000 IU of Vitamin D3 [6 mls of Invita D3 (Consiliant) 25,000 IU/ml oral solution] was administered under direct supervision. We chose this dose based on the work done by Oliveri et al. where the authors demonstrated the safety of a single dose of 150,000 IU of vitamin D to maintain appropriate levels of 25OHD without causing hypercalcaemia or hypercalciuria (9).

4.3.8 Laboratory methods

4.3.8.1 Serum total 25OHD

Serum total 25OHD levels were determined using an UPLC/Mass Spectrometer Semi-automated hexane extraction in the Acquity Ultra Performance LC/Quattro MS (Waters) analyser. Lower limit of detection for $25OHD_2$ was 6 nmol/L and for $25OHD_3$ 3.5 nmol/L. The interassay coefficient of variation (CV) for $25OHD_2$ and $25OHD_3$ were 5.7% and 5.4% respectively.

4.3.8.2 Serum free 25OHD

Free 25OHD levels were measured using an ELISA from Future Diagnostics Solutions. The interassay CV was 4.8%.

4.3.8.3 Serum Vitamin D Binding Protein (DBP)

DBP levels were measured using polyclonal ELISA from Genways Biotech Inc. The interassay CV was 5.8%.

4.3.8.4 Serum calcium, phosphate, albumin, and alkaline phosphatase; urine calcium, creatinine

Measured using Micro Slide Technology Colorimetric/Rate by Reflectance Spectrophotometry in the Vitros 5, 1 FS System (Ortho Clinical Diagnostics) analyser. The interassay CVs were: calcium (1.4%), phosphate (1.6%), albumin (2.9%), alkaline phosphatase (2.4%), urine calcium (1.7%) and urine creatinine (4.4%).

4.3.8.5 Intact Parathyroid hormone (PTH)

PTH was measured using Immunoassay (Chemiluminescent Microparticle Immunoassay) in the Architect *i* 1000 System (Abbot) [PTH analytical sensitivity ≤1 ng/L].

4.3.8.6 DBP genotyping

A pyrosequencing assay was developed in house, using PSQ assay design software version 1.0.6 (Qiagen), to detect two single-nucleotide polymorphisms (SNPs), rs4588 and rs7041 in the DBP gene, that give rise to 3 common variants of DBP (i) Gc1f (ii) Gc1s and (iii) Gc2**.** All subjects were genotyped for 6 different haplotypes - Gc1f-1f, Gc1f -1s, Gc1f-2, Gc1s-1s, Gc1s-2, and Gc2-2, ranked here in order of 25OHD binding affinity, highest to lowest.

4.3.8.7 PCR and sequencing primers

F: 5' -ATCTGAAATGGCTATTATTTTG-3',
R: 5' Btn -ACAGTAAAGAGGAGGTGAGTT-3',
Seq: 5' -AAAAGCTAAATTGCCTG-3'.

To ensure distinct pyrosequencing signals, (~10 ng) of human genomic DNA was amplified by 47× PCR cycles using One*Taq®* 2X Master Mix with Standard Buffer (NEB). For each genotype determination, single-stranded DNA was purified from 5 µl of PCR products using PyroMark Q96 Vacuum Prep Workstation (Qiagen). PCR products were denatured to single-stranded DNA (ssDNA) and annealed with sequencing primers. Pyrosequencing was then performed on a PyroMark Q96 MD Instrument according to manufacturer's instructions (Qiagen). Nucleotide dispensation order was as follows: DBP rs4588+rs7041 CATGTCACACACTG. SNP analysis was carried out using the SNP analysis software provided (Qiagen).

4.3.8.8 Calculated free and bio-available 25OHD

Free and bioavailable 25OHD levels were calculated using the mathematical model described by Chun et al (6). This model designates different affinity constants for the different DBP genotypes.

4.3.9 Statistical methods

This was an exploratory study to determine effect size and variance; hence no formal calculation of sample size was undertaken. We sought advice from the Yorkshire and Humber Research Design Service who suggested that a sample size of 30 per group was sufficient to undertake exploratory work of this nature. This was an arbitrary number.

Continuously distributed data was summarized by the median (25^{th}/75^{th} centiles); categorical data by n (%). Missing values are tabulated but not considered otherwise in our analysis.

Normality checks were carried out; data were generally normally or near-Normally distributed, hence parametric tests were used to assess differences between groups.

To compare the groups prior to dosing, 2 sample t-tests of baseline characteristics were performed and are reported in Table 4.1.

	Whites	Asians	P value
Study population	**30**	**30**	**-**
Age (years)	21.43 (1.54)	22.33 (1.44)	0.66
Weight (kg)	76.97 (9.16)	70.77 (8.34)	0.34
Height (cms)	179.95 (5.59)	175.77(5.6)	0.95
BMI	23.76 (2.51)	22.89 (2.30)	0.61
Waist : Hip ratio	0.81 (0.05)	0.88 (0.09)	0.30
Calcium intake (gms/day)	1065.87 (266.88)	1031.23 (382.6)	0.28
Vitamin D intake (iu/day)*	120 (20, 524)	140 (74,276)	0.29
Fitzpatrick skin grade	1 - 3	3 - 5	-
Serum Calcium (mmol/L)	2.31 (0.06)	2.30 (0.05)	0.49
Serum Phosphate (mmol/L)	1.38 (0.19)	1.36 (0.15)	0.74
Albumin (g/L)	44.2 (2.2)	43.7 (1.7)	0.32
Serum PTH (ng/L)	44.6 (14.2)	69.8 (38.6)	0.0019

Table 4.1 Baseline demographic data. The values are expressed in mean (SD) with the p values
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The main question of interest was whether ethnicity impacted on change in total and free 25OHD following dosing. Two sample t tests were carried out to compare the changes in total and free Vitamin D between the groups. Analysis of variance was also used to determine any other statistical differences between the groups and adjusted for covariates - ethnicity, vitamin D binding protein and vitamin D binding protein genotype. Generalized linear models were used to determine statistical differences between response variables that had error distribution models other than normal, and to assess the interactive effect of ethnicity and DBP on change in free vitamin D. Passing-Bablok

regression and Bland-Altman plots were drawn using the MedCalc statistical program for method comparison between directly measured and calculated free 25OHD.

Correlation was performed by Pearson's method. Fisher's exact test was used to compare the categorical data. Graphical presentation was made by Box and Whisker plot. P values were used sparingly with an arbitrary threshold of 0.05 (two tailed).

We performed all our analyses using Statistical Package for the Social Sciences version 22 (SPSS by IBM), Data Desk^{TM} v6.2.1 and Stata v14 (10)

4.4 Results

4.4.1 Baseline clinical characteristics

The baseline clinical characteristics of the study subjects are shown in Table 4.1. The white Caucasian and Asian men did not differ with respect to age, weight, height, BMI and waist to hip ratio. The mean (SD) Calcium intake (grams/day) was adequate and equal between the Asians [1031.23(382.6)] and white Caucasians [1065.87(266.88)]. The median (range) vitamin D intake (IU/day) in comparison with the UK recommended daily intake 400 (IU) (1) was low and similar between the groups, 140(74,276) and 120(20,524) in Asians and white Caucasians respectively. White Caucasians had a Fitzpatrick skin type of 1-3 and Asians 3-5. White Caucasians predominantly had Gc1s-2 haplotype and did not have Gc1f-1f and Gc1f-2 [Table 4.2]. South Asians predominantly had Gc1s-1s haplotype and East Asians Gc1f-1f haplotype. Gc2-2 and Gc1s-2 were not found in East Asians.

Haplotype	Caucasian	Asian	Total	Haplotype	Caucasian	Asian
					Median Baseline 25OHD concentration (nmol/L)	
Gc1f-1f	Obs 0 Exp 2.5	5 2.5	5	Gc1f-1f	-	-
Gc1s-1f	Obs 9 Exp 7.5	6 7.5	15	Gc1s-1f	31.6	20.4
Gc1s-1s	Obs 9 Exp 8.5	8 8.5	17	Gc1s-1s	40.8	20.4
Gc2-1f	Obs 0 Exp 1.5	3 1.5	3	Gc2-1f	-	-
Gc2-1s	Obs 11 Exp 8.5	6 8.5	17	Gc2-1s	33.2	24
4Gc2-2	Obs 1 Exp 1.5	2 1.5	3	Gc2-2	22.4	16.4
Total	30	30	60	Fisher's exact test, p=0.052		

Table 4.2 Haplotype frequencies by ethnicity and median 25OHD concentration at baseline
Reproduced with permission from Gopal-Kothandapani et al, 2018 (21)

The expected cell frequencies were calculated from chi square distribution. We show one row as an example. And the rest follow suit.

4.4.2 Chi-squared response

The data in Table 4.2 are the cross-classification between ethnicity and haplotype frequency. The rows and the columns are exclusive (can't be in two groups) and exhaustive (no more options). The expected cell frequencies are calculated assuming that the rows and columns are independent of one another. If there was no relationship between ethnicity and haplotype frequency then what we observe would be exactly the same as what we expect (under the independence assumption). The greater distortion between observed and expected the greater the degree of association between ethnicity and the haplotype.

To solve the expected cell frequency of Caucasian and Gc1f-1f haplotype (top left hand cell) is as follows. The probability of a Caucasian is 30/60=0.5. The probability of Gc1f-1f is 5/60=0.083. Assuming independence between ethnicity and haplotype 0.5x0.083=0.0415. The expected cell

frequency is 0.0415x60=2.5. The expected cell frequency of Asian and Gc1f-1f is obtained by total column subtraction (5-2.5=2.5). All other rows/columns follow suit. The total Chi-squared statistic is calculated as the sum of (observer-expected) 2/ expected for each row/column (12 summations). This number is referred to Tables of the Chi-squared distribution on the appropriate degrees-of-freedom.

Table 4.2 shows the relationship between haplotype frequency and ethnicity. There was a significant association between the two (p=0.052, fisher's exact test). There were more Asians with Gc1f-1f haplotype than expected [5 vs 2.5]. Similarly, more Caucasians with Gc2-1s haplotype than expected [11 vs.8.5]. Table 4.2 shows the differences in baseline vitamin D status between the different haplotypes and ethnicity. However, the numbers were too small for statistical comparison.

4.4.3 Influence of ethnicity on measured parameters at baseline and following intervention

Twenty-nine participants out of the 60 recruited had a vitamin D level of < 30mnmol/L at baseline, of which 60% (18/30) were Asian and 36% (11/30) were white Caucasians [Table 4.3]. Asians has significantly lower serum total 25OHD and DBP levels, but similar measured and calculated free 25OHD levels compared to white Caucasians at baseline [Table 4.3].

		Serum Total 25OHD (nmol/L)	Serum VDBP (umol/L)	Measured Free 25OHD (pmol/L)	Calculated Free 25OHD(pmol/L)	PTH (ng/L)
Baseline	Caucasians	34.1 (12.3)	6.6 (3.0)	17.8 (7.5)	13.6 (7.8)	44.6 (14.2)
	Asians	26.3 (13.7)	4.7 (2.3)	16.7 (10.4)	11.9 (6.8)	69.8 (38.6)
***p-value <0.05**	**p value**	***0.004**	***0.001**	**0.65**	**0.38**	***0.0019**
Increment	Caucasians	56.7 (18.3)	0.31 (2.0)	12.2 (13.3)	24.4 (14.5)	2.2 (14.2)
	Asians	56.2 (12.6)	0.24 (2.0)	18.1 (9.4)	29.4 (20.1)	- 4.7 (27.7)
***p-value <0.05**	**p value**	**0.90**	**0.90**	***0.0464**	**0.29**	**0.24**

Table 4.3 Serum total and free 25OHD levels, estimated 25OHD and PTH levels at baseline and increment post supplementation, mean and SD
Reproduced with permission from Gopal-Kothandapani et al, 2018 (21)

At four weeks the changes in serum total 25OHD, DBP, and calculated free 25OHD levels were similar between Asians and white Caucasians; however, the increase in directly measured serum free 25OHD

level was significantly greater in Asians 18.1(9.4) vs 12.2 (13.3) pmol/L in Caucasians (*p*=0.0464) [Figure 4.2 and Table 4.3]. Although the observed significance was marginal, we considered it to be acceptable for a preliminary study. There was a significant interaction (p=<0.01) between ethnicity and mean DBP in relation to change in free 25OHD following dosing. In Caucasians, a lower mean DBP was associated with a larger in increase in measured free 25OHD; this was not true for Asians, in whom the relationship of free 25OHD with mean DBP was positive, i.e. as mean DBP increased, the change in free 25OHD also increased.

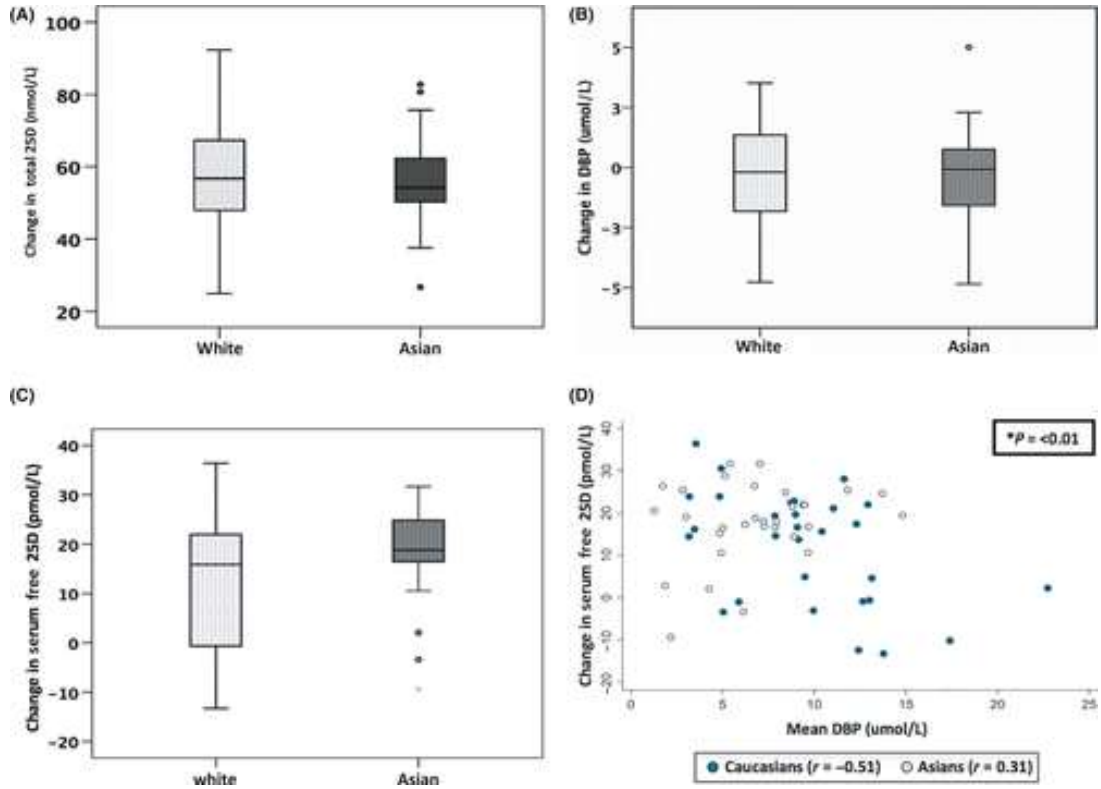

Figure 4.2 Change in total, free 25OHD and VDBP following vitamin D dosing
Reproduced with permission from Gopal-Kothandapani et al, 2018 (21)

In Figure 4.2. A, B, C shows the change in total, free 25OHD and VDBP following vitamin D dosing. Three-part box plot showing ethnicity in x-axis and change in total 25OHD, VDBP and free 25OHD in

y-axis. D, shows the change in serum free 25OHD according to mean D binding protein (DBP) with shaded circles representing Caucasians and clear circles for Asians.

We found no clear effect of DBP haplotype within ethnic group on change in either total or free 25OHD, either alone or in combination with other factors.

Baseline PTH concentration was higher in Asians than in white Caucasians (p=0.0019). Following intervention, no significant changes in PTH levels were noted in either of the groups. No increase in urine calcium: creatinine ratio was noted at either 1 or 4 weeks post intervention.

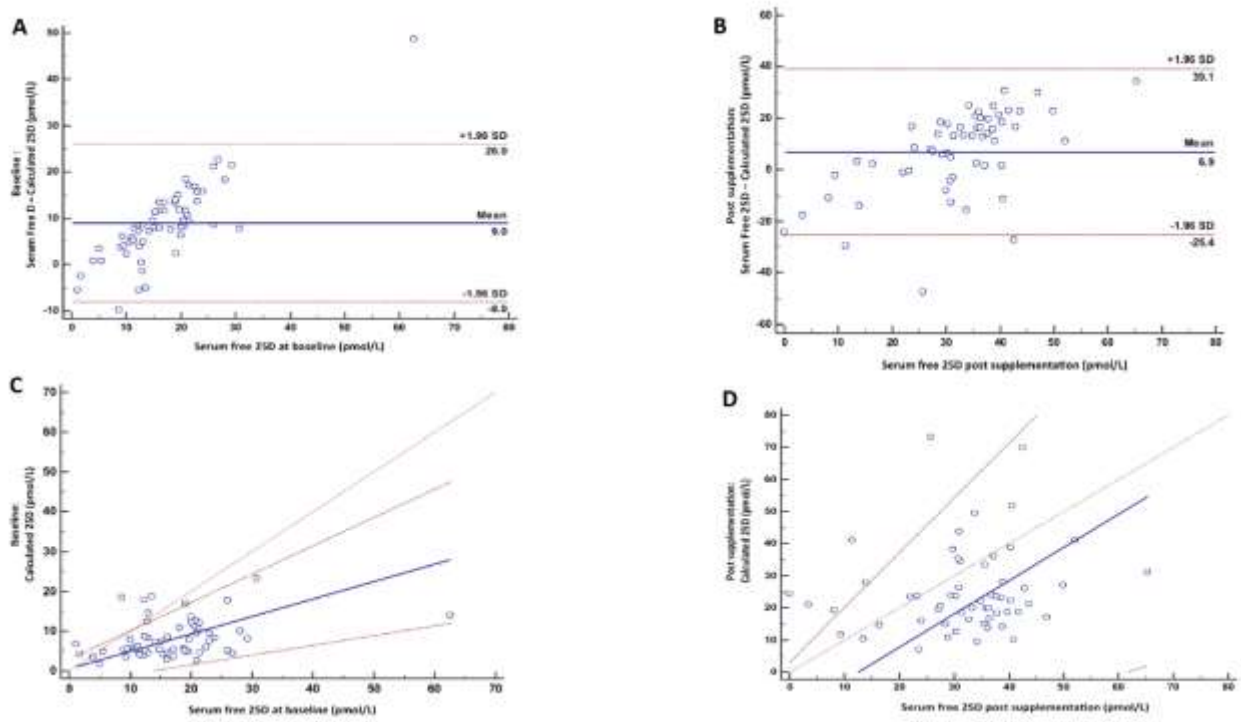

Figure 4.3 Method comparison: Direct vs. Calculated Free D-post supplementation
Reproduced with permission from Gopal-Kothandapani et al, 2018 (21)

Method comparison between directly measured and calculated serum free 25OHD concentration was undertaken using fixed affinity constant for the DBP genotype (Gc1f- 1f) (11).

Figure 4.3, A and B show the Bland Altman plots. Bland Altman plots measures agreement between 2 methods (A&B). On the Y axis the difference between 2 measurements is plotted (i.e A-B). If the

methods are the same A-B = zero. On the X axis the average of the 2 methods (A+B divided by 2) is plotted. If both methods are exactly the same, Bland Altman plots the line zero for all the x values. If method A is consistently higher than B, Bland Altman will have a line above zero across all the x values and vice versa. The average difference between A and B is referred to as bias. Also plotted on the same figure are 95% limits of agreement. These are measured as the mean difference +/- 1.96 times standard deviation of the differences. Values outsides of the two limits of agreement are outliers to the main data.

We now comment on the specific Bland Altman plots.

Figure 4.3 A. Pre vitamin D supplementation: There are no outliers indicated by all the values lying within the 95% limits of agreement. The average bias serum measure free 25D – calculated free 25D = 9 pmol/L. What is interesting is that for low levels of serum free 25D at baseline the bias is negative and vice versa. This means for low levels of serum free 25D at baseline serum free D measurement method underscores the calculated free 25D. At high levels the reverse is true.

Figure 4.3 B. Post vitamin D supplementation: There are 2 values just below the lower level of agreement outside the 95% limits of agreement (- 25.4 pmol/l). A 3rd value is well below the lower limit indicating an extreme value. The average bias is 6.9 pmol/L. The funnel shape is characteristic of Bland Altman plots. What this shows is increasing variability with increasing measurement size.

In Figure 4.3, C and D shows the Passing and Bablok plots. Passing and Bablok is a nonparametric regression for method comparison. It fits a straight line between two variables (X and Y) where both are measured with error. The intercept of the straight line represents the systematic bias between X and Y. The null hypothesis is zero intercept. If the 95% confidence (CI) crosses zero, the systematic bias is not statistically significant. The slope measures the amount of proportional bias. The null hypothesis is a slope of one. If the 95% CI cuts across on the proportional bias, it is not statistically

significant. If the two methods are identical, then they will share a common intercept (zero) and a common slope (one).

Bold line indicates the calculated regression. With 95% CI either side. The line of no difference is drawn at 45 degrees. C, demonstrates no systematic bias but a significant proportional bias at baseline. D, demonstrates neither systematic nor proportional bias post supplementation. Serum free 25OHD (directly measured) is represented in x-axis and the calculated free 25OHD (using fixed affinity constants) represented on the y-axis.

The Bland-Altman and Passing and Bablok plots demonstrating both systematic bias and poor agreement between calculated and directly measured serum free 25OHD at baseline, which became more pronounced following intervention. Thus, calculated serum free 25OHD concentrations were overestimated compared to directly measured free 25OHD in both the groups, more so in white Caucasians than in Asians, despite using a mathematical model utilising different affinity constants for the different DBP genotypes.

4.4.4 Relationship of PTH with total and free 25OHD

Baseline PTH concentration was higher in Asians than in white Caucasians (p=0.0019). Following intervention, no significant changes in PTH levels were noted in either of the groups. There was a statistically significant negative correlation between PTH and total 25OHD at baseline (r=-0.442; p=0.0006) and post intervention (r=-0.452; p=0.0004). However, the relationship of PTH at baseline with measured free 25OHD did not reach significance at baseline (r=-0.245; p=0.0634) or following intervention (r=-0.061; p=0.6446).

4.4.5 Relationship of skin type with total and free 25OHD

There was no statistically significant difference between skin types and baseline total 25OHD concentrations by one-way ANOVA (F (4, 54) =2.08, *p*=0.0956). Post-dosing total 25OHD also did not differ between skin types.

4.4.6 Relationship of DBP genotypes on total and free 25OHD

There were no significant differences by DBP genotype for baseline total 25OHD (F ratio =1.0075, *p*=0.4225) or serum free 25OHD (F ratio =0.4838, *p*=0.7868). Following intervention, subjects with Gc1f-1s haplotype (high affinity for 25OHD) showed the greatest increment in serum total 25OHD (increment 60.65 (17.3) nmol/L; baseline 32.9 (14.0) nmol/L). Subjects with the lowest affinity haplotype Gc2-2 had the smallest increment in serum total 25OHD (increment 48.9 (6.9) nmol/L, baseline 18.4 (3.6) nmol/L). The increment in serum direct free 25OHD levels was greatest in subjects with the Gc2-1s (16.6 (10.2) pmol/L; low affinity haplotype), and lowest in subjects with Gc1f-1f (10.1 (11.9) pmol/L highest affinity haplotype). None of these differences reached statistical significance, however.

4.4.7 Relationship of dietary calcium and vitamin D intake with PTH, DBP, total and free 25OHD

There was no relationship of dietary calcium intake at baseline with baseline PTH, DBP, total or free 25OHD, or change in any of those parameters, either for the whole group or by ethnicity.

4.5 Discussion:

We found that serum total 25OHD was low in young men of White Caucasian and Asian ethnic origin during winter in Sheffield and increased similarly in both groups following administration of 150,000 units of vitamin D3, but that measured free 25OHD increased more in those of Asian origin. We found a relationship between both baseline and mean DBP concentration and incremental change in

measured free vitamin D according to ethnicity. As mean DBP rose, the incremental increase in free 25OHD reduced in Caucasians, and increased in Asians. This suggests that the binding affinity of DBP may vary with ethnic origin. Calculated estimates of free 25OHD were found to be significantly different from direct measurements, and showed a systematic bias.

According to the free hormone hypothesis (5), the biological action of 25OHD is exerted by its freely-available form (<1% of the total), not by the total circulating amount which comprises DBP-bound (85-90%) and albumin-bound (10-15%) forms plus free. There is support also for the concept of bioavailable 25OHD (12), comprising the albumin-bound and free fractions, suggesting that measurement of free or bio-available 25OHD concentration may provide a more meaningful marker of vitamin D function than total (6).

The movement of DBP-bound 25OHD to bioavailable or free forms likely depends in part on the concentration of DBP and its binding affinity for 25OHD. Both are known to vary significantly both by and within ethnic groups. A recent report by Yao et al has also demonstrated significantly lower levels of DBP ($p < 0.001$) measured using a monoclonal assay (165.3+/-90.4 ug/ml) in comparison with the polyclonal assay (418.7+/_99.0 ug/ml) in a Chinese population (7). Powe et al in their cross sectional study reported that black Americans have lower total 25OHD and DBP resulting in similar concentrations of calculated bio-available vitamin D compared to white counterparts (12) . Bouillon et al showed similar results in Black Gambians compared to White Caucasians using the same DBP assay. In contrast, when they measured DBP using a polyclonal assay (as used here), in the same cohort, they did not find any difference between the groups (8). Similarly, Aloia et al. reported identical concentrations of DBP between US blacks and whites, using a polyclonal assay (13). Recently, Nielson et al. compared the DBP assays used in the MrOS (14) and MRC cohorts (15) and characterised the molecular forms of DBP (16) . The authors reported that the difference in DBP levels between the Africans and Whites identified using a monoclonal assay disappeared when measured using polyclonal

or proteomic methods (16). This contrasts with our results; we found a significant difference in DBP levels between the Asians and White Caucasians using the polyclonal assay. Studies using polyclonal immunoassays did not find racial differences in DBP levels (17). However polyclonal antibodies raised against DBP can cross-react with other proteins (18) which was confirmed by Powe et al. when they spiked serum with pooled DBP using a polyclonal assay (12) which may well be the case in our study (21). Similar to our results they also did not find a correlation between the directly measured and calculated DBP levels (12). Sollid et al. described significantly lower levels of DBP using a polyclonal assay in Caucasians with the Gc2/Gc2 haplotype (19). We found no clear effect of genotype either at baseline or following intervention on serum DBP, irrespective of ethnicity (21).

We found directly measured serum free 25OHD at baseline in Asians and Caucasians to be very similar despite lower total 25OHD in Asians. We hypothesized originally that the lower DBP concentrations found in the Asians in our cohort were likely to be the reason for their comparable levels of free 25OHD. Following intervention with vitamin D3, no change in DBP levels were observed in either Asians or White Caucasians when compared to baseline, indicating that the serum DBP concentration is not altered by single dose supplementation. Our findings agree with those of with Sollid, where no effect of vitamin D3 on serum DBP concentration was shown in an interventional trial (20,000 IU D3 weekly for a year) in Caucasians (19).

We found a higher increment in measured free 25OHD concentration in Asians following vitamin D3 supplementation, despite a similar increment in total 25OHD concentration and no significant change in DBP concentration. In addition, the incremental increase in Caucasians' free vitamin D was inversely related to both baseline and mean DBP, whereas in Asians it was not. This implies that DBP affinity for vitamin D and its metabolites may be a key factor in the response of Asians to vitamin D treatment. The lack of a clear relationship with DBP genotype could imply that additional factors may be at play, or may be due to the small sample size.

Alzaman et al. compared the differences in total and free 25OHD levels between Black and White Americans following daily D3 supplementation (2000 IU or 4000 IU) or placebo for a total of sixteen weeks in nearly 200 diabetic subjects. The authors found similar and dose-proportionate increases in both total and free 25OHD in both groups (20). Sollid et al. studied the relationships between serum total and free 25OHD (both directly and by calculation) in relation to age, sex, BMI, season and DBP genotype and their inter-relationship with the weekly administration of 20,000 units of D3 / placebo for a year in nearly 500 individuals. The authors found that serum DBP was not affected by vitamin D supplementation. They demonstrated that age, sex and DBP concentration did not affect increment in vitamin D parameters, following supplementation (19).

If serum free 25OHD increases disproportionately in some ethnic groups following vitamin D administration, is this a problem? There is another step beyond 25-hydroxylation in producing the biologically active form of vitamin D ($1,25(OH)_2D$), and this step is highly regulated to protect against hypercalcaemia. The finding here that PTH did not decrease as expected in White Caucasians as both free and total vitamin D increased is puzzling. However, PTH did decrease in the Asian group; this suggests that the greater increase in free 25OHD in Asians may have broader biological significance in terms of calcium metabolism, perhaps reflecting increased calcium absorption. Asians had lower baseline total serum 25OHD, and are more likely (as a population group) to receive treatment. The concern would be therefore that significantly increasing calcium absorption might have unexpected and undesirable consequences in an ethnic group already at higher risk for cardiovascular and renal disease.

4.6 Strengths and Limitations

Ours is the first interventional study reported so far, studying the effects of single dose of vitamin D3 on serum total 25OHD, DBP and free 25OHD concentration in both Asians and Caucasians. The main limitation of our study is the small sample size; hence we had an insufficient statistical power to

demonstrate the influence of skin types or DBP genotypes on the response to vitamin D3. However, our primary aim was to determine the size of the effect of vitamin D supplementation in different ethnic groups.

We also noted that secondary hyperparathyroidism in our Asian participants at the baseline. Serum PTH levels were significantly higher in Asians when compared to Caucasians as a result of significant vitamin D deficiency. This is worrying as having a low vitamin D level and subsequent secondary hyperparathyroidism for a long period of time will lead to poor musculoskeletal health. What is worrying is that all our study participants are healthy young adult males, attending University in the UK and are expected to have awareness on the dietary and or supplementary vitamin D intake at least during the winter months for their bone health. It would be interesting to explore the impact in this group and also when they are excluded from the Asian group. But the numbers were too small to justify the analysis.

We conducted the study exclusively in young healthy males; hence our study findings may not be applicable to conditions associated with variation in DBP levels such as gender, pregnancy, liver disease and infections, and may not necessarily be applicable across all ages. We also included no functional outcome measures.

4.7 Conclusion

In an era of “precision medicine” we should be able to better target vitamin D treatment. Rather than adopting a “one size fits all” policy, we should aim to develop measurements that accurately reflect vitamin D status across all ethnic groups, and use these to guide treatment for relevant functional outcomes.

4.8 Acknowledgements

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Chapter 5: Conclusion, Summary and Future Direction

5.1 Conclusion

The work conducted as part of my PhD and presented in this thesis highlights that vitamin D deficiency can have an impact at any age or stage of life and features

1. the potential for "programming" the foetal skeleton with vitamin D supplementation in-utero, with possible effects on later bone health and fracture risk
2. the need for higher dose of vitamin D supplementation 1000 IU during pregnancy than the currently recommended dose of 400 IU by Public Health England and NICE guidelines
3. the understanding of the relationship between vitamin D deficiency and fracture risk in the context of possible inflicted injury
4. the consideration of free as well as bound measurements to assess vitamin D status and the consideration of ethnicity in determining the response to a single high dose of vitamin D

The summary and directions for future research for each study as below.

5.2 VIVID study

5.2.1 Summary

The in-utero environment influences the foetal bone development and future bone health (1). Bone is a dynamic tissue that undergoes constant modelling and remodelling in children (2). It is highly responsive to loading and adapts its structure and size when mechanically stimulated (3). Whole body vibration (WBV) can be used as one of the methods to deliver mechanical stimulation in both adults and children (4).

As outlined in chapter 2, researchers from our team have used WBV as a means for mechanical stimulation. Harrison et al. demonstrated that short periods of WBV increased bone turnover

measured by P1NP (+25.1%) and CTX (+10.9%) in healthy pre-pubertal boys (5). Subsequently she studied the differences in bone response to WBV between healthy pre-pubertal boys with and without a history of fracture. She presented her work at the International Conference on Children's Bone Health in June 2019. She demonstrated that short periods of WBV increased the bone formation marker P1NP by 25.1% and resorption marker CTX by 10.9% in boy who did not have a history of fracture when compared with their fracturing counterparts (6). Borg et al. demonstrated that antenatal vitamin D depletion substantially reduces the loading-dependent increase in both cortical and trabecular bone mass of offspring mice close to skeletal maturity (7).

With this background, we explored the responsiveness of the skeleton to mechanical stimulation by using WBV in children exposed to higher levels of vitamin D in-utero through antenatal vitamin D supplementation.

The key finding of our study as detailed in chapter 2 is

1. ***Antenatal vitamin D supplementation increased the response of bone to mechanical stimulation as demonstrated by a significant increase in the bone formation marker PINP in those children whose mothers received such supplementation during pregnancy.***

Animal studies have reported that enhancing vitamin D activity within the osteocytes may mediate anabolic activities during mechanical loading. And enhancing vitamin D activity in mature osteoblasts promote greater mineralisation as well as differentiation of mature osteoblasts into osteocytes leading to increased sensitivity to mechanical loading and bone formation (8)). Our study offers an insight into the mechanism by which antenatal vitamin D supplementation may be having an effect on postnatal skeletal health.

Our study is however limited by the small sample size since we only recruited participants from one of three MAVIDOS study (9) centres. These results do highlight the potential for exploring the impact

of mechanical stimulus on an antenatal programmed skeleton across the whole cohort. We have initiated discussions on collaborating with the Southampton group and are looking for funding opportunities to conduct this work across all three sites.

These studies offer new information in a variety of research areas pertaining to the role of Vitamin D on bone throughout the life span. A number of these findings e.g. effect of mechanical vibration on disease states offer clear routes for future research in this field and some of which we have already began to explore as highlighted as above.

In summary, the use of short periods of WBV as a mechanical stimulation test needs to be validated across a broader age range, but has potential as a method to assess the effects of both diseases and interventions on an important aspect of skeletal function – the appropriate response to mechanical stimulation.

5.2.2 Future Direction

In this study, we investigated the skeletal response to mechanical stimulation in children who were aged 4 and born to mothers who received high dose vitamin D (1000 IU/day) supplementation during pregnancy in the placebo controlled MAVIDOS study.

We assessed the skeletal response by measuring their biochemical markers of bone turnover following short periods of mechanical stimulation [5 days of whole body vibration (WBV)]. We undertook this work in a small group of children (n=31).

We would further recommend a much larger and a longer study to try and understand the mechanisms underpinning the role of maternal vitamin D on offspring bone response to mechanical loading.

This evaluation could include:

1. Confirming our results in a larger cohort with older children. We endeavour to expand this work to all 3 centres of the MAVIDOS trial (Sheffield, Southampton and Oxford) during the next follow-up study of 11-14 year old children. We have initiated discussions with the wider team. Studying this in a bigger cohort and older children would help us understand further if the observed effects of WBV on increasing the bone formation marker PINP would remain the same as well as the effect on other bone measurements such as sclerostin. Of note, the most recent MAVIDOS 4 year follow-up study has just been accepted for publication by JBMR plus.

2. We would also recommend the measurement of PINP at 42 days (6 weeks) following WBV. Recent work undertaken by a colleague of our team on 'the effects of bisphosphonate treatment on skeletal response to mechanical stimulation in children with osteogenesis imperfecta' has demonstrated a significant increase in both PINP and CTX on day 8 and day 42 following a week of WBV and 5 weeks of monitoring without an intervention (10). WBV was delivered using the same platform, settings and protocol used in our study but for 7 consecutive days instead of 5 days like in ours. We reported that the initial increase in PINP and CTX after 7 days of WBV may indicate increased osteoid formation within the existing remodelling units but not increased mineralisation. It would be good to study, observe and prove similar anabolic effects by undertaking this study for a longer period of time to establish the robustness of these interpretations and determine the duration and magnitude of these effects.

3. We would also recommend measuring the functional outcome in respect of the response to whole-body vibration before and after vitamin D dosing by assessing physical performance such as short physical performance battery and handgrip muscle strength in further studies.

5.3 Vitamin D fracture study:

5.3.1 Summary

Diminished bone strength resulting from vitamin D insufficiency is being considered as an alternative mechanism for suspected child abuse in infants and young children with fractures (11) (12). However, evidence is lacking to support the notion that low vitamin D levels per se cause fracture (13).

Conversely, although it is said that a vitamin D level low enough to weaken and/or fracture a bone will be accompanied by altered bone biochemistry and rachitic radiological changes (14); evidence in support of this is lacking, with published studies either not designed to address the specific question (15) (16) or being expert opinion rather than original research British Paediatric and Adolescent Bone Group's position statement on vitamin D deficiency (17). The British Paediatric and Adolescent Bone Group [BPABG] issued a consensus statement in the context of suspected child abuse that the definition of VDD should only pertain to the sequelae of vitamin D on the skeleton and that isolated VDD not be accepted as the cause of unexplained fractures in children in the absence of biochemical and/or radiological rickets (17).

Physical abuse accounts for 12% of the several forms of child abuse as reported in the National Institute of Health and Care Excellence (NICE) guidelines on child abuse and neglect (18). Physical violence against children has several cost implications such as health costs, social services and legal services expenditure (19). A 2004 study by Rovi et al., to estimate the economic burden of hospitalisations associated with child abuse and neglect, reported that children diagnosed with child abuse or neglect were at significantly greater risk of dying during hospitalisation (4% vs 0.5%) and incurred double the hospital costs ($19266 vs $9513) of children hospitalised for other causes (20). The national average unit cost for non-elective admissions for childhood maltreatment or violence-related injuries in the UK was estimated to be £7,405,733 in 2015 (21). The central estimate of

discounted average life-time cost of non-fatal child maltreatment (including physical abuse) per victim in the UK in 2015 for criminal justice system costs incurred by perpetrators was £4316 (with a 95% certainty that the costs fall between £2500 and £6,165) and 31.2% of the victims were estimated to have a readmission which was associated with 11.5 outpatient and General Practice visits (22). Any debate in elucidating the aetiology of fractures in these patients may prolong hospital admission and will prolong the judicial process, further increasing the overall costs. Finally, the emotional distress experienced by the victims and their families during this entire process is immeasurable. Therefore, it is crucial to find a definitive answer to the issue of VDD and unexplained fracture in the absence of radiological rickets.

The key finding of our retrospective vitamin D fracture study as outlined in chapter 3 is

Infants with vitamin D deficiency but not radiological evidence of rickets do not have increased risk of fractures.

These findings add to the growing evidence on this highly sensitive subject matter and should play a crucial role in investigating and managing infants with unexplained fractures.

It is well recognised that radiology is more sensitive in identifying established rickets than it is in identifying the early changes of VDD (23) (24), and osteopenia can be recognised on radiographs only once a bone has lost a third of its mineral content (25).Therefore assessment of bone density using radiographs is insensitive and unreliable (26).None of the study participants had a dual-energy X-ray absorptiometry (DXA) scan, and we assessed their bone density solely from radiographs. Nevertheless, interobserver reliability was very good and given that DXA measurements may be more prone to movement artefact, and that there is limited normative data for the current generation of fan beam instruments for the “at risk” population i.e. those less than one year old. Thus, DXA is not routinely used in current clinical practice to measure bone mineral density in infants with unexplained fractures. We therefore simulated current clinical practice in this scenario.

5.3.2 Future Direction

Through this retrospective study, we reported that in children less than 2 years of age with unexplained fracture(s), isolated low serum vitamin D is non-contributory to the aetiology of the fracture(s). But a much broader evaluation would be recommended to provide more robust evidence to inform the influence of vitamin D status in both clinical management and legal deliberations in cases of suspected inflicted injury in children.

This evaluation could include several features such as

1. Conducting a larger and prospective multi-centre trial to establish the prevalence of rachitic changes and/or fractures in UK infants in vitamin D sufficiency and insufficiency and their relationship. The current streamlined guidelines for the management of rickets and fractures in infants and children would help with more accurate and relevant biochemical data acquisition for bone metabolism such as serum PTH and alkaline phosphatase.

2. Determine a child's fracture risk in the case of mild vitamin D deficiency versus that in florid clinically and radiographically evident rickets.

3. Determine the degree of vitamin D deficiency sufficient to cause deleterious skeletal effects (and what sort), and hence predispose to if any, fractures (what type)

4. Compare radiographic appearance of rickets with biochemical markers of bone metabolism and conclude their relationship, if any.

5. Professor Amaka Offiah's PhD student is currently working on a post-mortem CT/MRI study with pathologists and engineers. We will be developing finite element models of bones and correlating bone strength from these models with vitamin D levels in sudden unexplained death in infants. We hope to identify some infants with frank rickets through this collaborative work between Sheffield, Nottingham, Birmingham and Leeds. Preliminary results of this proposed small study (20 infants)

would then inform a larger prospective study in live children to help understand the points outlined in 3 and 4.

5.4 Vitamin D dosing study

5.4.1 Summary

The exponential rise in vitamin D testing and treatment in the current financial climate of the NHS is a real cause for concern. There is increasing recognition that individual characteristics and genetic makeup influence the response to vitamin D supplementation.

The key findings of our vitamin D dosing study (27) as outlined in chapter 4 are

1. ***A single high dose of vitamin D3 (150,000 units) is safe and effective for treating vitamin D deficiency in adults***

We demonstrated an increase of serum total 25OHD levels >50 nmol/L from baseline following the administration of 150000 IU of Vitamin D3. The mean increase in serum total 25OHD (nmol/L) following treatment was similar in both ethnic groups (white Caucasians 56.7 and Asians 56.4). Our findings demonstrate that single high dose of 150,000 IU of vitamin D3 is safe and effective in maintaining appropriate levels of serum total 25OHD levels without inducing hypercalcaemia or hypercalciuria irrespective of baseline serum total 25OHD levels even up to 120nmol/L.

Similarly, Niet el al randomised 60 subjects (male and female, aged between 18 and 55 years of age), with vitamin D insufficiency (25 - 50 nmol/L) to either receive 50,000 IU of vitamin D3 monthly for 3 months or 20,000 IU daily to reach a cumulative dose of 150,000 IU. The primary of the study was to achieve a target serum total 25OHD concentration of 20 ng/ml (50 nmol/L). The authors found the subjects in the daily group took 14 days to achieve the target Vitamin D concentration when compared to what the monthly group achieved in a single day (p=0.02). The authors reported a monthly, larger

dose of vitamin D administration is more effective, rapid and safe in normalising the vitamin D status. Similarly, other studies have reported the safety of large doses of vitamin D therapy (27). Binkley et al. reported that monthly administration of 50,000 IU of vitamin D for a year (cumulative dose 600,000 IU) is safe and effective in adults (29).

As discussed in chapter 1, Sanders et al reported a rise in serum total 25OHD concentration from a median baseline serum concentration of 49nmol/L to 120 nmol/L at 1 month following a single annual dose of 500,000 IU in older women (30). Similarly, Bischoff-Ferrari et al reported that higher monthly doses (60, 000 IU) of vitamin D were effective in reaching a threshold of at least 30ng/mL of serum total 25OHD concentration in a one year, double-blind randomised clinical trial conducted in 200 community dwelling elderly population (31). However, both these were conducted in a much older population where there is a pre-existing risk of increased fragility fractures and reported increased risk of falls and fractures. We can speculate that increased risk of fractures following high dose vitamin D supplementation in this age group may well be due to an improvement in their functional outcome resulting in increased activity levels. Nonetheless, these findings help devise a simple, safe and adherent management framework for vitamin D treatment that are not only based on scientific evidence but are also cost and time effective to be implemented in all healthcare settings, starting from primary to quaternary care.

2. Asians had a higher increment in free or bioavailable vitamin D levels following vitamin D supplementation when compared with their White Caucasian counterparts.

The contribution of ethnicity, skin colour, and vitamin D binding protein (VDBP) genotype is not something that is being considered in current clinical practice. We found significantly lower levels of serum total 25OHD and VDBP levels in Asians when compared to white Caucasians but similar free or bioavailable 25OHD levels at baseline. However, following vitamin D supplementation, we found a

higher increment in serum free 25OHD levels in Asians when compared to white Caucasians (27). This suggests a variation in VDBP binding affinity between various ethnic groups and measurement of the free or bioavailable form of serum 25OHD concentration may be more meaningful than measuring the serum total 25OHD levels when considering treatment with vitamin D. Our study is the first interventional study studying the effects of vitamin D on serum total and free 25OHD levels and VDBP concentration (27). Ours is a pilot study, conducted in a small age-limited and gender specific population and our results may not be applicable to the general population. Further research focusing on defining vitamin D status based on an individual's ethnic and VDBP genotype status across all ages and genders in a larger population is warranted for the establishment of targeted and personalised management strategies for vitamin D deficiency in the UK. More so, because of the limited sun source of vitamin D during the winter months and the growing changes in ethnic diversity of the UK population. Recent data (32) shows that Asian ethnic groups make up the second largest percentage of the population [7%] followed by African and African Caribbean origin [3%]. Asians are at risk of vitamin D deficiency due to their suboptimal dietary intake of vitamin D and socio-cultural practices such as sun-avoidance, and modest clothing. Darling et al conducted a large cohort study to analyse the vitamin D intake of UK South Asian adults using data from the UK Biobank. They reported a very low dietary vitamin D intake in this population (1-3 micrograms per day). This is well below the recommendations from the European Food Safety Authority (15 micrograms per day) and Public Health England (10 micrograms per day). Equally use of vitamin D supplementation is also low in this population with a higher percentage of vitamin D intake in Greater London (35 %) when compared to the rest of the country (18-25%) (33). In addition, darker skin complexion of individuals from South Asian, African and Afro-Caribbean origin puts them at risk of Vitamin D deficiency. Our study findings provide an insight into the consideration of ethnicity, skin colour and measurement of both total and

bioavailable vitamin D levels to assess vitamin D status. This calls for further studies in a larger population across all age and ethnic groups.

5.4.2 Future Direction

1. Our study addressed the effect of a single high dose vitamin D3 (150,000 IU) on serum total 25OHD, DBP and free 25OHD concentration in a small cohort of young adult males where we had insufficient statistical power to demonstrate the influence of skin types or DBP genotypes on the response to vitamin D3. We would recommend studying that effect in a bigger cohort in different ethnic groups incorporating the variation in DBP levels such as gender, pregnancy, liver disease and infections across all ages.

2. Equally we found a single high dose of 150,000 IU of vitamin D3 to be a safe dose for young adults. But there are more lessons to be learnt in terms of safety of high dose vitamin D supplementation. We would recommend looking at whether there are ethnically determined differences in calciuria in further studies which would also require consideration of the background dietary and supplemental calcium intake.

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Appendices

Appendix 1 VIVID study PPI report

RDSYH Public Involvement Fund

Award Report

Does antenatal vitamin D supplementation influence bone's postnatal acute response to mechanical stimulation?

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Introduction

We propose to determine whether vitamin D supplementation during pregnancy alters later response of bone to whole body vibration.

Fractures in children are common and the incidence is increasing. They are more common in children who have small, narrow and weak bones. Studies have shown that fractures in early childhood are associated with later bone strength.

Regular loading of bone during childhood has been shown to produce a consistent increase in bone strength and may reduce the risk of fracture in later life. Loading is achieved in the form of regular sport activities such as gymnastics and exercise programmes that can lead to increase in size and the quality of the bones at the hip, spine and pretty much the whole body. Studies have shown that standing on a vibrating platform for 10 minutes a day for 5 days in a row can make bones stronger which means they break less easily.

Contribution of vitamin D to fracture rates during childhood has been difficult to assess. Role of vitamin D is to increase the absorption of calcium from the gut. Low calcium is associated with an increased risk of fracture. Current Department of Health advice is targeted at the prevention of rickets, rather than altering fracture risk during childhood or later.

Maternal Vitamin D Osteoporosis Study (MAVIDOS) was a large study conducted recently at 3 different big centres (Sheffield, Southampton and Oxford). Results from this study have shown that giving a higher dose of vitamin D to pregnant women everyday from 14 weeks of pregnancy increased the strength of the bones in their infants. The same group of researchers have previously shown a reduction in bone strength in boys aged 8-9 years whose mothers had lower vitamin D levels during pregnancy or at the time of delivery.

Hence the role of diet and mechanical loading are of considerable interest in determining their role in skeletal health and the prevention of fractures.

We therefore propose to study the response to whole body vibration in children whose mothers participated in the MAVIDOS study in Sheffield. These children will be 4 years of age when they participate in this study. From our previous experience of conducting a similar study in children aged between 9 and 11 years of age, we know that children tolerate it very well.

Consent for the study will be obtained at an appointment prior to commencing the intervention. As the platforms are transportable, study visits will take place in the participant's own home or school. Participants will stand on the vibrating platform daily for 10 minutes on 5 consecutive days. Vibration is delivered in 4 short cycles of 2 minutes each, separated by 30 seconds rest, for 10 minutes each day. Delivering vibration in this pattern will allow the participants to get used to the platform in a more comfortable manner. Blood samples will be collected immediately before the vibration on day 1 and day 8 following an overnight fast. The researcher will be present at all study visits to instruct participants the use of the devices.

The results of this study will help inform public health policy on vitamin D intake during pregnancy. This will also help us identify a possible risk factor for poor bone health in children. The results will have immediate impact in terms of reinforcing new and existing advice on vitamin D supplementation.

Aim of the public involvement

The aim of the focus group is to

Find out about mothers' feelings and attitudes to tests being done in children aged 4-5 years for research.

This will help make sure our study is appropriate and acceptable to conduct in young children.

Method

We conducted a patient and parent involvement event in February 2016 to which we invited mothers with children (aged 4-5years old). These mothers were contacted via Sheffield Children's Hospital intranet and University of Sheffield's announcement composer. All respondents were sent an invitation letter and information poster about the event and were asked to contact Dr Gopal (chief investigator) if they wished to participate.

Six mothers were recruited and a parent attended with her 3 year old son. The event was held in a meeting room in the Clinical Research Facility at Sheffield Children's Hospital and it lasted 60 minutes. The session was jointly led by Dr Gopal (the researcher) and Ms Spooner (an experienced group facilitator) and key points of the conversations were recorded on a flip chart. Refreshments were provided, and mothers received £20 travel expenses.

Prior to the start of the discussion, we explained the purpose of the session as explained above. It was made clear how the information that group members shared would be used and that any personal information disclosed would be confidential and only shared in an anonymised format. The aim of this was to enable people to speak as freely and comfortably as possible and without fear of consequences. We engaged everyone in an introductory activity to help people feel at ease prior to a group discussion structured to elicit mothers' views and opinions on the study method, design, participant and parent information leaflets and consent forms.

Following the activities, the plans for future patient/parent involvement in the research project were explained and participants invited to join the project steering group. Prior to leaving, all participants were asked to complete a modified version of the RDSYH PPI evaluation form.

Parent contribution

The primary focus of the event was to help us better understand parents' experiences and opinions about children being recruited into research studies.

A common theme throughout the session from all the mothers were to conduct the study visits at home to reduce amount of time for children to be without food and make it less stressful for child, with minimum disruption to the family's morning routine. This confirmed our assumption that participants would like the option of conducting the study visits at home. However in the interest of other participants, we included in the information sheet for the study visit to be conducted either at home or school depending on family's choice. If it takes place at the school, prior permission will be sought from the head of the school.

Suggestions were made to include the vibration are with breaks (2.5 min, 30 sec break) as mentioned in the protocol and to make it clear that the children has to fast from midnight only on day 1 and day 8, when the blood tests are taken in the morning at a time convenient to the child and the family. The rest of the days are just standing on the vibration plate for 10 minutes in the morning. Also to mention that parents to use their own distraction strategies such as watching TV, listening to music or reading a book etc, for children to stand on the vibration platform These were felt appropriate and amendments made as necessary.

Mothers also felt strong about using the appropriate numbing spray or cream for an adequate time prior to the blood test. Some mothers felt that the information provided in the information sheet as ' a light breakfast will be provided to the child following a fasting blood test ' should be changed to ' breakfast will be provided to the child following a fasting blood test' as most children will need more than a snack to survive school until lunch time, which we felt was entirely appropriate and made changes accordingly.

When summarised, the key outcomes from the session were that:

Fasting blood test to be done as first thing in the morning

Study visits to be conducted at home

Make sure skin is made numb prior to the blood test

Mothers to adopt their own distraction strategies while the child is standing on the vibration platform

Difficulties

We found it difficult to have a detailed discussion within the allocated time frame of an hour; hence we decided to conduct the future meetings for 1.5 – 2 hours, which was also acknowledged by the members.

Evaluation of involvement

All mothers who participated in the event completed an evaluation form. The feedback received showed that all participants understood how their contribution was to be used in the research and 4 out of the 6 participants understood how this would make a difference to the research. Four out of the 6 participants said they enjoyed the experience and all would be willing to take part in future PPI activities. A copy of the modified evaluation form can be found in Appendix 1 and the completed forms are enclosed with this report.

Future patient involvement

Members of the public have an important role to play in our study. We plan to co-opt 3-4 mothers onto the project steering group (PSG). The PSG will meet every 3-4 months for the lifetime of the project (3 years). They will therefore be fully involved in the management of the project and will be able to bring a different perspective to that of the researchers and health professionals involved.

These activities will include designing and reviewing project information and creating and implementing a strategy for disseminating project outcomes through innovative means such as social media as well as the more traditional approach with posters and written information.

PPI expenditure

Travel expenses (including parking if required) £20 per family x 6 families = £120, **Refreshments** £ 5

Tot

Appendix 2 VIVID study protocol

Sheffield Children's NHS
NHS Foundation Trust

FULL STUDY TITLE
Does antenatal vitamin D supplementation influence bone's postnatal response to mechanical stimulation?

SHORT STUDY TITLE
Does vitamin D alter bone's response to vibration?

STUDY NUMBER

DATE AND VERSION NUMBER
(07.03.2016, V1.2)

LAY SUMMARY

Fractures in children are common and the incidence is increasing. They are more common in children who have small, narrow and weak bones. Studies have shown that fractures in early childhood are associated with later bone strength.

There are several (i) non-modifiable factors such as age, gender, race & genetic make-up and (ii) modifiable factors such as nutrition (vitamin D & calcium intake), lifestyle, body weight and exercise that can contribute to bone strength.

Low calcium is associated with an increased risk of fracture. Vitamin D plays a pivotal role in bone health by increasing the absorption of calcium from the gut. We know from the previous research that there is a reduction in bone strength in children whose mothers had lower vitamin D levels during pregnancy. Current Department of Health advice is targeted at the prevention of rickets, rather than altering fracture risk during childhood or later.

Bone growth can also be achieved by loading of bone during childhood in the form of regular sport activities such as gymnastics and exercise programmes. Equally it can be achieved by using whole body vibration (WBV). WBV is the application of vibratory stimulus to the body in a synchronous fashion by which the bones are made much stronger reducing the risk of fracture in later life. Thus WBV can be used as a means to assess bone responsiveness to mechanical stimulation. Studies have shown that standing on a vibrating platform for 10 minutes a day can significantly increase the bone mass. Our own research has also shown that healthy boys when made to stand on a vibration platform for 10 minutes daily for 5 days increased the strength and quality of their bones.

Thus the role of diet and mechanical loading are of considerable interest in determining their role in bone health and the prevention of fractures.

Maternal Vitamin D Osteoporosis Study (MAVIDOS) is a large study conducted recently at 3 different big centres (Sheffield, Southampton and Oxford). Results from this study have shown that giving a higher dose of vitamin D to pregnant women every day from 14 weeks of pregnancy increased the strength of the bones in their infants. In the proposed study we aim to show how vitamin D supplementation during pregnancy affects the response of bone to vibration in children whose mothers participated in the MAVIDOS study in Sheffield. These children will be 4 years of age when they participate in this study. From our previous experience of conducting a similar study in children aged between 9 and 11 years of age, we know that children very well tolerate it.

The results of this study will help inform public health policy on vitamin D intake during pregnancy. This will also help us identify a possible risk factor for poor bone health in children.

GENERAL INFORMATION

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GLOSSARY

WBV – Whole body vibration

PINP - Pro-collagen type 1 N-terminal propeptide

CTx - C-terminal cross-linked telopeptide of type I collagen

1.0 BACKGROUND

This project aims to determine whether antenatal vitamin D supplementation alters postnatal bone formation in response to mechanical stimulation.

Osteoporosis and fracture risk:

Osteoporosis is a major public health concern and is caused by the loss of bone mass and architectural deterioration with subsequent predisposition to fracture. It accounts for nearly 300,000 fractures in the UK each year (Book, 2007). These fractures typically occur at hip, spine and wrist (van Staa, Dennison, Leufkens, & Cooper, 2001). Nearly £1.7 billion of the NHS budget in the UK was spent on osteoporosis annually a decade ago, (Harvey N, 2004); current estimates are £3.4 billion(Hernlund et al., 2013). Peak bone mass, the amount of bone mass acquired by the end of skeletal maturation, predicts the risk of osteoporosis in later life (Heaney et al., 2000; Shaw & Mughal, 2013). Failure of peak bone mineral accrual in early life is associated with an increased risk of osteoporosis in later life (Hernandez, Beaupre, & Carter, 2003).

Peak bone mass depends on both genetic and environmental factors such as diet, exercise and sex steroid status (Bachrach, 2001). Although targeted interventions with Vitamin D in later life might stall or reverse the symptoms of osteoporosis to some extent (Verschueren et al., 2011) existing evidence does not support the sole use of vitamin D in fracture prevention (Abrahamsen B, 2010), possibly due to either low vitamin D levels at baseline or inadequate treatment doses (Reid, Bolland, & Grey, 2014).

Maternal vitamin D status and offspring bone mineral accrual:

Maternal vitamin D status is an important determinant of offspring bone development (Harvey et al., 2012). Lower maternal vitamin D levels during pregnancy and at birth are associated with reduced bone width and mass in the offspring aged 8-9 years, increasing their risk of fracture (Javaid et al., 2006). The Southampton Women's survey has also reported a positive correlation between maternal vitamin D status and neonatal bone mass (Harvey et al., 2008). The Maternal Vitamin D Osteoporosis Study (MAVIDOS) is a large-scale study conducted recently at 3 different tertiary centres (Sheffield, Southampton and Oxford) (Harvey et al., 2012). Results (unpublished data) from this study have shown that supplementation with 1000 units of vitamin D daily from 14 weeks of pregnancy increased bone mass at birth in winter/spring-born infants. Taken together, these data suggest that antenatal depletion of vitamin D alters the normal response of the offspring's skeleton to factors that influence its size, shape and mass post-natally. We recently undertook a study to determine the effect of reducing vitamin D intake during pregnancy and early life on the skeleton's response to mechanical loading using a mouse model. Our unpublished data show that antenatal vitamin D depletion substantially reduces the loading-dependent increase in both cortical and trabecular bone mass of offspring mice close to skeletal maturity.

Mechanical stimulation of bone:

The skeleton responds to mechanical stimulation by an increase in bone size and mass (Ref Rachel paper 9) and that increment can persist into adult life (Ducher, Daly, & Bass, 2009). In contrast, without mechanical stimulation, bone is lost. The mechanostat theory (Harold Frost) suggests that bone responds to the mechanical forces acting upon it by increasing or reducing mass until a state of equilibrium is reached in which bone mass and architecture are appropriate to the continuing demands placed upon it. As part of the proposed system there is a sensing mechanism that has a set point – akin to a thermostat in a heating system – that switches the system on and off. The available human and mouse data suggest that antenatal vitamin D deficiency in some way influences the determination of this set point, making bone less responsive to mechanical stimulation.

We have recently shown in healthy boys aged 9-11 years, whole body vibration experienced 10 minutes daily for five days increases the bone formation marker Pro-collagen type 1 N-terminal propeptide (PINP) by 25% and the bone resorption marker C-terminal cross-linked telopeptide of type I collagen (CTx) by 11% (Harrison et al., 2015). We can use this approach to assess the responsiveness of the skeleton to mechanical stimulation in the children whose mothers participated in the MAVIDOS study.

Study proposal:

In the proposed study we will use whole body vibration to elicit bone biomarker responses in children (n=56) whose mothers participated in the MAVIDOS study in Sheffield. These children are now aged between 2.5 and 3.5 years and will be between 4 and 5 years of age when they take part in the study. We believe that results of this study will help inform public health policy on vitamin D supplementation during pregnancy and also help identify a possible risk factor for sub-optimal bone health in childhood. The results will have immediate impact in terms of reinforcing new and existing advice on vitamin D supplementation.

2.0 STUDY OBJECTIVES AND PURPOSE

The main aim of the study is to investigate how antenatal vitamin D supplementation alters postnatal bone function in response to mechanical stimulation. Our hypothesis is that children whose mothers received vitamin D supplementation during pregnancy in the MAVIDOS study will show a greater increase in the bone formation marker P1NP in response to whole body vibration than children whose mothers did not receive supplementation, after adjusting for confounding factors such as vitamin D at baseline, season, gender, body size and age.

In the MAVIDOS study mothers whose baseline 25OH-vitamin D was >25nmol/l were randomised to receive either 1000 IU of vitamin D or placebo from 14 weeks of pregnancy. Infants had measurements of growth and bone mass undertaken at or close to the time of birth, and continue to be followed in the three participating centres.

We plan to measure (i) Pro-collagen type 1 N-terminal propeptide (PINP) and alkaline phosphatase [markers of bone formation] and (ii) C-terminal cross-linked telopeptide of type I collagen (CTx) [marker of bone resorption] (iii) Osteoprotegerin (OPG) [factor affecting bone resorption in serum. Measurement of these bone biomarkers will allow us to identify if the response of bone to vibration is influenced by antenatal vitamin D intake. A reduction in responsiveness would help explain the findings from the previous observational studies of reduced bone size and mass in children whose mothers had lower vitamin D levels during pregnancy and at delivery, and would be consistent with our murine data. Finding no difference between the groups would suggest that pathways involving mechanical loading in the growing human skeleton are not influenced by antenatal vitamin D exposure.
This study will be undertaken as part of a research-training programme (PhD).

3.0 STUDY DESIGN

This is a prospective single centre interventional study. Children of mothers (n=56) who participated in the MAVIDOS study will be eligible to take part. These children will form two groups; those whose mothers did (n=29), and those whose mothers did not receive antenatal vitamin D supplements (n=27). Power calculations suggest that 20 subjects (10 in each group) are sufficient to test the hypothesis that there will be a difference in the increase in pro-collagen type 1 N-terminal propeptide (P1NP) following vibration between the groups with 90% power at the 5% significance level. A validated questionnaire regarding exercise (Godin & Shephard, 1985) will be conducted to determine the amount of physical activity undertaken over the 7 days prior to standing on the platform and then again on day 8 to determine undergoing WBV has had an effect on participant's levels of physical activity. Fracture history will be recorded to include the date of fracture(s), site and degree of trauma. Assessment of dietary intake of vitamin D and calcium will be undertaken using a food frequency questionnaire. If any safeguarding issues become apparent, appropriate procedures will be undertaken as per the hospital policy. Basic anthropometry: height (to next 1mm by wall-mounted stadiometer), weight (wearing vest and pants to nearest 0.1 kg by electronic balance scales) will be measured and used to derive body mass index (BMI) and body surface area (BSA).
Participants will stand on a low magnitude vertical vibrating platform (LivMD) for 10 minutes on 5 consecutive days. From our previous experience we know that children could tolerate this well and we would also encourage them to hold the back of a chair or their parent for support. The period of vibration will be delivered in 4 cycles of 2 minutes 30 seconds each, separated by 30 seconds off the platform, providing 10 minutes of vibration in total each day. We would request the parents to adopt their own distraction strategies such as reading a book, watching television or listening to music etc. to engage their children while standing on the vibration plate.

Delivering vibration in this pattern will allow the participants to become accustomed to the platform in a more comfortable manner. Additionally it has been demonstrated that insertion of rest periods enhances the anabolic effect of loading on bone.

Fasting blood samples will be collected following an overnight fast immediately pre-vibration on day 1 and again on day 8. Biomarker investigations will be bone turnover markers (P1NP, CTx, OPG by Roche chemiluminescence immunoassay, automated chemiluminescence immunoassay and enzyme-linked immunosorbent assay (ELISA) respectively) serum 25OHD (tandem mass spectrometry assay), calcium, phosphorus, alkaline phosphatase, magnesium (colorimetric, by Elecsys), and parathyroid hormone (Abbott Architect immunoassay) will be performed.

As the platforms are transportable, study visits will take place in the participant's own home or school. The first study visit will take 45 minutes for collection of blood samples and vibration; 20 minutes on days 2 - 5 when the visit includes only vibration, and 15 minutes on day 8 when the final blood sample is collected. Each participant will complete 6 visits over 8 days. Breakfast will be provided to children on the days when the fasting bloods are taken.

The researcher will be present at all study visits to instruct participants in the use of the devices and to ensure compliance with the protocol.

Finding of any clinical significance will be notified to the GP and arrangements for further follow-up with the general paediatrician will be made as appropriate. The remaining blood samples will be stored for future research on bone health in a non-identifiable way, for a maximum of 5 years with the participant's consent.

A Gantt chart detailing the timeline for the project is enclosed with this application.

SELECTION OF PARTICIPANTS

Inclusion criteria:

All children born to mothers who participated in MAVIDOS trial in Sheffield free from any condition affecting bone health, general nutrition, growth and glucose metabolism will be invited to enrol in the study.

Exclusion criteria:

Children with (1) balance problems, (2) current or healing fractures (3) any chronic illness involving the bone, liver and kidney (4) current long-term use of steroids, anticonvulsants or any medication that might affect calcium and vitamin D metabolism will be identified from through a questionnaire and excluded.

4.0 PARTICIPANT RECRUITMENT

The principal investigator will contact families whose mothers and children have participated in the MAVIDOS trial by methods discussed and agreed by the patient and public involvement (PPI) focus group of this study. Information sheets detailing the study procedures and contact details for the study

team will be sent out to the families of potential participants who have expressed an interest in the study prior to their attendance. Parents and families will have the opportunity to discuss the study further with the research team prior to giving consent. It will also be made clear to the families that this study is an additional study to MAVIDOS, so they are free to decline without their participation in the core trial protocol being affected. Contact details of the PI will be provided for enquires. A minimum of 72 hours will be given for participants to consider their involvement. The PI or a specialist research nurse with appropriate experience and training in Good Clinical Practice will gain consent. Enrolment into the study will take in the Clinical Research Facility (CRF) at Sheffield Children's Hospital, the child's school or home where the study procedures will take place. A member of the research team will be present at each study visit and will witness compliance to the intervention. Participants are free to withdraw from the study at any time, without giving a reason. As this study involves 6 study visits over 8 days and no further follow up, participant withdrawal is expected to be minimal. The protocol has been designed to minimise the number of invasive procedures. Appropriate measures to numb the skin prior to venepuncture will be used. Study visits will be conducted in the participants' home or school for their convenience with the aim to reduce the number of withdrawals. Where two participants withdrew consent in the previous WBV study, this was due to cannulation difficulties. Each participant will be given a £25 voucher (on completion of the study). This amount is felt to suitably recognise the family's commitment without being an inducement. The reason for payment is in recognition of the time and dedication (fasting blood samples, engagement for approximately 3 hours altogether) that participation in this study requires. If the family has moved to another area, attempts will be made to contact the family to take part in the study. We expect recruitment to occur over a 3-month period.

5.0 DATA HANDLING AND RECORD KEEPING

The PI will be responsible for data collection, recording, quality and storage. Basic demographic details on participants to include: age, ethnic group, height, weight and calculated BMI, BSA on first attendance. Basic medical and medication history to confirm absence of exclusion criteria will be taken prior to consent. Data will be collected and retained in accordance with the Data Protection Act 1998. Data will be coded so that subjects are not immediately identifiable and double entered to maximize quality. Upon recruitment subjects will be allocated a unique identifying number, which will be used on all records. Any records with identifying information will be kept in a locked cabinet, within a locked office in the research department at Sheffield Children's Hospital. All data will be stored on encrypted media. Study documents (paper and electronic) will be retained in a secure location during and after the study has finished. All source documents will be retained for a period of 5 years following the end of the study.

6.0 ACCESS TO SOURCE DATA

The sponsor will permit monitoring and audits by the relevant authorities, including the Research Ethics Committee and the Medicines and Healthcare products Regulatory Agency (MHRA). The investigator will also allow monitoring and audits by these bodies and the sponsor, and they will provide direct access to source data and documents.

7.0 STATISTICAL ANALYSIS

Sample size was estimated (Stata V12.0 (Statacorp, Texas, USA) using the results from our previous WBV study done on healthy pre-pubertal boys, in which a mean difference of 175.6 (SD=162.7) in PINP between day 0 and day 8 was found. To detect 50% of the difference in PINP between children of mothers who were deficient in vitamin D versus those replete in pregnancy, at the 5% significance level with 90% power, would require 20 observations at day 8 (10 per group). We expect that of the 56 children born to mothers that participated in the original study, at least 70% will be willing to take part in this follow-up study. Allowing for a 15% drop out rate, we should recruit at least 32 children (16 per group), this would allow us to detect 50% difference in P1NP at 5% significance and >99% power if no one dropped out of the study.

Primary outcome: Difference in mean increase in the bone turnover marker P1NP between children born to antenatally vitamin D replete mothers versus antenatally vitamin D deficient mothers.

Secondary outcome: Change in CTx, OPG. Change in PTH. Safety outcomes.

Analysis will be performed using SPSS 22 and Data Desk™ v6.2.1. A two sample T-test will be used to compare the differences between the two groups and paired t-test to compare pre and post vibration values.

8.0 SAFETY ASSESSMENTS

Equipment:

There are no anticipated safety issues with the equipment. The platform has been extensively used in both clinical practice and sold 'over the counter' in countries within the EU including the UK. Minimal adverse events have bee reported in the use of vibrating platforms. In our previous study reported recently in JMNI (Harrison et al., 2015) children tolerated the intervention well with only mild effects being reported. A few participants were reported to have experienced itching type response in the feet and legs during the vibration and anxiety during cannulation. One subject felt dizzy whilst standing on the platform, and one felt faint after having blood taken – both issues resolved after subjects sat for a while.

Anticipated difficulties:

Families of mothers and children who participated in the MAVIDOS trial could have moved out of Sheffield making recruitment challenging. However all efforts will be made by the PI to contact the families by phone / email / post towards re-engagement /participation in this study.

It is possible that children could fall off the platforms (which are about the size and shape of a typical bathroom scales); hence we would encourage them to hold the back of a chair to steady them. Children and parents can become anxious towards cannulation. To overcome this we would apply appropriate cream to numb the skin prior to taking blood samples. We will also reassure the

participants and families that we would only have a maximum of 2 cannulation attempts to collect the blood samples per child per each visit.

9.0 ETHICAL CONSIDERATIONS

The study will be conducted in compliance with a Research Ethics Committee favourable opinion, including any provisions for Site Specific Assessment, and local Research and Development approval. The study will also be conducted in accordance with the International Conference for Harmonisation of Good Clinical Practice (ICH GCP), and the Research Governance Framework for Health and Social Care (2nd Edition).

10.0 FINANCE AND INDEMNITY

Participants will not be paid for their involvement in the study but will be offered a £25 voucher for reimbursement of their time and a voucher towards the cost of breakfast at the investigating site if they attend the CRF for any visits.

This is an NHS sponsored study. For NHS sponsored research HSG (96) 48-reference no. 2 refers. If there is negligent harm during the study when the NHS body owes a duty of care to the person harmed, NHS Indemnity will cover NHS staff, medical academic staff with honorary contracts and those conducting the study. NHS Indemnity does not offer no-fault compensation and is unable to agree in advance to pay compensation for non-negligent harm. Ex-gratia payments may be considered in the case of a claim.

11.0 JUSTIFICATION OF FINANCE

Fellowship applicant name	Jaya Sujatha Gopal-Kothandapani
Position and grade	Clinical Research Fellow

	Year 1	Year 2	Year 3	Total
Salary	44,034	44,915		£88,949
London Weighting				£0
Employers Costs (NI and pension contribution)	12,832	13,185		£26,017
Total	**£56,866**	**£58,100**	**£0**	**£114,966**

Consumables (exclusive of VAT) Do not include overheads or equipment.

Consumable	Year 1	Year 2	Year 3	Total
Blood tests (bone work-up)	11,039			£11,039
Sample storage and retrieval	120	120		£240
Printing costs	10			£10
Participant vouchers	1,250			£1,250
PPI costs	509	509		£1,018
Investigator travel	400	400		£800
Archiving costs		150		£150
Publication and dissemination costs		3,000		£3,000
				£0
Totals	**£13,328**	**£4,179**	**£0**	**£17,507**

Total amount requested **£132,473**

12.0 JUSTIFICATION OF RESOURCES

This study is to assess the responsiveness of the skeleton to mechanical stimulation in children whose mothers participated in the MAVIDOS study.

The consumables essentially reflect the costs of undertaking the biomarker assays (50 patients, 2 tests each, 100 tests in all; cost per sample P1NP £28; CTx £23; £51 x 100 = £5,100 plus the costs of consumables for sampling - needles, syringes, tubes; storing samples in the CRF); travel to and from patients homes and compensation for time spent by children and parents in participating in the study and dissemination costs. As detailed above, subjects will receive £25 voucher in order to compensate them for their time away from work or study, travel expenses and in recognition of the commitment the trial requires. Salary costs has been requested for the clinical research fellow undertaking this study as part of the Action Medical Research Training Fellowship scheme.

13.0 REPORTING AND DISSEMINATION

The results of the study will be reported in medical journals and will also be disseminated via the trust Research and Innovation (R&I) department and Children's Research Facility (CRF) website and in the R&D/CRF newsletters. We anticipate presenting results at national and international paediatric bone and endocrinology meetings. The study will also be presented at the Brittle Bone Society annual meeting. This work should also form the basis of a PhD.

14.0 IMPACT

The fundamental concept of this project is intrinsically linked with nation's health. The results of this study will help the clinicians to recognise children at risk for suboptimal acquisition of peak bone mineral accrual, and look at ways of intervention to optimise it for future prevention of osteoporosis. We expect the outcome of this study will have an immediate impact in terms of reinforcing new and existing advice on vitamin D supplementation during pregnancy in the UK and a long-term impact on future public health policies and guidance in this area across the globe.

15.0 OUTCOMES

We anticipate that we would be able to apply to a range of disease-specific national funders in the charitable sector; impaired skeletal health has been described in many disorders, including cystic fibrosis, diabetes, inflammatory arthritis, endocrinopathies, inherited metabolic disease, following the use of steroids and other drugs, as well as in inherited skeletal diseases. NIHR, Wellcome and MRC funding could also be sought for such studies, as well as more mechanistic studies to determine the pathways being activated during the WBV process. There is also the possibility of seeking pharma support for the studies in patients undergoing new treatments particularly in the area of rare skeletal disease, e.g. assessing the response to WBV in untreated mild hyphophosphatasia.
Initial applications are already under review with SPARKS and Children's Charity. Submissions to NIHR RfPB and Arthritis Research UK are planned in respect of the effects of steroids and methotrexate in inflammatory conditions.

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APPENDIX:


Study Design
Research team contact all potential participant's families
Families contact research team for more information
Check potential participants meet the inclusion criteria and send out information sheets
Follow-up contact to arrange study visit at home / school for consent
Day 1
Demography, auxology, exercise questionnaire
Fasting bone turnover markers & whole body vibration (WBV)
Day 2-5
Whole body vibration
Day 8
Fasting bone turnover markers & exercise questionnaire

Appendix 3 VIVID STUDY PARTICIPANT DOCUMENTS

Sheffield Children's NHS
NHS Foundation Trust

Participant study number:

PARENT/LEGAL GUARDIAN CONSENT FORM

Title of project: Does antenatal vitamin D supplementation influence bone's postnatal response to mechanical stimulation?

Name of researchers: Dr Suji Gopal / Professor Nick Bishop

	Please initial box
I confirm that I have read and understand the information sheet dated, 24.02.2016 (version 1.2) for the above study. I have had the opportunity to consider the information ask questions and have had these answered satisfactorily.	☐
I understand that my child's participation is voluntary and that I am free to withdraw my child at any time, without giving any reason, without my child's medical care or legal rights being affected.	☐
I understand that relevant sections of any of my child's medical notes and data collected during the study, may be looked at by researchers and those involved in the running and supervision of the study from Sheffield Children's NHS Foundation Trust.	☐
I give permission for the regulatory authorities, to have access to my child's records where it is relevant to my child taking part in research.	☐
I agree to my child having blood test to be done for this study after an overnight fast i.e. not giving the child anything to eat overnight until the blood samples are taken first thing in the morning either at my home or my child's school.	☐
I agree for my child's remaining blood samples to be stored for future research on bone health in a non-identifiable way, for a maximum of 5 years.	☐
I agree to my child's GP being informed of participation in this study.	☐
I agree to my child taking part in the above study.	☐

Name of Parent/Guardian	Date	Signature

Name of Person taking consent	Date	Signature

Sheffield Children's NHS
NHS Foundation Trust

FFQ for Rapid Assessment of Dietary Calcium Intake
MAVIDOS-VIVID study

Name:

Product	Total Daily Intake	Calcium Intake
Milk 1ml=1mg		
Hard Cheese (1 ounce = 200mg)		
Yoghurt (1 medium 100-150-gram pot): = 150 mg		
Bread 1 Slice = 35 mg		
Vegetable Bowl (250 ml): = 80mg		
Total Daily Calcium Intake		

Sheffield Children's NHS
NHS Foundation Trust

FFQ for Rapid Assessment of Dietary Calcium Intake
MAVIDOS-VIVID study

Name:

Product	Total Daily Intake	Calcium Intake
Milk 1ml=1mg		
Hard Cheese (1 ounce = 200mg)		
Yoghurt (1 medium 100-150 gram pot): = 150 mg		
Bread 1 Slice = 35 mg		
Vegetable Bowl (250 ml): = 80mg		
Total Daily Calcium Intake		

Sheffield Children's NHS
NHS Foundation Trust

7 day diary of all food and drink consumed

HOW TO USE THIS RECORD BOOK
Please keep this diary of EVERYTHING you eat and drink over 7 days. You will be told which days to keep the diet record.

Please remember to:
Start each day with a new page, and at the top of each new page, write down the date and the day of the week.

Use as many pages as you need for each day, and use as many lines as you like for each meal or part of a meal or snack.

Remember to include everything you eat or drink (including tap water and bottled water), and also any 'non' food items, such as vitamin pills, cod liver oil etc.

At the end of each day, please check that you have not forgotten any snacks or drinks, even the smallest item. For example a single polo mint, is very important!!!!

Please remember to eat and drink as you would normally do so.

All information that we collect about what you eat is confidential and there are no right or wrong answers. The more detailed your food record the better!

Please see the example given of a typical food diary entry

Food Portion Booklet:

- The food portion booklet contains photographs of some foods that can be used to help you to estimate the portion size.
- Refer to the contents page of the food portion booklet for an alphabetical list of the foods used in the photographs.
- Find the page, then simply select the photograph which best resembles the amount of food prepared. Write the photograph number in the portion size column (Column D).
- This is to help us estimate your portion size but it is not essential that you use it, so do not worry if your food is not found in the food portion booklet.

HOW TO DESCRIBE THE FOOD AND DRINK

Column A: Write down the time when food or drink was eaten.

Column B: List all of the food items, individually, that form your meal or snack. PUT EACH ITEM ON A SEPARATE LINE.

Column C: Describe each food item. Please give as much information as possible – type of food, brand name, and how it was cooked.

Column D: Please give an approximate size or weight of the food or drink portion. If possible, please use the blue food portion booklet that has been provided to help you to decide the estimated weight of your food portion.

E.g. A bowl of cornflakes:

On page 6 of the food portion booklet there are four photographs numbered 45-48 of varying amounts of cornflakes in a breakfast bowl.

Choose the photograph which best represents the amount you have prepared and record the photograph number in column D.

Please do not forget to add the amount of milk you have used.

E.g. ¼ pint of Semi-skimmed milk or 150ml of semi-skimmed milk.

The pictures can also be used for equivalent foods that are not shown in the food portion booklet. For example, the portion size of a slice of cheesecake that you have prepared may be of a similar size to a portion of sponge pudding that is found in the food portion booklet. This can also be noted in column D.

Column E: If you have any leftovers, describe what was left e.g. ½ the chop or ¼ of chips.

WHEN DESCRIBING FOOD AND DRINK IN COLUMN C, please give as much detail as you possibly can:

The type of food – "battered cod from a chip shop" (not just "fish"), "jam sponge" (not just "cake"), "Spaghetti Bolognese with 1 tablespoon of Parmesan" (not just "Spaghetti Bolognese").

The brand name – "flora margarine" (not just "margarine"), "Sainsbury's cornflakes" (not just "cornflakes")

The cut of meat – "lamb chop" (not just "lamb"), "lean beef mince" (not just "beef" or just "mince")

How the food is cooked – boiled, roasted, grilled, fried, casseroled. If anything is added during cooking, for example fat or oil, then give the brand name of the fat or oil as well, if you can.

Homemade recipes – write the name of the dish and include **all** of the ingredients

Date: ___25/09/12__Example____ Subject Initials: _____SB_____

Day: Tuesday_____ Subject Number:___2040_____

Please start each day with a fresh page. Use as many pages as you need to each day

A. Time	B. Food or Drink Please enter each food/drink item on a new line.	C. Description, brand name, method of cooking E.g. 2 large fried eggs, 2 slices of Hovis bread.	D. Approx. Weights/ portion size E.g. cup, slice or food portion booklet photo number.	E. Leftovers E.g. ½ a cup, ¼ of chicken breast, 5 chips.
0815	Toast	3 slices of toasted Hovis whole meal bread + Flora Margarine	3 thick slices Light spread of flora margarine similar to photo 13 in food portion book	None
1030	Instant coffee with sugar	Nestle	1 regular tea cup + splash of milk 1 teaspoon coffee 1 teaspoon of sugar	None
1215	Mushroom Soup	Heinz	300g tin	¼ of the tin
	Strawberry yoghurt	Tesco's own brand - light	1 x 100g pot	None
	Apple	Granny Smiths	1 x medium sized	None
1500	Mars bar		1 x normal sized bar 58 grams	None
1545	1 glass of water		Half pint glass	¼ of glass
1830	Chicken breast	Local butcher, grilled. Added pinch basil. Added Philadelphia cheese (light)	1 medium fillet 1 tablespoon of Philadelphia cheese	None

Date: _____ Subject Initials: _____

Day:_____ Subject Number:_____

Please start each day with a fresh page. Use as many pages as you need to each day

A. Time	B. Food or Drink Please enter each food/drink item on a new line.	C. Description, brand name, method of cooking E.g. 2 large fried eggs, 2 slices of Hovis bread.	D. Approx. Weights/ portion size E.g. cup, grams, teaspoon/tablespoon, slice or food portion booklet photo number	E. Leftovers E.g. ½ a cup, ¼ of chicken breast, 5 chips.

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Exercise Questionnaire

Study No. _____ Age_____

Height _____ Weight _____

1. Considering a 7-day period (a week) how many times on the average do you do the following kinds of exercise **for more than 15 minutes** during your free time?

   - strenuous exercise (heart beats rapidly): number of times in a week _____
   - moderate exercise (not exhausting): number of times in a week _____
   - mild exercise (minimal effort): number of times in a week _____

2. Considering a 7-day period (a week) during your leisure time how often do you engage in any activity long enough to work up a sweat (heart beats rapidly)? (please tick)

   - often [ ]
   - sometimes [ ]
   - never or rarely [ ]

Strenuous exercise: running, jogging, hockey, football, soccer, squash, basketball, cross country skiing, judo, roller skating, vigorous swimming, long distance bicycling

Moderate exercise: fast walking, baseball, tennis, easy bicycling, volleyball, badminton, easy swimming, alpine skiing, popular and folk dancing.

Mild exercise: yoga, archery, fishing from river bank, bowling, horseshoes, golf, snow mobiling, easy walking

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Dr

Date

Dear Dr

Ref: (patient name and date of birth)

This is to inform you that your patient (xxxxxxx) has agreed to participate in a research study to compare the acute response of bone to whole body vibration (WBV) in children whose mothers participated in the Maternal Vitamin D Osteoporosis Study (MAVIDOS).

MAVIDOS is a large study conducted recently at 3 different big centres (Sheffield, Southampton and Oxford). Results from this study have shown that giving a higher dose of vitamin D to pregnant women every day from 14 weeks of pregnancy increased the strength of the bones in their infants.

In the proposed study we aim to determine whether vitamin D supplementation during pregnancy affects the response of bone to vibration in children whose mothers participated in the MAVIDOS study in Sheffield.

You patient is a child of one of the MAVIDOS study participants and our study will involve him / her standing on a LivMD vibrating platform device for 10 minutes on 5 consecutive days along with the measurement of serum bone turnover markers at day 1 (baseline) and day 8 of the study.

The objective is to compare the difference in bone turnover marker response to WBV between the 2 groups. The primary outcome of the study is change in serum bone turnover markers - P1NP (marker of bone formation) and CTx (marker of bone resorption).

He / she will also be asked questions about his /her physical activities in 7 days prior to the study visits. Study visits will take place in the child's home or at school and will last approximately 20-45 minutes.

Please do not hesitate to contact the study team on 0114 2717228 if you would like further information regarding this study.

Yours sincerely

Dr Sujatha Gopal
Research Fellow in Paediatric Metabolic Bone Disease

Sheffield Children's NHS
NHS Foundation Trust

VIBRATION AND VITAMIN D (VIVID STUDY)

PARENT/LEGAL GUARDIAN INFORMATION SHEET

Study title
Does antenatal vitamin D supplementation influence bone's postnatal acute response to mechanical stimulation?

Part 1 – to give you first thoughts about the project

1. Invitation paragraph
You and your child are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish.

Part 1 tells you the purpose of this study and what will happen to you and your child if you take part.

Part 2 gives you more detailed information about the conduct of the study.

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you want your child to take part.

2. What is the purpose of the study?

Children who have narrower bones and a low bone mass are more likely to break a bone than children with larger bones and a higher bone mass.

The results from the MAVIDOS study that you took part in showed higher bone mass for babies born during winter when their mother had received extra vitamin D during pregnancy than those children whose mothers did not receive extra vitamin D during pregnancy.

We want to know if these differences persist into later childhood, and if the changes might be related to how bones respond to mechanical stimulation. Normally that would mean exercise, but we can also stimulate bones using vibration.

Separately to MAVIDOS, we found that when boys aged 9-11years stand on a vibrating plate for 10 minutes a day for 5 days, the rate at which they make bone, measured on a blood sample, goes up by 25%.

The aim of this study is find out if vitamin D intake during pregnancy influences the bone's response to vibration during early childhood.

If vitamin D given during pregnancy does do this, then the study will help us be clear about the benefits of vitamin D during this important time of life. It may also help us understand why some people are more likely to develop bone problems during childhood and later life.

3. Why has my child been chosen?

Your child has been chosen because you and your child took part in the MAVIDOS study and have consented to be contacted for taking part in further follow-up studies.

4. Does my child have to take part?

No. It is up to you to decide whether or not your child can take part. You are free to withdraw from the research at any time and without giving a reason. Your decisions about this will not influence how we look after your child whenever they need to come to hospital.

If you are happy to take part, and are satisfied with the explanations from the research team, you will be asked to sign a consent form. Your child will be given an information sheet. You will be given a copy of both the information sheets and the signed consent form to keep for your records.

5. What will happen to my child if we agree take part?

We will measure your child's height and weight, and ask you questions about the dietary intake of vitamin D / calcium; amount and type of exercise they have done in the 7 days prior to the study and then again on day 8 to determine undergoing whole body vibration has had an effect on participant's levels of physical activity. We plan for your child to stand on a vibrating plate. Your child will not feel the motion of the vibration plate although will hear a slight buzzing noise when it is switched on. We would request you to adopt your own distraction strategies such as reading a book, watching television or listening to music etc. to engage your children while standing on the vibration plate.

Fasting blood samples will be taken from your child on 2 days in total: one on day 1 before the vibration begins and then again on day 8.
Fasting means your child will not eat or drink anything except water from midnight until early next morning when we do the blood test. We will work with you in timing these tests in such a way that your child's breakfast routine is preserved as much as possible based on your preference. This is really important for us to do these tests at a fasting state; because eating and drinking have major effects on the things in the blood we want to measure.

A small needle will be inserted into a vein in your child's hand or arm for the blood sample to be taken. We will use a special cream or spray to numb the area where the cannula is to be inserted so that your child will not experience any pain. 2 - 4mls of blood (approximately ½ - 1 teaspoon) will be taken for each sample.

Day 1:	Measure height and weight. Take fasting blood sample; stand on vibrating platform for 10 minutes (4 cycles of 2.5 minutes each with 30 seconds break after each cycle). Exercise questionnaire.
Day 2:	Stand on vibrating platform for 10 minutes (4 cycles of 2.5 minutes each with 30 seconds break after each cycle).
Day 3:	Stand on vibrating platform for 10 minutes (4 cycles of 2.5 minutes each with 30 seconds break after each cycle).
Day 4:	Stand on vibrating platform for 10 minutes (4 cycles of 2.5 minutes each with 30 seconds break after each cycle).
Day 5:	Stand on vibrating platform for 10 minutes (4 cycles of 2.5 minutes each with 30 seconds break after each cycle).

Day 8: Take fasting blood sample and exercise questionnaire

You can choose to have the study visit either at your home or at your child's school with the head teacher's permission, as first thing in the morning so as not to disrupt their school day. The visits will take approximately 15-45 minutes to complete. A member of the study team will stay with your child for the whole visit, if you wish to stay with your child too you can. If you are not able to remain with your child and wish for us to arrange a chaperone we will do so.

All children who take part in the study will be offered a £25 voucher as a thank you for helping us with our research. We will provide a breakfast on the days that blood samples are taken, or alternatively your child could bring in something to eat and drink from home.

6. What will we have to do?

All study procedures will take place during the study visits. Other than nothing to eat or drink except water after midnight on the days that the blood samples are taken no preparation is required.

7. What are the possible disadvantages and risks of taking part?

In our previous studies of 64 boys aged 9-12 years, the only reported side effect was some itching in the calf muscles. However, the manufacturer does list the following as possible side effects:

a. Skin lesions/blisters on contact surface
b. Itching in trained body parts
c. Nausea and dizziness
d. Quick temporary drop of blood pressure
e. Drop in blood sugar level in diabetics due to high physical activity.

Your child will have a small needle placed into a vein for the taking of blood samples. This can cause discomfort to your child. To reduce this, a local anaesthetic in the form of a cream or a cold spray will be applied to the skin prior to insertion of the needle.

8. What are the possible benefits of taking part?

We do not anticipate that this study will be of direct benefit to you or your child. We hope the study will help us understand if vitamin D intake during pregnancy influences the bone's response to vibration during early childhood.

9. What happens when the research study stops?

We collect all the information together and we will use this to design longer-term studies in the use of vibration platforms in children with bone health problems.

10. What if there is a problem?

Any complaint about the way you or your child has been dealt with during the study or any possible harm you or your child might suffer will be addressed. The detailed information on this is given in Part 2.

11. Will my child's taking part in the research project be kept confidential?

Yes. We will follow ethical and legal practice and all information about your child will be handled in confidence. The details are included in Part 2.

12. Contact for further information

If you would like any further information about this study you could contact:

Name: Suji Gopal
Designation: Research Doctor
Hospital/Department: Room C7, Stephenson Wing, Sheffield Children's Hospital, Western Bank, Sheffield, S10 2TH
Tel: 0114 271 7228
Email: Sujatha.gopal@sheffield.ac.uk

This completes Part 1 of the Information Sheet.

If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

Part 2 - more detail – information you need to know if you still want to take part.

13. What if new / findings of significant clinical information becomes available?

Sometimes during the course of a research project, new / significant clinical information becomes available. As your child is involved for only 2 weeks this is unlikely. However, if new information relating to vibration and bone health does become available someone from the research team will tell you and your child about it and discuss with you whether you want your child to continue in the study. We will also contact you via your GP and will be invited to attend an appointment with the general paediatrician if necessary. If you change your mind this will not affect any care your child receives now or in the future whilst in hospital. If you decide to continue in the study you will be asked to sign an updated consent/assent form.

14. What will happen if we don't want to carry on with the research?

If you withdraw from the study, we will destroy all your child's identifiable samples if you wish, but we will need to use the data collected up to their withdrawal.

15. What if there is a problem?

Complaints

If you have any cause to complain about any aspect of the way in which you or your child has been approached or treated during the course of this study, the normal National Health Service complaints mechanisms are available to you and are not compromised in any way because you have taken part in a research study. If you have any complaints or concerns please contact either the project co-ordinator:

Name: Suji Gopal
Designation: Research Doctor
Hospital/Department: Room C7, Stephenson Wing, Sheffield Children's Hospital, Western Bank, Sheffield, S10 2TH
Tel: 0114 271 7228
Email: Sujatha.gopal@sheffield.ac.uk

Otherwise you can use the normal hospital complaints procedure and contact the following person:

Mrs Linda Towers
Patient Advice & Liaison Co-ordinator
Sheffield Children's NHS Foundation Trust
Tel: 0114 271 7594
Email: Linda.Towers@sch.nhs.uk

Harm

If your child is harmed by taking part in this research project, there are no special compensation arrangements. If your child is harmed due to someone else's fault, then you may have grounds for a legal action – but you may have to pay for it.

Safeguarding

If any safeguarding issues become apparent during the fracture history, the chief investigator is fully trained to recognise and manage them appropriately as per the hospital safeguarding policy.

16. Will 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 your child which leaves the hospital will have their name and address removed so that your child cannot be recognised from it. Once the study is complete all information including questionnaires and blood samples will be kept for five years.

Our procedures for handling, processing, storage and destruction of data are compliant with the Data Protection Act 1998.

Access to data will be restricted to members of the study team, the study sponsor and Sheffield Children's Hospital Research and Development

department. Monitoring and audit may be carried out by the relevant authorities.

We will also ask for permission to inform your family GP that your child will be taking part in the study.

Your child's medical notes may also be looked at by other people within the hospital involved in the running and supervision of the study to check that it is being carried out correctly.

As study visits may take place at your child's school, the teachers may know that your child is taking part. They will not have access to any of the study data collected.

17. What will happen to any samples my child gives?

All samples collected will be anonymised with a study number and will be stored in a freezer in the Clinical Research Facility (CRF) at Sheffield Children's Hospital until they are ready to be analysed in the laboratory. We will ask your permission to use any left-over samples for future research and this will only be after further ethical approval has been given. Access to the samples will be limited to the same people who have access to study data.

18. Will any genetic tests be done?

No.

19. What will happen to the results of the research study?

When the study has finished we will present our findings to other doctors, and we will put the results in medical magazines and websites that doctors read. We would also like to put a brief summary on the hospital research website so that you will be able to read about our results too. This will be available at the end of the study, on our website www.sheffieldchildrenscrf.nhs.uk. The results will also be included as part of the chief investigator's educational qualification. They will be anonymous, which means that your child will not be able to be identified from them.

20. Who is organising and funding the research?

The research is being organised by Sheffield Children's NHS Foundation Trust and paid for by [*add name].*

21. Who has reviewed the study?

This study was given a favourable ethical opinion for conduct in the NHS by *[add name]* Research Ethics Committee. It has also been approved by the Research Department at this hospital.

22. How can we find out more about research?

The Clinical Research Facility at this hospital has an **Information for family's** section on its website www.sheffieldchildrenscrf.nhs.uk or you could contact the hospital Clinical Research Facility:

Mrs Wendy Swann
R&D Manager
Sheffield Children's NHS Foundation Trust
Tel: 0114 226 7904
Email: wendy.swann@sch.nhs.uk

If you and your child decide to take part in this study, you will be given this information sheet and signed consent and assent forms to keep.

Thank you for taking the time to read this information sheet.

Sheffield Children's NHS
NHS Foundation Trust

PARTICIPANT INFORMATION SHEET FOR YOUNG CHILDREN

This information is to be shown and read by a parent/carer

Study title:

What does vitamin D taken by your mum when you were inside her tummy do to your bones when you stand on a vibration plate?

I am a doctor who is studying about "what makes our bones strong"?

Before you were born, when you were inside your mummy's tummy, she took some special medicine called 'vitamin D'.

I would like to find out if this has made your bones stronger when you stand on a vibrating plate"?

To help us find out more, I would like to

(i) See how big you are?

(ii) Ask you to stand on a vibrating plate for 10 minutes, 5 days in a row

We will do a blood test once before you stand on the vibrating plate and then again a week later.

I would also like to ask you and your mummy and daddy some questions

Thanks for reading this

Appendix 4 Vitamin D fracture study protocol

Vitamin D, Rachitic Radiographic Changes and Fractures in Infants: What is the Relationship?

Lay summary

In children who have suffered from non-accidental trauma, suboptimal vitamin D status has been offered as an alternative explanation to unexplained fractures. The presence of low vitamin D has been successfully used as a defense in Court cases of suspected child abuse where an infant has fractures despite only mild or no radiological evidence of rickets. As such; Vitamin D status in suspected non-accidental injuries has been a contentious issue in recent years. There is a shortage of research into the relationship between vitamin D, radiographic changes and fractures in infants, especially in the UK.

We identify a wide range of research questions that could be addressed. The first of these we would like to answer is the extent of rachitic changes on radiographs and fracture risk in relation to different statuses of vitamin D. Members of the British Paediatric and Adolescent Bone Group (BPABG) have come together and agreed to a study that will pool images and clinical data from children below 2 years of age presenting to their hospitals over the past 5 years. We would like to analyse their data to ascertain relationships between vitamin D, calcium, phosphate, radiographic features of rickets (Thacher score) and fractures. In addition, we wish to test the hypothesis that vitamin D deficiency alone (when radiographic and biochemical markers are normal) can predispose infants to fractures, with particular attention paid to a child's fracture risk in the case of mild vitamin D deficiency versus that in florid clinical and radiographically evident rickets.

General Information

With support from: British Paediatric and Adolescent Bone Group (BPABG)

Investigators: Elaine J Pang, Amaka C Offiah, Zulf Mughal

Background

Once a public health concern of the past (industrial revolution), there has been a resurgence of vitamin D deficiency in the past decade [1]. In the United Kingdom, the prevalence of vitamin D deficiency in children (25-hydroxyvitamin D level ≤25 nmol/L) is estimated to be 29% [2]. A low vitamin D level ultimately leads to an increased osteoclastic activity, leading on to an altered phosphorus-calcium product and decreased mineralization of collagen. There is then the development of rickets in the immature skeleton [3].

In infants younger than 6 months, vitamin D deficiency presents as hypocalcemic seizures, whereas older infants and toddlers classically present with clinically and radiographically evident rachitic changes alongside biochemical changes: elevated parathyroid hormone (PTH)

and alkaline phosphatase (ALP) levels [4]. In addition, the severity of vitamin D deficiency is proportional to the rachitic changes seen on radiographs [5].

As highlighted by the recent high profile case *LB Islington v Al Alas and Wray* (2012), there is a dearth of research into the ramification of vitamin D deficiency and rickets. Rickets is the deficiency of bone mineralization, most recognizable at the growth plate, which results in radiographically evident abnormalities. Currently, there is little data regarding a child's fracture risk in the case of a mild vitamin D insufficiency versus that in florid clinical and radiographically evident rickets. If suboptimal vitamin D status does in fact increase susceptibility to fractures, there is a potential for misdiagnosis of otherwise unexplained fractures as abuse. Conversely, abused children may pass under the radar if suboptimal vitamin D status is inappropriately linked to a predisposition to fractures [6-9]. The morbidity and mortality of a missed diagnosis of child abuse has been well documented [8], although the emotional, psychological and financial burdens to both the family and child of false accusations (in the context of underlying bone pathology) can be presumed detrimental as well.

One previous study examined vitamin D levels in 118 young children presenting with fractures, however was limited by the absence of a control group [10]. Another looked into the relationship between rachitic changes, demineralisation and fracture risk in 40 toddlers with vitamin D deficiency via only bilateral wrist and knee computed radiographs [11]. The same research paper emphasized the lack of understanding in the relationship between radiographic appearance of rickets and biochemical markers of bone metabolism (eg. PTH, ALP) in infants and young children.

While all research points to the conclusion that there is little association between suboptimal vitamin D status and fractures related to abuse, this study looks to further fill the gap of knowledge on a larger sample population recruited retrospectively in the UK and reviewing entire skeletal surveys, as well as cementing the understanding on rachitic changes on radiographs and biochemical markers of bone metabolism. Radiographic changes and fractures in infants will be correlated with vitamin D levels, with the aims of testing the hypothesis that vitamin D deficiency alone (when radiographic and biochemical markers are normal) can predispose infants to fractures and, documenting patterns of fractures in infants with and without confirmed rickets.

We believe that this will provide an evidence base for estimating fracture risk in infants and young children who are vitamin D deficient, and lend support to medical opinions offered in medico legal proceedings in which child abuse is alleged.

Study objectives and purpose

This application is to obtain the participants list from 5 centres in the UK that will:

1. Establish the prevalence of rachitic change and/or fractures in UK infants within the following categories of vitamin D:
    a. normal
    b. insufficient
    c. frankly deficient vitamin D

    and hence their relationship.
2. Demonstrate any association of vitamin D deficiency (even if mild) with a predisposition to fracture, further more so in the absence of any radiographic evidence of bone dysplasia (including rickets).
3. Determine the degree of vitamin D deficiency sufficient to cause deleterious skeletal effects (and what sort), and hence predispose to if any, fractures (what type)
4. Determine a child's fracture risk in the case of mild vitamin D deficiency versus that in florid clinical and radiographically evident rickets.
5. Compare radiographic appearance of rickets with biochemical markers of bone metabolism and conclude their relationship, if any.
6. Allow EJP to gain experience in obtaining and interpreting rachitic changes on radiographs, through sessions with ACO.
7. Lead to presentations and publications that will provide credibility to funding a large-scale research project.

Study Design

We plan a retrospective case review. Participants and their families/carers will not be involved in its design as demographic information and relevant data will be obtained from the patient's case notes at five medical centers.

We are comparing vitamin D status (deficient, insufficient, normal levels) of infants and young children to rachitic changes on skeletal surveys and any fractures recorded. Our main goal in this study is to establish the prevalence of rachitic radiograph changes and/or fractures in UK infants within the different categories of vitamin D (normal, insufficient, deficient), and hence determine their relationship. We would also like to demonstrate the degree of vitamin D deficiency sufficient to cause deleterious skeletal effects such that it increases a child's fracture risk, even in the absence of clinically diagnosed rickets. Lastly, we plan to compare radiographic evidence of rickets with biochemical markers of bone metabolism to conclude any relationship.

Selection of Participants

This is a five medical centre study in which a participant's list will be obtained from selected members of BPABG who have volunteered their centres as contributors to the study. ACO and EJP will travel to the hospitals and obtain the required information. It should be noted that the

methodology used in all centres will be standardized. If insufficient numbers are procured, we will recruit children diagnosed with rickets who have had entire skeletal surveys.

The inclusion criteria are as follows:

1. Infants and young children aged up to 24 months
2. Who firstly have had an Computed Radiography (CR) performed in the preceding 5 years
3. And had both a vitamin D measurement and the CR done within 2 weeks of each other.

Within this group, the exclusion criteria are as follows:

1. if received any medication known to affect vitamin D metabolism 3 months before enrollment (oral glucocorticoids, anticonvulsants etc.)
    a. vitamin D supplements are not implicated

Participant Recruitment

We aim to reach a minimum sample target of 100, with an ideal of over 200 participants. A sub-group of children will be those who did not have a full skeletal survey but did have at least one radiograph of at least one joint to allow the diagnosis of rickets.

Within this group, data to be collected from the case notes:

1. current age (plus age at birth if current age below 3 months)
2. age at which skeletal survey was conducted
3. sex
4. ethnicity
5. biochemical levels of 25(OH)D, calcium, phosphate, alkaline phosphatase, parathyroid hormone
6. medications, if any
7. breast or bottle fed
8. fractures:
    a. site of fracture(s)
    b. number of fracture(s): single/multiple
    c. injury classification: accidental/non accidental/indeterminate
9. discharge diagnosis
10. corresponding radiographs to be evaluated for the presence of rachitic changes and fractures
    a. documentation of the severity of rickets will be made by using the Thacher score.
11. co-existing chronic disease, if any

Factors 1-6 are known to influence 25(OH)D levels [12].

Presence of rachitic changes on radiographs is demonstrated by impaired mineralization of the growth plates evident by widening of the growth plate, and fraying and cupping of the margins of the metaphyses [9].

In addition, vitamin D status will be classified according to the table below [12]:

25(OH)D concentration	Classification
≤25 nmol/L	Deficient
25-50 nmol/L	Insufficient
>50 nmol/L	Optimal

Table. **Classification of Vitamin D status by 25(OH)D Concentration***

*25(OH)D = 25-hydroxyvitamin D.

Data-Handling and Record Keeping

The investigators will acquire the data and store and collate the anonymized data on an Excel spreadsheet/Surveymonkey system, both password-controlled. All data will be backed-up to an external hard drive. ACO will analyse the images and instruct EJP on this.

STANDARD STATEMENT ON DATA PROTECTION

Data will be collected and retained in accordance with the Data Protection Act 1998.

STANDARD STATEMENT ON STORAGE OF RECORDS

Study documents (paper and electronic) will be retained in a secure location during and after the study has finished. All source documents will be retained for a period of 5 years following the end of the study.

Statistical Analysis

Descriptive for age, sex ethnicity, discharge diagnosis. Chi-square for categorical data, with the possibility of regression analysis on advice of a statistician.

- Rachitic changes will be considered to be present if ACO assigns a Thacher score of more than 0 and absent if the score was 0.

Safety Assessments

We do not foresee any issues with regard to safety due to the retrospective basis on which the research will be carried out.

Ethical Considerations

Site-specific ethical approval to be obtained for ACO and EJP to review case notes at the various UK centres.

There are no other ethical issues that might arise from this project.

Ethics and R&D approval

The study will be conducted in compliance with a Research Ethics Committee favourable opinion, including any provisions for Site Specific Assessment, and local Research and Development approval. The study will also be conducted in accordance with the International Conference for Harmonisation of Good Clinical Practice (ICH GCP), and the Research Governance Framework for Health and Social Care (2nd Edition).

Duration of the Project

2 years

Problems Anticipated

Type and scope of data obtainable/incomplete

RISK OF BIAS???

Reporting and Dissemination

Our intentions are twofold; 1) to submit the work for presentation at national and international conferences and for publication in high impact peer-reviewed journals 2) to use our results to support a larger scale vitamin D study the year after.

Expected Outcomes of the Study

1. Hypothesize that radiographic evidence would correlate with biochemical abnormalities but that fracture risk will be low.
2. Dispute the proposition that vitamin D deficiency (even in milder degrees) can predispose infants to rachitic fractures mimicking those caused by non-acoidental trauma of infants. Hence, vitamin D deficiency is an unlikely explanation for unexplained fractures when radiographic and biochemical markers are absent.
3. Apply for a grant for an even larger scale study to be offered for the next round of University of Sheffield's BMedSci programme.

Outcomes & Impact

As a result of this retrospective study, the impact of such a programme of work cannot be overstated. Firstly, a better understanding of the relationship between vitamin D status and fractures will result. Secondly, we will be able to answer questions that have led to significant national and international debate concerning vitamin D status, fractures and child abuse. Thirdly, presentations and publications will be brought about on behalf of BPABG.

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Appendix 5 Vitamin D Dosing Study Protocol

FULL STUDY TITLE
Determining the effects of race, skin colour and genotype on the response to vitamin D therapy

SHORT STUDY TITLE
Getting vitamin D dosing right

STUDY NUMBER SCH/14/053

DATE AND VERSION NUMBER (29/09/2014, V3)

LAY SUMMARY

The Department of Health and the Chief Medical Officer have identified vitamin D deficiency as a key area of interest and concern for public health. The main function of vitamin D is to improve the uptake of calcium from the diet; calcium is essential for the normal activity of nerves, muscle and bone. Severe lack of vitamin D causes bones to become soft and grow more slowly in children, resulting in a disease called rickets. In adults, the bone softening is called osteomalacia; in older people, this can make bone loss and osteoporosis worse and increase the risk of hip fractures.

Vitamin D occurs naturally in the diet in foods like oily fish; vitamin D can also be given as a supplement, either on its own or as part of a multivitamin tablet. A lot of vitamin D is made from the action of sunlight on natural chemicals in the skin.[1,23-30] Vitamin D levels tend to be lower in people who are overweight, in those with darker coloured skin or if the skin is covered by clothing. Vitamin D levels can also be affected by a person's individual genetic make-up.[1,2]

There is natural variation from one person to another in how well the system controlling vitamin D blood levels works. This variation is determined in part by a person's genetic makeup, and recent large studies have identified specific genetic variations that are linked to blood levels of vitamin D; some of these vary with the person's ethnic origin.[3-7]

Vitamin D is carried round the body by a specific protein in the blood stream called "vitamin D binding protein" (VDBP). We will find out whether lower or higher levels of this protein affect the response to giving extra vitamin D.

At present if someone has low vitamin D levels that puts them at increased risk of bone problems, a course of vitamin D treatment is given. When we assessed our regular treatment given to children recently, we found some individuals developed very high blood vitamin D levels and others didn't.

We don't know how genetic variation, VDBP levels and other factors affects the response to treatment with vitamin D. Some variation in the response to giving extra vitamin D may occur because of the distribution of vitamin D into fat tissue, so as part of this study we will measure height and weight and calculate body mass index (BMI), which is often used to decide if someone is overweight or obese.

We want to make sure that people get the right dose of treatment. We will therefore investigate how skin colour, BMI, ethnicity, VDBP and genetic variation affect the response to a single dose of vitamin D in young adults. **We will also evaluate whether blood or saliva tests give better information about vitamin D levels.** The information about how these factors affect the response to vitamin D will help us choose the right dose of vitamin D for studies in younger children who are still growing.

GENERAL INFORMATION

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GLOSSARY

25OHD	25-hydroxyvitamin D; circulating form of vitamin D, made in the liver, measured to assess overall vitamin D status
1,25D	1,25-dihydroxyvitamin D; active metabolite of vitamin D, made from 25-hydroxyvitamin D in the kidney
VDBP	vitamin D binding protein
GC gene	group-specific complement gene

1.0 BACKGROUND (max 600 words))

Vitamin D is not only important for bone health but also for immune function, insulin sensitivity, muscle strength and cardiovascular function. As sunlight is the main source of vitamin D, in the UK we are dependent on our body stores and dietary sources of Vitamin D during winter months.

Studies have shown that people of South Asian, Afro –Caribbean and middle eastern origin are at increased risk of vitamin D deficiency defined by low serum 25 OHD. COMA (DH 1991), and NICE (2012) have issued evidence based guidelines on vitamin D supplementation for at risk groups; however these recommendations are not fully implemented and there has been a resurgence of rickets in UK.

The same amount of vitamin D synthesis requires increased sunlight exposure in dark skinned compared with light skinned individuals. Studies have recommended a 15 min unshaded noon time sunlight exposure 3 times a week with 35% skin exposure is adequate for improving 25OHD concentrations in white population whereas this is inadequate in South Asian participants.
Even a three-fold increment in sun exposure failed to achieve sufficiency in more than half of the South Asian cohort, suggesting that sun exposure advice needs to be tailored based on the degree of skin pigmentation.

At present there is no clear guidance on the recommended daily intake of vitamin D for both general and at-risk groups over the age of 4 years. The age groups with the highest frequency of low circulating vitamin D levels (25OHD < 25nmol/l) are older teenagers/young adults, and the elderly. (SACN 2007).

The vitamin D binding protein (VDBP) encoded by the group-specific complement (GC) gene on chromosome 4 acts as the main transporter for vitamin D and its metabolites. There is emerging evidence in the literature that single nucleotide polymorphisms (SNP) in GC contributing to significant variation in circulating Vitamin D (25 OH D) levels. What is not known is the extent to which such variation results in altered response to standard treatment. Powe et al has recently shown in their cross-sectional study that black Americans have low levels of total vitamin D and VDBP resulting in similar concentrations of estimated bio-available (free) vitamin D compared to their white counterparts. The black population had a higher BMD and calcium levels, only slightly higher parathyroid hormone levels compared to whites. This observation raises the question of whether standard treatment with vitamin D based on the total 25OHD levels and VDBP is appropriate across all ethnic groups. The authors also proposed that bio-available 25OHD may be a more appropriate cross-racial marker of vitamin D deficiency.

We recently undertook an audit of the application of the existing guidelines for the treatment of vitamin D deficiency in 66 infants and children referred to the metabolic bone disease service at Sheffield Children's Hospital and found that although the increase in serum 25OHD largely correlated with the dose/kg of vitamin D administered (range 3,400-35,000 U/kg), 7% children remained vitamin D insufficient (vitamin D 25-50 nmol/l) and 14% (all aged <18 months) developed serum vitamin D > 200nmol/l.

Variation in serum 25OHD after vitamin D ingestion can occur because of the distribution of vitamin D into fat tissue; a recent published abstract showed high levels of 25OHD occurring only in adults with a low BMI (16.6-18.5) following monthly dosing with 100,000 IU for 3 months. We will measure height and weight, and waist and hip circumference and calculate Body Mass Index, body surface area (BSA) and waist:hip ratio as proxy measures of fat mass.

There is a huge variation in recommendations regarding treatment for vitamin D deficiency.
Both forms of vitamin D i.e vitamin D2 (ergocalciferol) or vitamin D3 (cholecaloiferol) can be used for treatment. Whilst some have raised concerns about the effectiveness of ergocalciferol compared with cholecalciferol in increasing 25OHD levels during treatment, and about a precipitate fall after completion of treatment, other studies undertaken in paediatric population have demonstrated that there is no difference in between the two forms in increasing 25OHD levels post treatment. Jeans and Steam in 1934 has reported that there is no difference in functional outcome (longitudinal growth) between the two forms.

Oliveri B et al in 1996 demonstrated that after 150,000 IU of vitamin D2 administration, serum 25OHD levels at the end of winter were similar to those at the beginning of autumn, but significantly higher from those obtained in a previous study without vitamin D. The authors also concluded that a single dose of 150,000 IU of vitamin D maintained appropriate levels of 25OHD without causing hypercalcaemia or hypercalciuria.

Serum calcium rises with increasing 25OHD and hypercalcaemia typically occurs when 25OHD is greater than 200nmol/L. Stosstherapy with 600,000 IU of vitamin D can result in hypercalcaemia and nehprocalcinosis.

The British National Formulary for Children guidelines 2013 recommends a stosstherapy of 300,000 IU of either ergocalciferol or cholecalciferol as a single dose or two divided doses over 12 hours for children and young adults aged between 12 and 18 years who are vitamin D deficient. (Ref BNFc 2013) We have chosen to use 150,000 vitamin D3 (cholecaloiferol) given as a single dose, regardless of baseline vitamin D status for our study. It is unlikely to cause a rise in 25OHD above 200nmol/l (based on Oliveri's work).

Vitamin D in circulation in blood is bound to proteins, and the levels of the proteins affect the vitamin D measurement. The free hormone hypothesis suggests that it is unbound (or 'free') vitamin D that is biologically active, and so free vitamin D may be a better measure of vitamin D status. Free 25OHD can be measured in blood, but the available methods are very complex or not very reliable. An alternative is to measure vitamin D in saliva, where there are no binding proteins. As part of this study we will evaluate the use of salivary vitamin D measurements in response to vitamin D treatment.

The investigation of the mechanisms underlying variation in response to administered vitamin D is the purpose of this study.

2.0 STUDY OBJECTIVES AND PURPOSE (max 300 words)

The main aim of the study is to investigate how skin colour/ethnicity and genetic variation affect the response to 150,000 units of vitamin D administered to young adults who are either White or South Asian origin and to use the data derived from this study to plan a large scale intervention study in children.

The specific objectives are to

1. determine whether a single dose of 150,000 IU of Vitamin D3 [6 mls of Invita D3 25,000 IU oral solution (1 ml in ampoule)] can increase serum 25OHD by at least 25nmol/l in the majority of those receiving this dose.

2. determine if dark skin colour or South Asian heritage reduces the increase in serum 25OHD

3. determine if variation in GC genotype is associated with variation in the increase in serum 25OHD

4. determine the change in bioavailable ("free") vitamin D **in blood and saliva** after dosing and the influence of the above factors on this.

5. determine the extent of PTH suppression in relation to overall increases in total and free vitamin D.

3.0 STUDY DESIGN (max 600 words)

This is an exploratory study to assess the size of the effect engendered by administering a standard dose of vitamin D to young adults from different ethnic groups and genotypic variation.

We aim to recruit 30 subjects of both White Caucasian and South Asian origin i.e. an equal number of participants from each ethnic group.

Data from the National Diet and Nutrition Survey indicate that over 50% of 19-24 year olds have a serum 25OHD < 50 nmol/l and 25% have a level of <25nmol/l. Since <25nmol/l is currently defined by NICE as being "deficient" and 25-50 nmol/l as being "suboptimal", we want to know if a single dose of vitamin D can increase serum concentrations by at least 25 nmol/l. We are not stratifying the study in any way, as we want to have a clear idea of the effect of a range of starting concentrations of 25OHD on its subsequent rise in response to vitamin D administration; however baseline 25OHD and VDBP will be covariates in the analysis we perform.

Potential participants will be invited to the CRF and baseline investigations (all done fasting) including serum 25OHD [tandem mass spectrometry assay], bioavailable vitamin D [calculated][20], VDBP (ELISA assay), **salivary vitamin D,** GC (vitamin D binding protein) genotype by Pyrosequencing, calcium, phosphorus, alkaline phosphatase, magnesium (colorimetric, by Elecsys), bone turnover markers (P1NP, CTx by Roche chemiluminescence immunoassay & automated chemiluminescence immunoassay respectively) and

parathyroid hormone (Abbott Architect immunoassay) and urine (second void) calcium:creatinine ratio will be performed. Basic anthropometry: height (to next 1mm by wall-mounted stadiometer), weight (wearing vest and pants to nearest 0.1 kg by electronic balance scales), giving calculated BMI, BSA; waist:hip circumference ratio (paper tape measure). Skin colour grading using the Fitzpatrick grading scale[21] and dietary intake of calcium using the DIETQ food frequency questionnaire (FFQ) will be undertaken.

A single dose of 150,000 IU of Vitamin D3 [6 mls of Invita D3 25,000 IU oral solution (1 ml in ampoule)] will then be administered under direct supervision.

Nivea Sun Lotion Factor 50 (200 ml pump, hypoallergenic, fragrance-free) will be provided to all participants for regular use during the study period of a total of 4 weeks to avoid influence of sun exposure if carried out in summer months. Ideally we would prefer to conduct the study this winter i.e from October to February to avoid sun exposure that allows skin synthesis of vitamin D.

Repeat saliva samples and spot urine calcium: creatinine ratio will be performed on second void fasting urine 1 week after dosing to reassure that no hypercalciuria has occurred in any subject.

The subjects are then requested to return 4 weeks after baseline for repeat blood **and saliva** samples (excluding genotype, but including VDBP).

The time line for project is outlined in the attached Excel spread sheet

4.0 SELECTION OF PARTICIPANTS (max 300 words)

A population based cohort of healthy young male adults 18 -25 years from two ethnic groups – White Caucasian and South Asian origin will be selected for this study. Subjects will essentially be medical students and their friends. Whilst it is anticipated that gender should not impact on outcome, the administration of a large dose of vitamin D might have unexpected effects on an early stage pregnancy and young women are therefore excluded.

Inclusion criteria:

Healthy young male adults aged 18 -25 years, free from any condition affecting bone health, general nutrition, growth and glucose metabolism will be enrolled for the study.

Exclusion criteria:

Subjects with any chronic illness involving the liver and kidney, current use of steroids, anticonvulsants or any medication that might affect calcium and vitamin D metabolism are excluded from the study.

5.0 PARTICIPANT RECRUITMENT (max 300 words)

Interested volunteers will have to take a minimum of 24 hours to consider the information sheets before enrolling into the study. To avoid translation costs and the concerns about misunderstanding of important information only English speakers will be recruited. All volunteers will be asked for their consent to write to their

General Practitioner to inform them of their role in the study. A total of 60 subjects will be recruited (30 White, 30 South Asian origin).

In keeping with single dose PK-type studies we will recruit only male participants to avoid the risk of drug administration in early stage pregnancy. Recruitment will take place within the University using a mixture of email, posters/leaflets and pre-lecture 2-minute talks. We will also use local newspaper advertisement if necessary. Each subject will be given a £50 voucher (on completion of the study). This amount is felt to suitably recognise the subject's commitment without being an inducement. The reason for payment is in recognition of the time and dedication (fasting repeat blood samples, attendance for approximately 3 hours altogether) that participation in this study requires. At the time of recruitment candidate subjects will receive printed information about the study, its aims and what will be required of them should they consent to participate. Contact details of the PI will be provided for enquires.

Interested volunteers will be asked to contact the PI by telephone or email and suitability for inclusion into the study will be assessed, questions answered and contact details sought. We expect recruitment to occur over a 3-month period.

6.0 DATA HANDLING AND RECORD KEEPING (max 300 words)

The PI will be responsible for data collection, recording, quality and storage. Basic demographic details on participants to include: age, ethnic group at first attendance and basic auxological data: height, weight and calculated BMI, BSA on first attendance. Basic medical and medication history to confirm absence of exclusion criteria will be taken prior to consent.
Data will be coded so that subjects are not immediately identifiable and double-entered to maximize quality. Upon recruitment subjects will be allocated a unique identifying number, which will be used on all records. Any records with identifying information will be kept in a locked cabinet, within a locked office in the research department at SCH. All data will be stored on encrypted media.
Contact details of participants collected at recruitment will be used to facilitate reminders of the 4 week follow-up appointment. They will be stored separately to the study data.

STANDARD STATEMENT ON DATA PROTECTION

Data will be collected and retained in accordance with the Data Protection Act 1998.

STANDARD STATEMENT ON STORAGE OF RECORDS

Study documents (paper and electronic) will be retained in a secure location during and after the study has finished. All source documents will be retained for a period of 5 years following the end of the study.

7.0 ACCESS TO SOURCE DATA (max 300 words)

The sponsor will permit monitoring and audits by the relevant authorities, including the Research Ethics Committee and the Medicines and Healthcare products Regulatory Agency (MHRA). The investigator will also allow monitoring and audits by these bodies and the sponsor, and they will provide direct access to source data and documents.

8.0 STATISTICAL ANALYSIS (max 300 words)

This is an exploratory study to determine effect size and variance; hence no formal calculation of sample size has been undertaken. Advice from Yorkshire and Humber RDS indicates that a sample size of 30 per group is usual in undertaking exploratory work of this nature. Statistical analysis will be performed by Dr Alan Rigby C Stat, CSci, University of Hull. We anticipate that the results will enable us to undertake a larger scale multicenteric study in 2015.

9.0 SAFETY ASSESSMENTS (max 300 words)

Monitoring

The study will be monitored and audited in accordance with the Monitoring Standard Operating Procedures of the Clinical Research Facility. All study related documents will be made available on request for monitoring and audits by the Sponsor, the relevant Research Ethics Committee and for inspection by the MHRA or other licensing bodies.

10.0 ETHICAL CONSIDERATIONS (max 300 words)

The study will be conducted in compliance with a Research Ethics Committee favourable opinion, including any provisions for Site Specific Assessment, and local Research and Development approval. The study will also be conducted in accordance with the International Conference for Harmonisation of Good Clinical Practice (ICH GCP), and the Research Governance Framework for Health and Social Care (2nd Edition).

11.0 FINANCE AND INDEMNITY (max 300 words)

This is an NHS sponsored study. For NHS sponsored research HSG (96) 48 reference no. 2 refers. If there is negligent harm during the study when the NHS body owes a duty of care to the person harmed, NHS Indemnity will cover NHS staff, medical academic staff with honorary contracts and those conducting the study. NHS Indemnity does not offer no-fault compensation and is unable to agree in advance to pay compensation for non-negligent harm. Ex-gratia payments may be considered in the case of a claim.

12.0 JUSTIFICATION OF FINANCE (max 300 words)

Item/subject	Cost/subject	No. of episodes	Total cost per item	Total cost for study
CRF visit	£17.50	2	£35	£2100
CRF sample preparation	£17.50	2	£35	£2100
GC genotype inc preparation	£13	1	£13	£780
VDBP	£26	2	£52	£3120
25 (OH) Vitamin D	£10.50	2	£21	£1260
PTH	£5	2	£10	£600
Bone profile	£5	2	£10	£600
Urine Ca :Cr ratio	£5	2	£10	£600
Payment for participation	£50	1	£50	£3000
Vitamin D3 [6mls of Invita D3 25,000IU (1ml in 1 ampoule)]	£1	1	£60	£60
P1NP	£28	2	£56	£3360
CTX	£23	2	£46	£2760
DIETQ software - calcium FFQ	-	-	-	£500
Nivea Sun Lotion Factor 50	£8.33	1	£8.33	£500
Salivary vitamin D	To be done by the Academic Unit of Bone Metabolism labs, funded by existing grant.			
Total				**£21,340.00**

13.0 JUSTIFICATION OF RESOURCES

This is an exploratory study that aims to define the variability in the response to the administration of a single dose of vitamin D according to ethnicity and genotype. We anticipate that the results will inform a submission to NIHR/MRC for a larger scale multi-dose study subsequently.

As detailed above, subjects will receive £50 voucher in order to compensate them for their time away from work or study, travel expenses and in recognition of the commitment the trial requires.

14.0 REPORTING AND DISSEMINATION (max 300 words)

The results of the study will also be disseminated on the R&D/CRF website and in the R&D/CRF newsletters. We anticipate presenting results at national and international paediatric and endocrinology meetings.

15.0 IMPACT (max 400 words)

Over-dosing and under-dosing of vitamin D in children and young people appears to be common, based on our audit of current practice, but the contribution of ethnicity and genotype has not been fully explored at younger ages. This study will provide data that will enable us to look for external funding to conduct a formal trial of vitamin D administration based on both phenotype and genotype the so-called "personalised medicine" approach now being advocated for many conditions and their treatments. Because vitamin D deficiency is common in the UK, we expect that these data will have impact for all children, but most especially those with darker skin, or who have restricted exposure to sunlight either through choice (clothing) or situation, e.g. children with chronic immobilising conditions.

16.0 OUTCOMES (max 400 words)

We expect the outcomes to be relatively simple in terms of whether there are significant differences that need to be accounted for on the basis of ethnicity and genotype when considering administration of vitamin D; we expect to submit a further grant proposal based on the outcome of these preliminary study that is likely to be multi-centred in nature and externally funded.

17.0 Authorisations

Finance Department Representative:

Finance Department Signature: **Date:**

Applicant's Manager:

Applicant Manager's Signature: **Date:**

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04/12/2014

Dr Sheila Fisher
NRES Committee Yorkshire & The Humber –Leeds West
Room 002, Jarrow Business Centre
Rolling Mill Road
Tyne and Wear
NE32 3DT

Dear Dr Fisher

Study Title: **Determining the effects of race, skin colour and genotype on the response to vitamin D therapy**

REC reference: **14/YH/1149**

Protocol number: **SCH/14/053**

IRAS project ID: **14781**

Substantial amendment

One of the objectives of this study is to assess the response of 'free' (not bound to protein) vitamin D levels to vitamin D treatment. Existing methods to measure free vitamin D in blood are not very accurate, or are too complex for general use.

Our colleagues in the Academic Unit of Bone Metabolism have developed a method to measure free vitamin D in saliva, and we would like to add this to our study protocol. It will add value to the study by giving a different method of free vitamin D measurement to confirm our results, and to evaluate how the salivary test performs in response to a high dose of vitamin D.

We will ask the participants to give a saliva sample at each of the three study visits. The participants will be asked to rinse their mouth with water, then spit into a tube. There is no extra visit or risk associated with the collection of this extra sample.

We attach the amended documents for your review

Protocol – version 4 – 04/12/2014

Participant information sheet – version 3 – 04/12/2014

Consent form – version 3 – 04/12/2014

Email advert – version 3 – 04/12/2014

Thank you for considering this amendment.

Should you require any further information please get in touch with me on sujatha.gopal@sheffield.ac.uk.

Yours sincerely

Appendix 6 Vitamin D Dosing Study Participants Documents

Sheffield Children's NHS
NHS Foundation Trust

ADULT PARTICIPANT INFORMATION SHEET

Getting vitamin D dosing right

We would like to invite you to take part in our research study. Before you decide we would like you to understand why the research is being done and what it would involve for you. **One of our team will go through the information sheet with you and answer any questions you have.** Talk to others about the study if you wish. You don't have to take part if you don't want to. You can stop at any time without giving a reason.

Part 1 tells you the purpose of this study and what will happen to you if you take part.
Part 2 gives you more detailed information about the conduct of the study.

Ask us if there is anything that is not clear.

Part 1 – to give you first thoughts about the project

1. What is the purpose of the study?
The aim of this study is to make sure that people get the right dose of vitamin D treatment and to find out if skin colour, ethnicity, and genetic variation affect the response to a single dose of vitamin D in young adults.

2. Why have I been invited?
You have been chosen because you are a healthy male aged between 18-25 years.

3. Do I have to take part?
It is up to you to decide to join the study. We will describe the study and go through this information sheet with you either by phone or face to face. If you agree to take part, we will then ask you to sign a consent form the first time you come to the hospital, once you have told us that you have no further questions. You will be given a copy of the information sheet and the signed consent form to keep. You are free to withdraw at any time, without giving a reason.

4. What will happen to me if I agree take part?
Taking part in the study will involve you making 4 visits to our clinical research facility at Sheffield Children's Hospital.

Visit 1 – time estimate 15-30 min
During this visit we will answer any questions in relation to the study, obtain consent for the study from you and provide you a urine bottle and instructions for 2nd void urine samples. To provide this sample, you pass urine once shortly after you get up/wake up, drink tap water and then provide a second sample – this second sample is called a "2nd void urine sample". If you haven't had anything to eat or drink from midnight except tap water, then it is a "fasting 2nd void urine sample".

Visit 2 – time estimate 60-90 min
For this visit we will ask you to not to have anything to eat or drink except tap water from midnight before the day of your visit.

We will ask you to bring the fasting 2nd void urine sample with you, or provide it whilst here.
We will then do the following

1. Measure your height, weight and waist: hip circumference ratio.
2. Grade your skin colour (using the Fitzpatrick grading scale).
3. Assess your dietary intake of calcium using the DIETQ food frequency questionnaire (FFQ).
4. Take a blood sample from you for vitamin D studies. (You will be requested to attend fasting (i.e. no food at all, only tap water from midnight of the night before) until the blood sample (10 ml, approx 2 teaspoons) is taken).
5. **Take a saliva sample from you. (You will be asked to rinse your mouth out with water then spit into a tube).**

Finally we will give you a single dose of 150,000 IU of Vitamin D3 (6 mls of Invita D3 25,000 IU/ml oral solution) to be taken orally under direct supervision. We will also provide you with another urine bottle to bring in the 2nd void urine sample for the third visit.

Visit 3 – time estimate 10 -15 min
This visit is one week after the initial visit. We will ask you to bring in the repeat sample of your fasting second void urine sample to measure the calcium: creatinine ratio during this visit, **and to give another saliva sample.**

Visit 4 – time estimate 60-90 min
This visit is 4 weeks after the initial visit and we will repeat all the blood samples **and the saliva sample** during this visit.

5. Expenses and payments

You will be paid £50 voucher in total (paid on completion of the study) in recognition of your time and dedication to take part in our study.

6. What will I have to do?

1. Sun light exposure:
Ideally we would prefer to conduct the study this winter i.e from October to February to avoid sun exposure that allows skin synthesis of vitamin D. Nivea Sun Lotion Factor 50 (200 ml pump, hypoallergenic, fragrance-free) will be provided to you for regular use during the study period of a total of 4 weeks to avoid influence of sun exposure if carried out in summer months.

2. Travel and Vitamin D supplements:
You will be requested not to make any plans to travel abroad during the study period. If you are planning to go abroad, please tell us so we can schedule your participation to a later date. You will also be asked to refrain from taking vitamin supplements during the study period.

7. What are the possible disadvantages and risks of taking part?

There is a risk, as with many other clinical research projects, of exposing previously unknown or unexpected clinical findings in you. You will be made aware prior to enrolling in the study that any information of this type will, with your permission, be communicated to your general practitioner for further action. We will also contact you to tell you what is happening.

8. What are the side effects of any treatment received when taking part?

The frequency of developing an adverse reaction to vitamin D is uncommon. Taking a single large dose of vitamin D might lead to an increase in vitamin D levels in the blood. This might then lead onto an increased level of calcium in the blood and its excretion in the urine. The common symptoms and signs related to high levels of calcium in the blood are nausea, vomiting, loss of appetite, flushing, muscle weakness, excessive thirst, increased urine output, constipation, abdominal pain and inflammation of the pancreas. Excessive amounts of calcium in the urine might lead to kidney failure.

However, there is no evidence in the literature suggesting a rise in calcium levels in blood (hypercalcaemia) or urine (hypercalciuria) following a single dose of 150,000 IU of vitamin D3 in otherwise normal individuals. To ensure your safety we will check for calcium levels in the urine with the spot urine testing that is scheduled for 1 week after vitamin D dosing.

The figures for side effects quoted by the producer of the Invita D3 solution are – hypercalcaemia and hypercalciuria occurring in less than 1 in 100 individuals; rash, pruritus (itching) and urticaria (allergic rash, hives) occurring in less than 1 in 1,000 individuals. These figures may represent a broader (in terms of both age and other illnesses or ill-health) population who may therefore be more at risk of such complications than in healthy young adult men.

8. What are the possible benefits of taking part?

There is no expected direct benefit for you but the information we get from this study will help improve the treatment of people with vitamin D deficiency. If your vitamin D levels are low before taking the vitamin D dose, taking 150,000 IU of vitamin D will help you increase your body vitamin D stores.

With your consent, we will inform your GP if your vitamin D level is low. There are no recommendations in place at present in the UK to supplement young adults with vitamin D on a routine basis. Your GP may suggest that you take a multivitamin that contains vitamin D during the winter months. For most people this is sufficient to keep vitamin D levels up.

9. What happens when the research study stops?

We will collect all the information together and we will decide if it is useful in telling us if the doctors can manage Vitamin D deficiency better in the future.

10. What if there is a problem?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in Part 2.

11. Will my taking part in the study be kept confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. The details are included in Part 2.

This completes Part 1.

If the information in Part 1 has interested you and you are considering participation, please read the additional information in Part 2 before making any decision.

Part 2 of the information sheet

12. What if relevant new information becomes available?

Sometimes we get new information about the treatment being studied. If this happens, someone from the research team will tell you and discuss whether you should continue in the study. If you decide not to carry on, arrangements will be made for your care to continue. If you decide to continue in the study you may be asked to sign an agreement outlining the discussion.

13. What will happen if I don't want to carry on with the study?

You can withdraw from treatment any time you wish. Information collected may still be used. Any stored samples that can still be identified as yours will be destroyed if you wish.

14. What if there is a problem?

Complaints
If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions.

Name:	Dr J Sujatha Gopal-K
Title:	Clinical Research Fellow
Hospital/Department:	Department of Human Metabolism Room C7, C floor, Stephenson wing Sheffield Children's Hospital Sheffield
Telephone:	0114 271 7228

If you remain unhappy and wish to complain formally, you can do this by contacting:

Mrs Linda Towers
Patient Advice & Liaison Co-ordinator
Sheffield Children's NHS Foundation Trust
Tel: 0114 271 7594

Mrs Linda Towers will liaise with our Research & Innovation department manager to help solve the issue.

Harm
In the event that something does go wrong and you are harmed during the research and this is due to someone's negligence then you may have grounds for a legal action for compensation, but you may have to pay your legal costs. The normal NHS complaints mechanisms will still be available to you.

15. Will my taking part in this study be kept confidential?
All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital will have your

name and address removed so that you cannot be recognised from it. All study data will be retained for a period of 5 years following the end of the study.

Our procedures for handling, processing, storage and destruction of data are compliant with the Data Protection Act 1998.

We will also ask for permission to inform your family GP that you will be taking part in the study.

16. What will happen to any samples I give?

Once tested for your vitamin D levels along with the other related metabolites, the residual blood (serum/plasma) **and saliva samples** not used in the analyses will be transferred to an appropriate Biobank to be saved for up to 5 years with your consent.
If you have given consent the sample will be used for future research. This would be in relation to future studies of younger people/ children's bone metabolism. Professor Bishop will act as custodian and control access to the samples.

17. Will any genetic tests be done?
Genetic testing to look at factors affecting vitamin D metabolites will be carried out in the blood samples collected as a part of the study.

18. What will happen to the results of the research study?

When the study has finished we will present our findings to other researchers, and we will put the results in medical magazines and websites that researchers read. We would also like to put a brief summary on the hospital research website so that you will be able to read about our results too. This will be available at the end of the study, in *January 2015* on www.sheffieldchildrens.nhs.uk/research-and-innovation.htm
They will be anonymous, which means that you will not be able to be identified from them.

19. Who is organising and funding the research?

The research is being organised by Sheffield Children's NHS Foundation Trust and paid for by Sir Halley Stewart Trust

20. Who has reviewed the study?
All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given a favourable opinion by Leeds West Research Ethics Committee.
It has also been given approval by the Research Department to run at this hospital.

21. How can I find out more?
If you would like to find know more about research in general, the Clinical Research Facility at this hospital has an **Information for families** section on its website www.sheffieldchildrens.nhs.uk/research-and-innovation.htm or you could contact the hospital Clinical Research Facility:

Ms Wendy Swann

R&D Manager
Sheffield Children's NHS Foundation Trust
Tel: 0114 3053478

If you would like to know more specific information about this research project, please contact the project co-ordinator:

Name:	Dr J Sujatha Gopal-K
Title:	Clinical Research Fellow
Hospital/Department:	Department of Human Metabolism Room C7, C floor, Stephenson wing Sheffield Children's Hospital Sheffield
Telephone:	0114 271 7228

If you would like advice as to whether you should participate you could contact the project team, or one of your health care professionals.

If you have any concerns during the study, you should contact the project team.

If you decide to take part in this study, you will be given this information sheet and signed consent form to keep.

Thank you for taking the time to read this information sheet.

Sheffield Children's NHS
NHS Foundation Trust

Participant study number:

PARTICIPANT CONSENT FORM

Getting vitamin D dosing right

Name of researcher: Dr J S Gopal-K

Please initial box

1. I confirm that I have read and understand the information sheet dated 25.09.1414 (version 2) for the above study. I have had the opportunity to consider the information ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of any of my medical notes and data collected during the study, may be looked at by researchers and those involved in the running and supervision of the study from Sheffield Children's NHS Foundation Trust or from regulatory authorities, where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐

4. I agree to my GP being informed of participation in this study. ☐

5. I agree to take part in the above study. ☐

6. **I agree for my blood and saliva samples to be stored and used for future research for up to 5 years.** ☐

_____	_____	_____
Name of Participant	Date	Signature
_____	_____	_____
Name of Person taking consent	Date	Signature

When completed: 1 for participant; 1 for researcher site file

Appendix 7 Vitamin D Dosing Study Advertisements

Email template

Subject: Are you a male aged between 18 and 25 years of age? If so, this is for you.....

Hi

I am a clinical research fellow in Paediatric Endocrinology, conducting research on "Determining the effects of race, skin colour and genotype on the response to vitamin D therapy" and would really appreciate your help.

Taking part in the study will involve you making 4 visits (3 hours in total) to our clinical research facility at Sheffield Children's Hospital. The study includes collection of anthropometry data, blood, urine and **saliva samples for Vitamin D studies** and administration of a stoss dose of vitamin D.

You will be paid £50 voucher in total (paid on completion of the study) in recognition of your time and dedication to take part in our study.

If you are interested and would like to get more information please email me at sujatha.gopal@sheffield.ac.uk

My research supervisor is Prof Bishop, who you can email at n.j.bishop@sheffield.ac.uk.

Thanks and I look forward to hearing from you.

Regards

Sujatha Gopal

Dr Sujatha Gopal
Research Fellow in Paediatric Endocrinology
Room C 2, C Floor, Academic Unit of Child Health
Department of Human Metabolism
University of Sheffield
Stephenson Wing
Sheffield Children's Hospital
Western Bank
Sheffield
S10 2TH

Phone : 0114 271 7228

Appendix 8 Fitzpatrick Skin Types

Eye colour

0. Light colours
1. Blue, gray or green
2. Dark
3. Brown
4. Black

Natural hair colour

0. Sandy red
1. Blond
2. Chestnut or dark blond
3. Brown
4. Blac k

Your skin colour (unexposed areas)

0. Reddish
1. Pale
2. Beige or olive
3. Brown
4. Dark brown

Freckles (unexposed areas)

0. Many
1. Several
2. Few
3. Rare
4. None

If you stay in the sun too long?

0. Painful blisters, peeling
1. Mild blisters, peeling
2. Burn, mild peeling
3. Rare
4. No burning

Do you turn brown?

0. Never
1. Seldom
2. Sometimes
3. Often
4. Always

How brown do you get?

0. Never
1. Light tan
2. Medium tan
3. Dark tan
4. Deep dark

Is your face sensitive to the sun?

0. Very sensitive
1. Sensitive
2. Sometimes
3. Resistant
4. Never have a problem

How often do you tan?

0. Never
1. Seldom
2. Sometimes
3. Often
4. Always

When was your last tan?

0. +3 months ago
1. 2 - 3 months ago
2. 1 - 2 months ago
3. Weeks ago
4. Days

Score		
0 - 6	Skin Type I	Always burns, never tans (pale white skin)
7 - 13	Skin Type II	Always burns easily, tans minimally (white skin)
14 - 20	Skin Type III	Burns moderately, tans uniformly (light brown skin)
21 - 27	Skin Type IV	Burns minimally, always tans well (moderate brown skin)
28 - 34	Skin Type V	Rarely burns, tans profusely (dark brown skin)
35+	Skin Type VI	Never burns (deeply pigmented dark brown to black skin)