Vitamin D deficiency, nutritional rickets and osteomalacia: Hidden disease burden and implications for public health prevention policies

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ABSTRACT

Vitamin D deficiency remains the most common cause of nutritional rickets in the UK with considerable rise in reported cases in the last decade, however the true extent of disease burden remains unexplored. The thesis contains three prospective studies investigating the hidden burden of vitamin D deficiency in: 1) infants presenting with symptomatic deficiency and their family members, 2) a large multi-ethnic newborn cohort and 3) Sudden Unexpected Death in Infancy and Childhood (SUDIC).

In infants with severe deficiency, significant cardiac dysfunction, growth plate abnormalities and osteomalacia was uncovered in infants with severe deficiency; and biochemical osteomalacia in 77% of their asymptomatic family members. Only a third of the UK newborns (n=919/2999) had sufficient 25 hydroxyvitamin D (25OHD) levels, with babies born in the winter to dark-skinned mothers most affected. At post-mortem examination (n=33, 29 infants) 70% had histological rickets at the growth plate but only 40% manifest biochemical vitamin D deficiency and only 9% radiological rickets. Lower 24,25 dihydroxyvitamin D_3 [$24,25(OH)_2D_3$] levels and elevated ratio of $25OHD_3$:$24,25(OH)_2D_3$ were better indicators of histological rickets when compared to $25OHD_3$. Elevated osteoid surface, volume and thickness were noted in roughly 33%, 62% and 14% respectively. On three-point bending test, rib samples with histological rickets displayed disproportionate displacement in response to load indicating increased plasticity and had a tendency towards higher fracture toughness.

The findings of this thesis reveal the disease burden which has significant implications for public health prevention policies on antenatal and postnatal vitamin D supplementation, additionally it informs future research.

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“If we knew what it was we were doing, it would not be called research, would it?”

— Albert Einstein

“We know very little, and yet it is astonishing that we know so much, and still more astonishing that so little knowledge can give us so much power.”

— Bertrand Russell

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2nd Vitamin D Rank forum, Surrey, UK, 2019: Nutritional rickets: UK prevalence and policies.

21st Workshop on Vitamin D, Barcelona, 2018: Life threatening complications of vitamin D deficiency in infants: Hypocalcaemia dilated cardiomyopathy, cardiac arrest and death.

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Media Engagement

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Abbreviations

25OHD	25 hydroxyvitamin D or calcidiol
$25OHD_2$	25 hydroxyvitamin D2
$25OHD_3$	25 hydroxyvitamin D3
1, 25 $(OH)_2D$	1, 25 dihydroxyvitamin D or calcitriol
24,24$(OH)_2D$	24,25 dihydroxyvitamin D
3-epi-25OHD	3-epi-25 hydroxyvitamin D
AI	Adequate Intake
ALP	Alkaline Phosphatase
ASTM	American Society for Testing and Materials
BAME	Black, Asian and Minority Ethnic
BMDD	Bone Mineral Density Distribution
BPSU	British Paediatric Surveillance Unit
DBS	Dried Blood Spot
EAR	Estimated Average Requirement
IOM	Institute of Medicine
IU	International units
NBS	Newborn Screening
NICE	National Institute for Health and Care Excellence
PHE	Public Health England
PTH	Parathyroid hormone or parathormone
qBEI	Quantitative backscattered electron imaging
RNI	Reference Nutrient Intake

SACN	Scientific Advisory Committee on Nutrition
SIDS	Sudden Infant Death Syndrome
SUDI	Sudden Unexpected Death in Infancy
SUDC	Sudden Unexpected Death in Childhood
SUDIC	Sudden Unexpected Death in Infancy and Childhood
TNSALP	Tissue non-specific alkaline phosphatase
YPSG	Young Person's Steering Group

Chapter 1: **Introduction to the thesis**

1.1 Introduction

Rickets and osteomalacia occur as a consequence of defective mineralization of the skeleton [1]. Such reduced mineralization results in soft bones, which deform and break more easily, and stunting of growth. Defective mineralization occurs due to:

1) Lack of mineral supply to the body (calcium or phosphate),

2) Lack of the mineral supplier calcitriol (active form of vitamin D) which controls the intestinal absorption of calcium and phosphate and

3) Less commonly due to lack of the tissue non-specific alkaline phosphatase (TNSALP) enzyme which facilitates mineralization.

The leading cause of rickets and osteomalacia worldwide are solar vitamin D deficiency and/or dietary calcium deficiency, collectively termed as 'nutritional' [2]. In high latitude countries such as the United Kingdom, solar vitamin D deficiency predominates, especially in the Black, Asian and Minority Ethnic (BAME) population groups [3]. Infants and adolescents with vitamin D deficiency can present with complications of low calcium (hypocalcaemia), due to the high demands during phases of rapid growth [4]. Hypocalcaemic complications can be life threatening and include seizures, tetany [5] and cardiac failure secondary to dilated cardiomyopathy in infants [6]. Hypophosphataemic complications occur following prolonged vitamin D or dietary calcium deficiency and include muscle weakness, rickets and osteomalacia. Osteomalacia, the softening of bone tissue, mostly remains undiagnosed due to the less specific clinical manifestations such as malaise, fatigue and tiredness [2].

In the UK, despite the presence of antenatal and infant vitamin D supplementation policies for prevention of rickets, several reports of symptomatic vitamin D deficiency in infants and children have emerged over the last decade [4-9]. Yet, these reports of symptomatic vitamin D deficiency only capture the tip of the iceberg. The true burden of rickets and osteomalacia remains unexplored to date. Studying the hidden iceberg of the deficiency is important not only to assess the effectiveness of the current public health policies but also to influence the course of future prevention policies.

1.2 Hypothesis

Nutritional vitamin D deficiency, occult rickets and osteomalacia in UK infants and children are more widely prevalent than currently reported.

1.3 Aims and objectives

1.3.1 Aim:

The overall aim of this thesis was to investigate the true burden or the hidden iceberg of nutritional vitamin D deficiency, occult rickets and osteomalacia in UK children, with a focus on infants.

1.3.2 Objective:

The main research objectives were to:

- Detail the hidden pathology in a cohort of infants presenting to a single paediatric tertiary centre with symptomatic vitamin D deficiency

- Determine the prevalence of biochemical osteomalacia in family members, of the above cohort, of infants presenting with symptomatic vitamin D deficiency
- Identify the prevalence of vitamin D deficiency in a multi-ethnic newborn cohort on blood spot screening
- Study the ethnic and seasonal variations and determinants of newborn blood spot vitamin D levels
- Determine the prevalence of vitamin D deficiency, occult rickets and osteomalacia in cases of Sudden Unexpected Death in Infancy and Childhood (SUDIC).

1.4 Thesis outline

The current thesis reports the findings of a series of three prospective studies (**Figure 1.1**) which utilise different methodologies and collectively explore the hidden burden of vitamin D deficiency, rickets and osteomalacia in UK infants and young children.

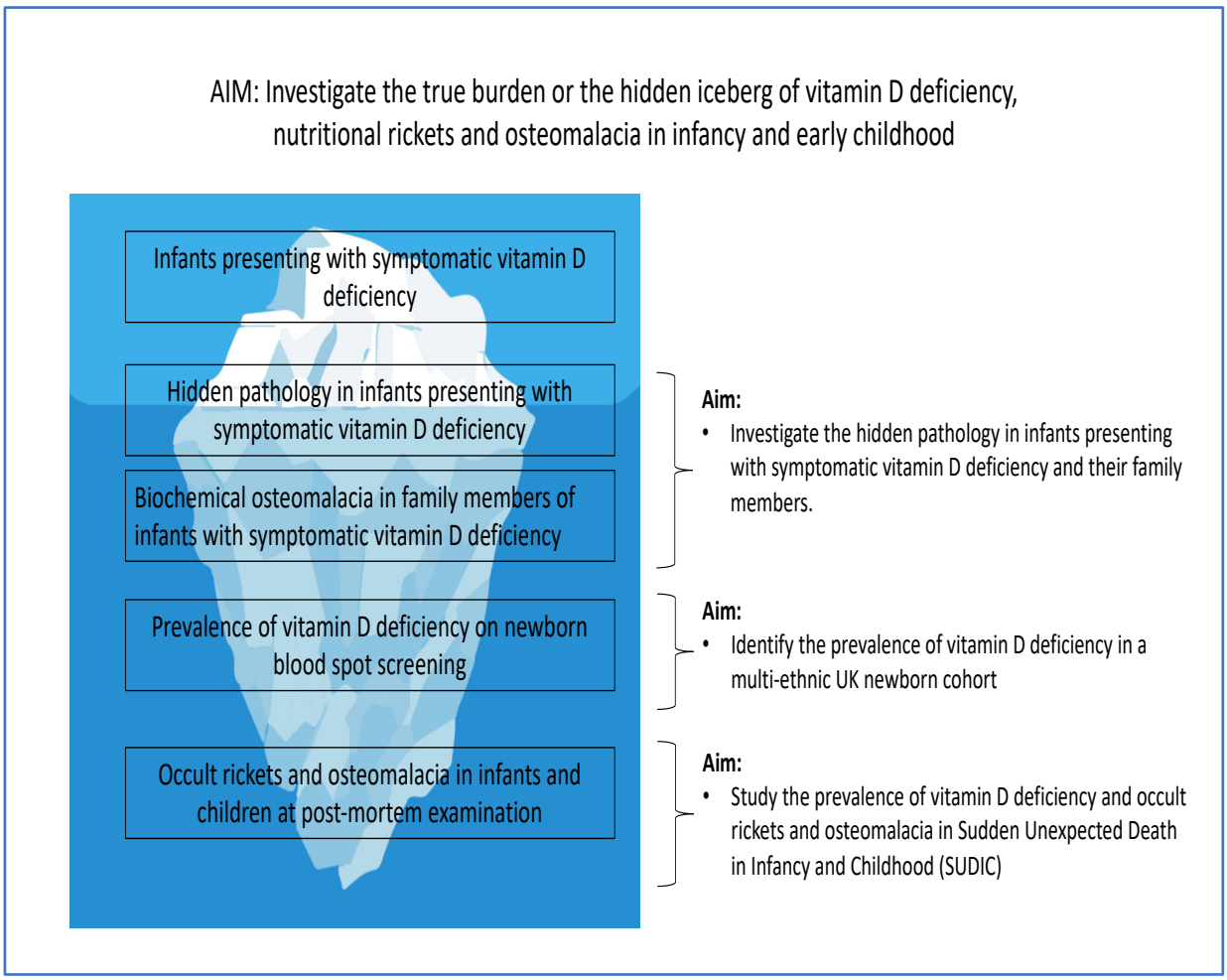


Figure 1.1: Thesis outline

A summary of the content of subsequent chapters is as follows:

Chapter 2: This introductory chapter clarifies relevant terminologies and details the pathophysiology of mineralization and classification of rickets. The clinical features and management of nutritional rickets are discussed in brief.

Chapter 3: In this background chapter, the burden of nutritional rickets in UK infants and young children is reviewed. The background work, comparing UK infant vitamin D supplementation policy to policies across 28 European countries, which led to the thesis is detailed.

Chapter 4: This chapter describes a prospective series of cases with symptomatic vitamin D deficiency presenting to a single paediatric tertiary centre. The hidden pathology in these infants and prevalence of biochemical osteomalacia in family members are detailed.

Chapter 5: This chapter describes a newborn blood spot screening study determining the prevalence of vitamin D deficiency in a multi-ethnic newborn cohort. The utility of blood spot 25 hydroxyvitamin D (25OHD) in population screening is reported.

Chapter 6: This chapter describes a prospective cohort study exploring the prevalence of vitamin D deficiency and occult rickets and osteomalacia, at post-mortem examination, in Sudden Unexpected Death in Infancy and Childhood (SUDIC).

Chapter 7: In this discussion chapter, the findings from the three studies are summarised and implications for public health policies discussed.

Chapter 2: **General introduction: pathophysiology and clinical management of rickets**

Parts of this chapter has been published as:

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Uday S, Högler W. Spot the silent sufferers: A call for clinical diagnostic criteria for solar and nutritional osteomalacia. J Steroid Biochem Mol Biol. 2019;188:141–6.

2.1 Pathophysiology of bone mineralization

An understanding of endochondral ossification and mineralization is essential to appreciate the pathogenesis and clinical features of rickets and osteomalacia.

2.1.1 Clarification of terminologies:

Table 2.1: Clarification of terminologies relating to pathophysiology of bone mineralization

Terminology	Clarification
Bone formation	Describes the process of synthesis of extracellular organic osteoid followed by its mineralization, with later integration of osteoblasts which transform into osteocytes.
Mineralization	Relates to the integration of hydroxyapatite $[Ca_{10}(PO_4)_6(OH)_2]$ into osteoid.
Ossification	Relates to the two major modes of bone development: intramembranous ossification seen in flat bones (e.g.skull) and endochondral ossification seen in long bones (e.g.femur) [10].
Calcification or pathological mineralization	Deposition of calcium-phosphate crystals in extra-skeletal tissues (kidneys, skin, arteries, muscle, brain, tumours) [11].
Phosphopaenia	Deprivation of body phosphorus
Calcipaenia	Deprivation of body calcium
Hypocalcaemia	Low serum calcium
Rickets	Defective mineralization of the growth plate cartilage and adjacent primary and secondary spongiosa in the metaphysis (‘new bone’) and thus can only affect growing children with open epiphyses.
Osteomalacia	Defective mineralization of newly formed osteoid in existing bone during remodelling (‘old bone’). Can affect humans of all ages, thus always co-exists with rickets in children.
Osteoporosis	Reduced bone mass and structural quality. It is not associated with reduced bone mineralization of bone tissue. Osteoporosis occurs when bone resorption exceeds bone formation (adults and children) or from reduced bone mass accrual (children).

2.1.2 Bone composition:

The bone tissue or matrix is composed of 50 to 70% mineral (predominantly calcium and phosphate), 20 to 40% organic material (predominantly type 1 collagen), 5 to 10% water, and <3% lipids [12]. The three main types of bone cells are:

1) osteoblasts which form new bone,

2) osteoclasts which resorb old bone and calcified growth plate cartilage, and

3) osteocytes which constitute 90% of the cells in the matrix and are responsible for biomechanical sensing and hormone production [13].

2.1.3 Endochondral ossification

Longitudinal growth occurs through endochondral ossification in long bones. Condensation of mesenchymal cells to form cartilage precedes their differentiation into chondrocytes [14]. These cells then secrete cartilage extracellular matrix components. The cartilage structure is invaded by cells to form the primary ossification center at the centre (midshaft) followed by secondary ossification centers at the ends (epiphyses). The primary and secondary ossification centers are separated by the growth plate which is responsible for longitudinal growth and drives expansion of the primary ossification center [14]. The articular epiphyseal growth cartilage (AEGC) is responsible for growth and shaping of the epiphyses and drives expansion at the secondary ossification center. The primary and secondary ossification centers encroach onto each other, gradually replacing the cartilage with bone tissue except at the articular surface. **Figure 2.1** depicts the events leading to the development of endochondral bones [14].

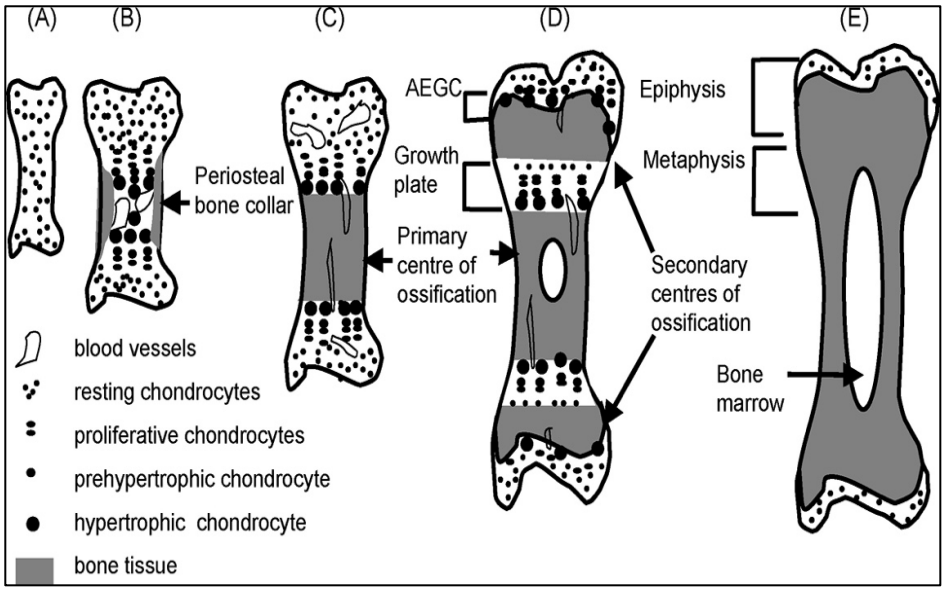


Figure 2.1: Schematic diagram showing the events leading to replacement of an embryonic cartilage model of bone

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(A) Cartilage model. (B) Initiation of formation of the primary centre of ossification in the centre of the cartilage model, with chondrocyte hypertrophy and vascular invasion. (C) Well established primary centre of ossification; blood vessels are present in cartilage canals in the remaining cartilage. (D) The secondary centres of ossification have formed and are separated from the primary centre of ossification by the growth plate, which is responsible for longitudinal growth. Articular-epiphyseal growth cartilage (AEGC) remains under the permanent articular cartilage and is responsible for growth and shaping of the epiphysis. (E) In the adult bone, the metaphyseal and epiphyseal bone have fused to each other, leading to the disappearance of the growth plate. The AEGC has been replaced by bone, and the only remaining cartilage is the permanent articular cartilage at each end of the bone.

The chondrocytes in the growth plate are arranged in morphologically distinct zones **(Figure 2.2**) [15]. At the epiphyseal end of the growth plate is the reserve zone or resting zone. Cells in the resting zone then enter the proliferative zone where they undergo division and produce large amounts of extracellular matrix protein [16]. The proliferative cells lose their capacity to divide and then start to increase in size (pre-hypertrophic zone) until they are fully differentiated to form hypertrophic chondrocytes (in the hypertrophic zone) [16] close to the

ossification center. Intracellular calcium concentration continues to increase in the hypertrophic chondrocytes and at some point they start to mineralize the longitudinal septa in the surrounding matrix to form the zone of provisional calcification [16]. The final apoptosis of hypertrophic chondrocytes triggers mineralization of the matrix and vascular invasion (facilitating osteoblast invasion) at the metaphyseal/growth plate junction [primary spongiosa].

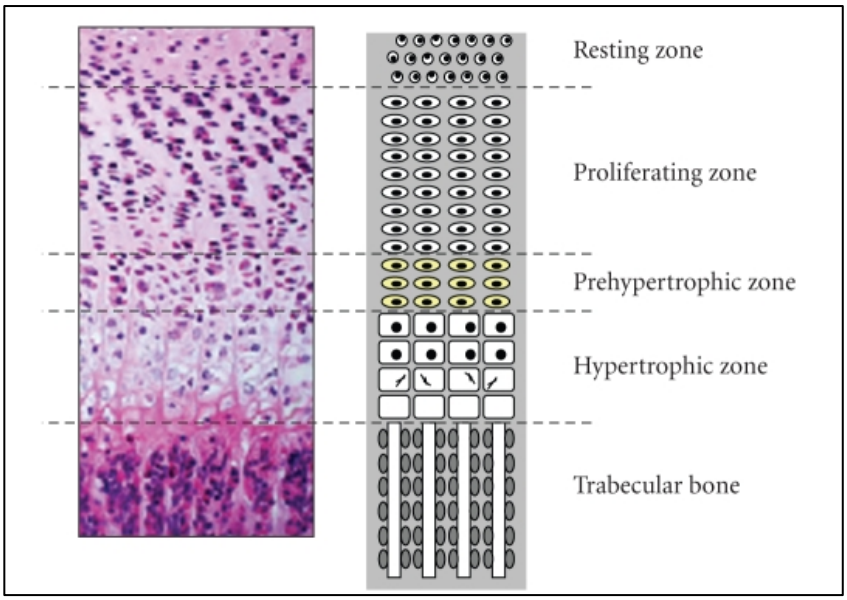


Figure 2.2: A histological (haematoxylin and eosin stained) image of an adult mouse growth plate and schematic representation of differentiation zones in the tissue

Figure reproduced from [15] in accordance with the Creative Commons Attribution License. In the growth plate several distinct structural zones can be identified, reflecting the gradual transition of cells through different stages of differentiation. The resting zone acts as a reserve of precursor cells for the proliferating chondrocytes in the columns. The proliferating zone is where the cells flatten and divide, laying down a cartilage extracellular matrix that will later serve as a scaffold for bone formation. In the prehypertrophic zone, the cells enter the maturation zone and begin to enlarge. In the hypertrophic zone, the chondrocytes and their lacunae become 5–12 times bigger. These cells eventually die, triggering vascularisation and bone formation.

Lack of phosphate supply at the growth plate [17] prevents apoptosis of hypertrophic chondrocytes and thus inhibits both mineralization and growth in rickets. The enlarged hypertrophied chondrocyte layer contributes to the clinical appearance of swollen joints (wrists and ankle) and radiological appearance of widened growth plates in children with rickets. Eighty percent of the longitudinal septa of the growth cartilage are rapidly resorbed in the metaphyseal zone immediately behind the invading blood vessels and the remaining longitudinal septa serve as scaffolds, on which osteoblasts deposit further bone matrix [secondary spongiosa] [16].

2.1.4 Mineralization

Bone is mineralized connective tissue. The most abundant minerals in bone matrix are calcium and phosphorus, present in the form of hydroxyapatite crystals [$Ca_{10}(PO_4)_6OH_2$]. The mineralization process therefore requires adequate supply of substrates, calcium and phosphate, which are supplied by Calcitriol (1,25 dihydroxy vitamin D) through intestinal absorption.

The mechanism of initiation and regulation of cell mediated mineralization is not entirely clear, however several theories have been proposed [18]. Matrix vesicles (MVs) protruding from osteoblasts play an important role in mineralization [19]. One theory suggests that MVs bud off the parental plasma membrane, another suggests that MVs represent the rearrangement of the apoptotic cell membrane [20].

The first step in mineralization is the formation of hydroxyapatite in MVs of mature chondrocytes and osteoblasts [20]. During Phase I, there is increased activity of the MV phosphatases (including alkaline phosphatase, adenosine triphosphatase, pyrophosphatase and

PHOSPHO1) which generate and transport phosphate; as well as calcium binding compounds such as the annexin family and phosphatidylserine [21]. Calcium and phosphate are attracted into the MVs by these compounds, until the threshold for their precipitation is reached [19]. In Phase II, the hydroxyapatite crystals penetrate the MV membrane and are elongated into the extracellular space [19], which requires appropriate concentrations of calcium and phosphate outside the MVs. Tissue non-specific alkaline phosphatase (TNSALP) plays a crucial role in the formation of hydroxyapatite crystals by hydrolysing inorganic pyrophosphate (PPi), an inhibitor of hydroxyapatite formation, to inorganic phosphorus (Pi) [22]. The ratio of PPi to Pi is of critical importance in the promotion or indeed restriction of mineral in physiological tissues [20]. TNSALP, encoded by the *ALPL* gene, is abundant on the surface of MVs derived from osteoblasts and hypertrophic chondrocytes [23]. Reduced TNSALP activity leads to hypomineralization as seen in hypophosphatasia [24].

2.2 Pathogenesis and classification of rickets

Based on the primary pathology, rickets and osteomalacia can be classified broadly into defective mineral supply (calcipaenia, phosphopaenia) and defective mineralization (hypophosphatasia). Irrespective of the primary pathology, the underlying mechanism of all forms of rickets is low phosphate availability to the hypertrophic chondrocytes in the growth plate [17] resulting in lack of their apoptosis [25].

2.2.1 Calcipaenia

Deprivation of body calcium results in calcipaenic rickets. The most common cause is vitamin D and/or dietary calcium deficiency leading to nutritional rickets and osteomalacia.

2.2.2 Phosphopaenia

Deprivation of body phosphorus results in phosphopaenic rickets. The most common cause is renal phosphate wasting, due to excess Fibroblast Growth Factor 23 (FGF23), as seen in X-linked hypophosphataemic rickets (XLH) which is an inherited condition caused by *PHEX* gene mutation.

2.2.3 Hypophosphatasia

Hypophosphatasia (HPP) is caused by low alkaline phosphatase (ALP) resulting from lack of TNSALP due to mutations in the *ALPL* gene.

Table 2.2 details the pathophysiology and clinical and biochemical features of the three main forms of rickets using nutritional rickets, XLH and HPP as examples. The radiological findings in different forms of rickets are also distinctive and are presented in **Figure 2.3**.

Table 2.2: Pathogenesis, clinical and biochemical features of different forms of rickets

Nutritional rickets (NR)	**X-linked hypophosphataemic rickets (XLH)**	**Hypophosphatasia (HPP) rickets**
Pathogenesis		
Calcipaenia secondary to vitamin D deficiency and /or dietary calcium deficiency	Phosphopaenia due to renal phosphate wasting	Hypophosphatasaemia due to defective TNSALP
Distinguishing features		
Presence of risk factors (i.e. dark skin, covered clothing, indoor living, high latitude or reduced dietary intake of calcium or lack of vitamin D supplementation)	Family history of hypophosphataemic rickets, rarely *de-novo* mutations can occur. Usually present with bowing deformities of the legs.	Most severe forms present at birth with respiratory failure due to pulmonary hypoplasia, seizures and hypotonia. Older children have early tooth loss.
Biochemical features		
<u>Features common to calcium and vitamin D deficiency</u> ↔↓ Serum calcium, phosphate ↑ Alkaline phosphatase (ALP) ↑ Parathyroid hormone (PTH) <u>Vitamin D deficiency</u> 25OHD↓ and 1,25 dihydroxyvitamin D [$1,25(OH)_2D$] ↔↓ <u>Calcium deficiency</u> 25OHD ↔ $1,25(OH)_2D$ ↑	↓ Serum phosphate ↑ ALP ↔ Calcium, 25OHD ↑ ↔ PTH ↓ ↔ 1,25 dihydroxy vitamin D (inappropriately low) ↑ FGF23 ↓ TMP/GFR (Tubular Maximum Reabsorption of phosphate to Glomerular Filtration Rate)	↑↔ Serum calcium, phosphate ↓ ALP ↔ ↓PTH ↔ 25OHD ↑ Serum Pyridoxal-5-Phosphate (PLP) ↔↑ urinary calcium ↑ urinary phosphoethanolamine (PEA)

Nutritional rickets	**X-linked hypophosphataemic rickets**	**Hypophosphatasia rickets**
		
Growth plate widening, metaphyseal cupping and fraying. Generalised hypomineralization and periosteal elevation due to hyperparathyroidism.	Fraying and widening of metaphyses	Generalised hypomineralization, irregular zone of calcification; large tongue like radiolucent areas at the metaphyses

Figure 2.3: Radiological features of different forms of rickets

2.3 Nutritional rickets and osteomalacia

Rickets is defective mineralization of the growth plate cartilage and osteomalacia is the defective mineralization of newly formed osteoid in existing bone. The leading cause of rickets worldwide is nutritional, caused by deficiency of vitamin D and/or calcium [26]. Classification of deficiency/sufficiency of vitamin D and calcium, pathophysiology of calcium deprivation and its clinical manifestations are discussed here.

2.3.1 Classification of vitamin D and calcium status - from deficiency to sufficiency

Vitamin D status of an individual is established by measuring calcidiol or 25 hydroxyvitamin D (25OHD) which is the most stable form of vitamin D. Previously, vitamin D deficiency was regarded as the main cause of nutritional rickets with exaggerated disease seen in the presence of concurrent calcium deficiency. However, subsequently it was uncovered that dietary calcium deficiency in isolation can also result in rickets in the absence of vitamin D deficiency or insufficiency [27]. Calcium deficiency can result from low calcium intake in diet or poor intestinal absorption. Low calcium intake can result from reduced intake of dairy products due to intolerance or due to cultural dietary habits. Poor absorption of calcium can result from lack of vitamin D or other malabsorptive conditions. Classification of vitamin D status [28,29] and calcium requirements [29] are detailed in **Table 2.3.**

Table 2.3: Classification of vitamin D status and dietary calcium intake

	Serum 25OHD levels	Daily calcium intake
Sufficient	> 50 nmol/L (20 μg/L)	> 500 mg
Insufficient	30-50 nmol/L (12-20 μg/L)	300-500 mg
Deficient	< 30 nmol/L (12 μg/L)	< 300 mg

2.3.2 Physiologic response to low calcium in the body

The main function of calcitriol (active vitamin D) is to maintain calcium homeostasis in the body by increasing intestinal absorption of calcium and phosphate. Therefore, any decrease in dietary calcium or calcitriol action results in calcipaenia. Calcium deprivation in the body is sensed by the calcium sensing receptor in the parathyroid gland's chief cells which stimulate the release of Parathyroid hormone (PTH) [30].

High PTH increases: 1) osteoclastic bone resorption to release stored calcium,

2) renal calcium absorption and

3) 1α-hydroxylase activity to maximize calcitriol synthesis.

These compensation mechanisms maintain serum calcium at the expense of high bone resorption and renal phosphate loss leading to low serum phosphate **(Figure 2.4)**.

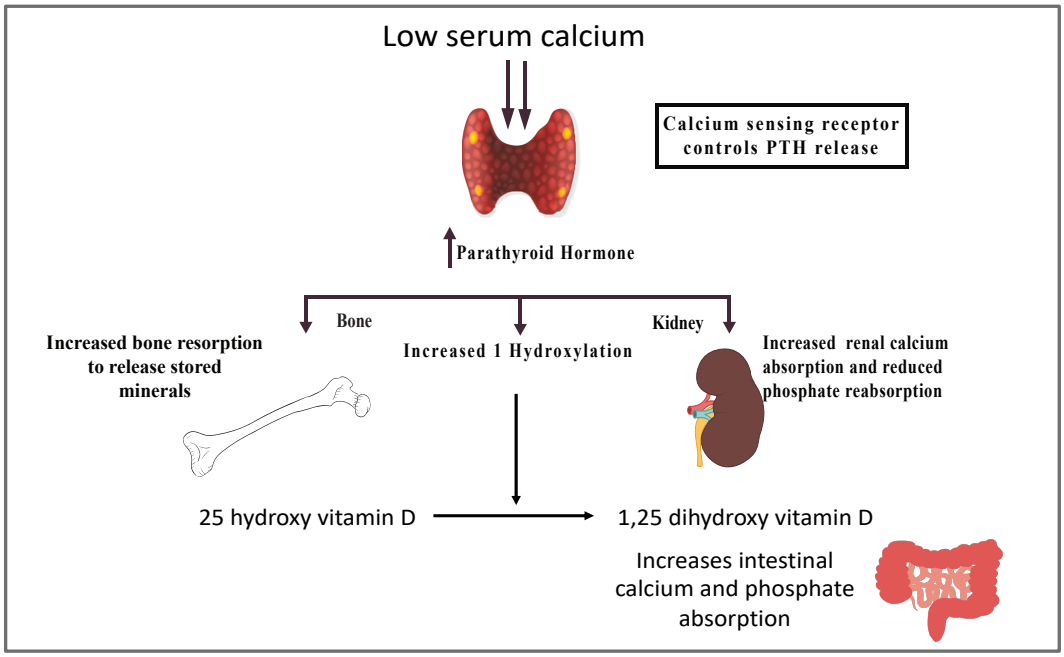


Figure 2.4: Physiologic response to low calcium in the body

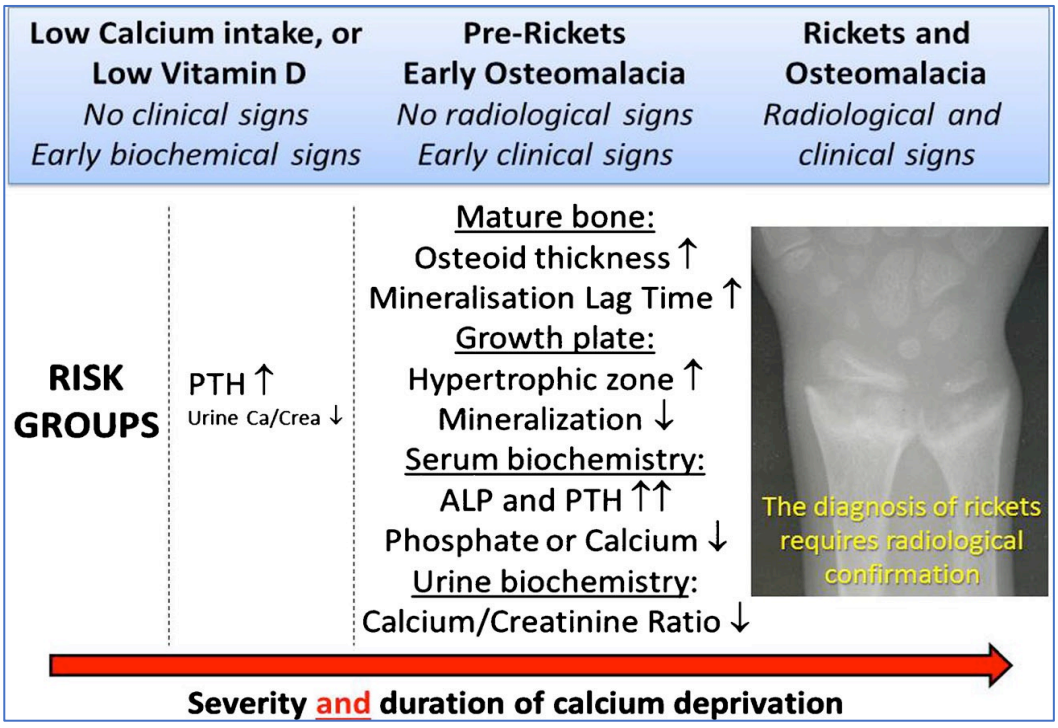


Figure 2.5: Stages of calcium deprivation leading to nutritional rickets and osteomalacia

2.3.3 Stages of calcium deprivation

In the initial stages of calcium deprivation, serum calcium is maintained by secondary hyperparathyroidism as detailed above. This state can be identified biochemically with raised PTH and reduced renal excretion of calcium [30]. Prolonged secondary hyperparathyroidism leads to hypophosphataemia, except in a vitamin D replete state with isolated calcium deficiency where there is elevated serum phosphate due to end organ resistance to PTH [31].

Hypophosphataemia results in lack of apoptosis and accumulation of hypertrophic chondrocytes in the growth plate resulting in typical changes of rickets [32]. Lack of apoptosis results in accumulation of osteoid (osteomalacia), alongside a rise in serum ALP, which is the first of the three histological stages of hypovitaminosis D as summarised in Bhan et al [33]. As the degree and duration of deficiency progresses there is increased accumulation of osteoid with preservation of mineral apposition in stage ii (as indicated by the presence of tetracycline uptake) which eventually ceases in stage iii (no tetracycline uptake)[33]. Radiological changes of rickets are said to be evident in stage iii of hypovitaminosis D [34]. The presence of radiological rickets in only 67% (n=72/107) [9] and 77% (23/30) [5] of subjects presenting with symptomatic vitamin D deficiency in the national surveys supports the histological staging and late appearance of radiological rickets. Similar to appearance of rickets, complete healing of radiological rickets following treatment also demonstrates a lag and on average takes around 3 months [35].

The concept of calcium deprivation and its evolution into rickets and osteomalacia is depicted in **Figure 2.5**. Because secondary hyperparathyroidism maintains normal serum calcium levels until compensation fails, serum calcium is a poor marker of calcium status.

2.3.4 At-risk population

Nutritional rickets can be caused by vitamin D and/or dietary calcium deficiency, therefore individuals at risk of both are listed in **Table 2.4.** Individuals of Black, Asian and Minority Ethnic (BAME) background with darker skin pigmentation are at high risk of vitamin D deficiency. Melanin in skin prevents UVB penetration, therefore individuals with darker skin require longer duration of exposure compared to those with lighter skin in order to produce the same amount of vitamin D [36]. People who practice whole body cultural clothing are at high risk as sunlight remains the main source of vitamin D in the absence of supplementation [37]. However, lack of recommendations specific to skin pigmentation and latitude of residence precludes reliability on sun exposure to optimise vitamin D status [36].

Table 2.4: Risk factors for vitamin D and dietary calcium deficiency

Vitamin D deficiency risk	**Calcium deficiency risk**
• Skin pigmentation - Dark skin produces less vitamin D due to excess melanin which reduces UVB penetration • Sun avoidance - Whole body clothing - Excessive sunscreen use - Indoor living/Institutionalisation • Lack of sunlight - High latitude residence - Environmental pollution • States of high physiological demand - Infancy - Pregnancy and lactation - Old age	• Dairy restricted diet - Cultural reasons - Food allergies - Lactose intolerance - Malnutrition • Malabsorption
Individuals with more than one risk factor are at highest risk	

Individuals with multiple predisposing factors are naturally at the highest risk of rickets and osteomalacia; such as the dark-skinned immigrants residing in high latitude countries who retain cultural practices such as whole-body clothing, sun avoidance and a diet restricted in dairy [38].

Pregnant women are classed as high risk due to increased demands during pregnancy. For adequate foetal bone development, a foetus must be provided with approximately 30g of calcium during gestation, which requires increased resorption of maternal bone, and increased intestinal calcium absorption [39]. The third trimester is the time where most foetal phosphorus and calcium deposition occurs [39].

2.3.5 Clinical presentation

The clinical features of rickets and osteomalacia depend largely on the age of presentation [4] and also the severity and duration of deficiency [40] (**Figure 2.5**) The presentation at various stages of life are depicted in **Figure 2.6** [2]. Infants and adolescents, who grow rapidly, frequently present with hypocalcaemic manifestations (seizures, tetany) [4]. Other childhood presentations include, limb deformities, delayed development and dentition, muscle pain and weakness. Adults with osteomalacia present with non-specific symptoms of malaise, fatigue or tiredness and often go undiagnosed [2].

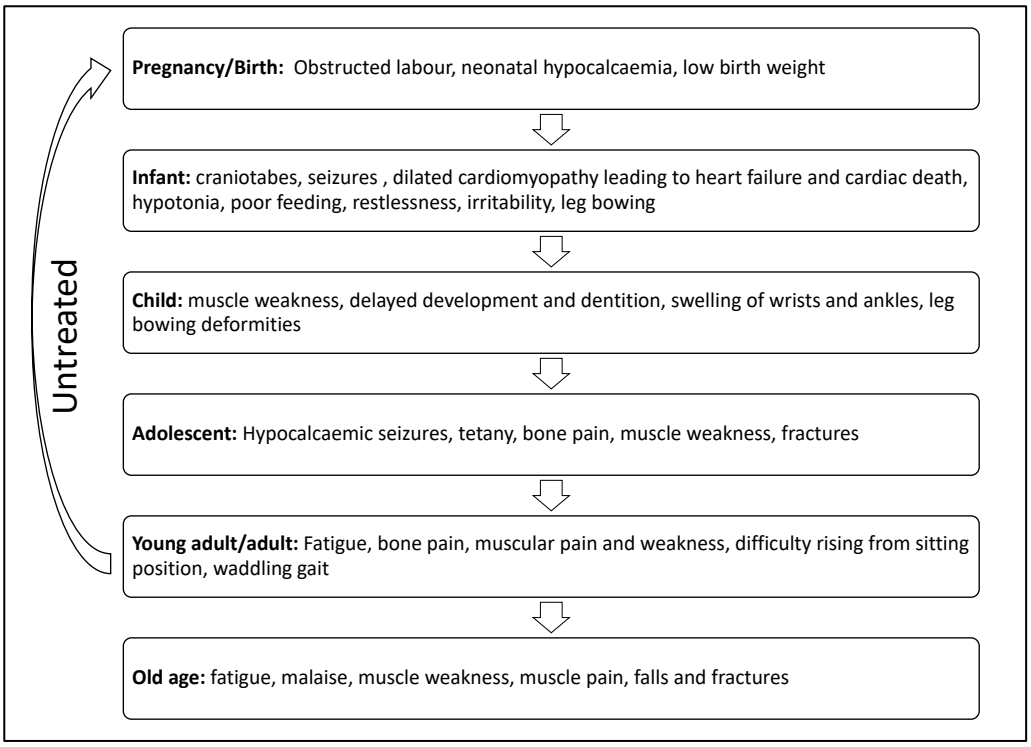


Figure 2.6: Clinical presentation of rickets and osteomalacia

2.3.6 Diagnosis

A diagnosis of nutritional rickets/osteomalacia is suspected based on pre-disposing risk factors, dietary history and clinical presentation. The diagnosis is established on typical biochemical markers of vitamin D and/or calcium deficiency (high serum PTH and ALP, low phosphate). Definitive diagnosis in children requires radiographic confirmation and in adolescents and adults would require bone histomorphometric confirmation.

Abnormal biochemical markers such as raised PTH and ALP are seen before serum calcium and/or phosphate drop and long before radiographic changes of rickets become evident. In the presence of the above biochemical features, a low serum 25OHD and/or low dietary calcium intake strongly suggest a diagnosis of nutritional rickets/osteomalacia.

Rickets is confirmed on radiographs of the knee and/or wrist in the presence of splaying, fraying, cupping, coarse trabecular pattern of metaphyses, widening of the growth plate and osteopenia [29]. Confirmation of osteomalacia requires a transiliac bone biopsy. Whilst the classic radiological features of osteomalacia include Looser's zone fractures, these only occur at a late stage in the disease course. Osteomalacia is diagnosed on bone histomorphometry and is defined by a combination of osteoid thickness>12.5 μm and a mineralization lag time of >100 days following tetracycline labelling in adults [33]. There is lack of normative histomorphometry data and tetracycline labelled studies in children under 2 years of age to enable a definitive diagnosis of osteomalacia.

2.3.7 Treatment

Rickets treatment incorporates replacement of vitamin D ± calcium as detailed in **Table 2.5** [29]. Both D2 (ergocalciferol) and D3 (cholecalciferol) can be used for daily oral supplementation, however D3 is preferred for stoss therapy, i.e a single high dose treatment, due to its longer half-life [41]. Stoss therapy should however be restricted to individuals where compliance or follow up is an issue as there is a risk of hypercalcaemia [35]. A minimum of 12 weeks treatment is recommended [29]. Calcium (intravenous or oral) is used in the management of children presenting with acute hypocalcaemic complications such as seizures or heart failure. In acute situations, intravenous calcium gluconate bolus followed by an infusion is recommended until serum calcium normalises. Once the child is stable and normocalcaemia is achieved a switch to oral calcium supplements can be made.

Table 2.5: Treatment of nutritional rickets and osteomalacia

	Infants	**12 months to 12 years**	**>12 years**
Daily treatment dose for a minimum of 12 weeks (with D_2 or D_3)	2000 IU	3000-6000 IU	6000 IU
Stoss therapy (preferably with D_3)	50,000 IU only in infants >3months	150,000 IU	300,000 IU
Calcium through diet or supplements where necessary	200 - 300 mg/ day	500 mg/day	500 mg/day
Minimum prevention dose of daily vitamin D	400 IU	600 IU	600 IU

Vitamin D conversion factor 1 IU = 0.025 μg

There is no role for phosphate supplements in the treatment of nutritional rickets; giving phosphate worsens secondary hyperparathyroidism. Reversal of secondary hyperparathyroidism improves serum phosphate levels. Calcitriol or alfacalcidol do not have a role in the treatment of nutritional rickets. Alfacalcidol may be used for a brief period in children presenting acutely with hypocalcaemic seizures to enhance calcium absorption in the acute phase. However, its long-term use is not indicated. The use of these active agents should be reserved for conditions where hydroxylation is likely to be affected such as in chronic liver or renal disease.

Chapter summary

Pathophysiology of mineralization

- Adequate mineralization of bone tissue requires: minerals (calcium and phosphate), mineral supplier (active vitamin D or calcitriol or 1,25, dihydroxy vitamin D) and a facilitator (alkaline phosphatase enzyme)
- Low phosphate availability to the hypertrophic chondrocytes in the growth plate prevents apoptosis and the mineralization that follows

Pathogenesis and classification of rickets

- Based on the aetiology, rickets can be classed into 3 forms calcipaenic, phosphopaenic and hypophosphatasia
- The leading cause of rickets worldwide is calcipaenic rickets caused by solar vitamin D and/or dietary calcium deficiency

Nutritional rickets and osteomalacia

- Nutritional rickets and osteomalacia are widely prevalent in high latitude countries, especially in the dark skinned immigrant population.
- Serum calcium is a poor marker of calcium deprivation in the body
- Rickets is a radiological diagnosis and is evident only in the late stages of the disease.

Chapter 3: **A review of nutritional rickets burden and health policies in the UK**

Parts of this chapter has been published as:

Uday S and Högler W. Prevention of rickets and osteomalacia in the United Kingdom: Political action overdue. Archives of Disease in Childhood.2018;103(9):901-906.

Uday S, Kongjonaj A, Aguiar M, Tulchinsky T and Högler W. Variations in infant and childhood vitamin D supplementation programmes across Europe and factors influencing adherence. Endocrine connections. 2017;6(8):667-675.

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3.1 Aims and objectives

3.1.1 Review aims

The overall aim was to review the burden of nutritional rickets in UK children and the factors contributing to disease burden.

3.1.2 Review objectives

Section 3.2

- Review the burden of nutritional rickets in UK infants and young children since the introduction of the Healthy Start vitamin scheme in 2006
- Detail the ethnic mix, radiological changes of rickets, vitamin D supplement use and feeding mode in symptomatic vitamin D deficiency

Section 3.3

- Review the history of rickets in the UK
- Evaluate the changes in UK population demographics over the last decade
- Detail the current UK public health policies relating to vitamin D supplementation and food fortification

Section 3.4

- Report the findings from the study on infant vitamin D supplementation policies across Europe [42] which led to this thesis
- Report the factors associated with improved adherence to infant supplementation

- Highlight the key differences in policy features and implementation strategies between the UK and other European countries with better adherence to infant supplementation.

3.2 A review of the burden of nutritional rickets in the UK

3.2.1 Introduction

Over the last two decades several UK studies have highlighted the burden of symptomatic vitamin D deficiency and nutritional rickets in children. The increased susceptibility of BAME groups to develop nutritional rickets has also been widely described. In 2006 the 'Healthy Start Scheme' replaced the previous welfare food scheme to offer vouchers for cows' milk, fruit and vegetables or infant formula, and coupons for vitamin supplements, to pregnant women under 18 years and to low-income pregnant women and families with children under 4 years of age [43]. The Healthy Start vitamin tablets for pregnant and lactating women contain vitamin D (400 IU), vitamin C and folic acid. The Healthy Start vitamin drops for children contain vitamin D (300 IU or 7.5 μg in 5 drops), vitamin A, and vitamin C. In April 2019, children's Healthy Start was updated to incorporate 400 IU (10 μg) of vitamin D per 5 drops in keeping with the government's recommendations. The scheme however is extremely complex and is ill-implemented [44,45] leading to poor uptake of vitamins by eligible families [43]. Poor policies and changing population demographics have resulted in a rise in the incidence of symptomatic vitamin D deficiency and nutritional rickets in the UK with infants and young children being the most affected. Reports on nutritional rickets in infancy and early childhood in the UK are reviewed in this section.

3.2.2 Methods

PubMed and Medline were searched to identify published literature on 'vitamin D deficiency rickets' in the UK since the introduction of the Healthy Start Scheme in 2006. The reference lists of studies that met the inclusion criteria, and of relevant review articles (identified by the search or already known to the author), were also hand-searched to identify additional relevant work. The search terms used are presented in the box below:

Search 1: 'vitamin d deficiency', 'nutritional rickets', rickets, osteomalacia, 'nutritional osteomalacia'
Search 2: child*, infant*, infancy, neonat*, newborn*, baby, babies, toddler*
Search 3: 'United Kingdom', UK, England, London, Britain, 'Great Britain'
Final search: 1 AND 2 AND 3

After exclusion of duplicates, the titles and abstracts of the remaining references were screened to include studies only from the UK reporting on vitamin D deficiency rickets, with the exception of case reports and case series. The studies were reviewed with specific focus on ethnic mix, radiographic evidence of rickets, supplement use and feeding mode where these were reported.

3.2.3 Results

A total of 8 studies, with varying aims and objectives, which reported on vitamin D deficiency rickets in the UK population were reviewed. Three studies were retrospective case

note reviews, one was a national database review, two were prospective surveillance studies and two retrospective post-mortem studies. The main findings of these studies are detailed in **Table 3.1** and the key points are summarised below.

Table 3.1: Reports of nutritional rickets in UK infants and young children since the introduction of the 'Healthy Start' scheme in 2006

Author, year of publication	Aim/ objectives	Methodology	Study numbers Ethnic mix Age group	Main findings, radiology, supplement use and feeding mode	Comments
Maiya et al 2007 [6]	Review the prevalence of hypocalcaemic dilated cardiomyopathy (DCM) in paediatric cardiology units of southeast England and determine the prognosis	Retrospective review of cases with hypocalcaemic DCM due to vitamin D deficiency (VDD) in southeast England (2000 and 2006), London, UK	Total 16 infants 10=black 6=Indian subcontinents Median (range) age at presentation was 5.3 months (3 weeks– 8 months)	**Main findings:** Presentation: 6 cardiac arrest and 10 cardiac failure. 10/16 presented at the end of the British winter (February–May). Outcome: 3= Death, 2= Mechanical ventilation, 2= circulatory support and 2= referral for cardiac transplantation **Radiology:** 10/16 (62.5%) had evidence of rickets. **Supplement use:** None were on supplements (mother or baby) **Feeding mode:** 12/16 = exclusively breast fed, 4/16 = breast feeds + solids	Significant morbidity and mortality associated with DCM. All mothers were investigated and reported to have normal calcium and phosphate. 3/5 mother had raised PTH and low 25OHD. No investigations in family members are reported.
Ahmed et al 2010 [8]	Identify the incidence of symptomatic VDD in the context of continuing	Retrospective case review (2002 and 2008), of children presenting with symptomatic vitamin D	Total 160 cases Ethnic split by year represented graphically (n not	**Main findings:** Annual incidence of 23 cases/year. Absolute annual incidence increased over the study period, reaching a peak of 42 cases in 2008	No specific reference was made to supplement uptake or maternal 25OHD status. A high

	demographic changes and the introduction of new public health measure (Healthy Start scheme in 2006)	deficiency at the Royal Hospital for Sick Children, Glasgow, UK	defined): The majority were South Asian or North African followed by Middle Eastern and Sub-Saharan African origin- 94% of total. The median age of the cohort was 24 months (range 2 weeks-14 years)	Presenting features: 64 (40%) = bowed legs and 19 (12%) = seizures **Radiology:** Among the 132 cases with radiographs, 42 (32%) = no sign of rickets, 70 (53%) = clear signs of rickets 20 (15%) = equivocal signs of rickets **Supplement use:** Not reported **Feeding mode:** Not reported	proportion of cases had no radiological evidence of rickets
Moy et al 2012 [46]	Evaluate the effectiveness of a free universal (pregnant and lactating women and children <5y) vitamin D supplementation programme in reducing the number of cases of nutritional rickets in West-Midlands region of the UK	Retrospective review of laboratory records for low vitamin D levels (< 25 nmol/L) in children under 5 years. Cross reference was made against medical records to confirm the diagnosis of symptomatic vitamin D deficiency.	Total 12 cases Ethnicity and age not reported	**Main findings:** Case incidence rate fell from 120/100 000 in 2005 (n=29) to 49/100 000 in 2009-2010 (n=12) **Radiology:** 10/12 cases had rickets and two were hypocalcaemic seizures **Supplement use:** Uptake rate increased to 17% (baseline rate was not specifically reported in this study. Relevant references were reviewed and a baseline uptake of 2-4% was identified) **Feeding mode:** Not reported	Breakdown of uptake for mother/child not reported. No information on maternal 25OHD status of the identified cases. Cases with 25OHD levels between 25-50 nmol/L will have been missed due to the strict inclusion.

Goldacre et al 2014 [7]	Identify rates of hospital admissions for rickets in England, across five decades	Retrospective national database review using International Classification of Diseases codes for active rickets. Review of all England hospital episodes for 1968– 2011 (no national data for 1986–90). Review of episodes from the Oxford record linkage study (ORLS) which includes data for all day cases and inpatients (1963 and 2011)	32% = white (vs 83% in the general population for children <15 years) 33% = south Asian (vs 8%), 33% = black (vs 3%), 2% = other non-white (vs 6%) Age < 15 years with most patients being younger than 5 years.	**Main findings:** In all England in 2007–11, the episode-based rate was 4·78 (4·58–4·99) and the person- based rate was 3·16 (3·00–3·33) per 100 000 aged under 15. In ORLS, rates were lowest in 1991–96 at 0·34 children per 100 000 under 15 years (95% CI 0·13–0·55) and highest in 2007–11 at 1·78 (1·27–2·29). **Radiology:** Not reported **Supplement use:** Not reported **Feeding mode:** Not reported	Nature of the study meant there were no details of radiological findings and all forms of rickets were included. Extent of morbidity was not known.
Basatemur et al 2015 British Paediatric Surveillance Unit (BPSU) survey [5]	Determine the incidence of hypocalcaemic seizures secondary to VDD in children in the UK and Ireland. Describe the demographic and clinical features of cases.	Prospective surveillance (September 2011-2013) of children presenting with hypocalcaemic seizures through reporting by secondary/tertiary care paediatricians.	Total 91 cases 38% (n=35) Pakistani 25% (n=22) Black 12% (n=11) Indian 12% (n=11) Any white 9% (n=8) Other South Asian 4% (n=4) Mixed 95% = 0–2 years, of whom 85% (n=77) were infants 5%= 11–15 years.	**Main findings:** Overall annual incidence of hypocalcaemic seizures was 3.49 per million children (0-15 years), South Asian = 26.04 per million Blacks = 20.70 per million Peak presentation was in Feb (range Nov-March) **Radiology:** 23/30 (77%) cases with radiographs had features of rickets **Supplement use:** Not reported	Where maternal 25OHD was available, 71% had levels below 25nmol/L. A high proportion of children (50%) required anti-epileptic medications for seizures. 7% (n=10) of cases could not be ascertained and were excluded. Radiographs

				Feeding mode: Only 55% exclusively breast fed, 19% were exclusively formula fed, 9% on mixed feeds and 17% weaned onto solids.	were only available on a third of the cases.
Julies et al 2020 BPSU survey [9]	Determine the incidence of nutritional rickets in the UK and Ireland in children under 16 years of age	Prospective surveillance (March 2015 – March 2017) of children diagnosed with nutritional rickets through reporting by secondary/tertiary care paediatricians.	Total 125 cases (116 were < 5 years) 43% (n=54) Black 38% (n=47) South Asian 5% (n=6) White 6% (n=7) mixed Median age at diagnosis was 18 months (IQR 11.25–24.0, range 2–181 months)	**Main findings:** Annual incidence was 0.48/100,000 for total, 0.03 for whites, 2.75 for South Asians and 5 for Blacks. 4 = dilated cardiomyopathy 2= deaths related to nutritional rickets 41/125 (32.8%) presented in spring, 29/125 (23.2%) in summer and 28/125 (22.4%) in winter **Radiology:** 107/125 (85.6%) had radiographs and 69/107 (64%) had evidence of rickets at diagnosis, 4/107 had healing rickets on treatment. **Supplement use:** 77.6% not on supplements **Feeding mode:** 85/125 (68%) were on solids, 22/185 (12%) were exclusively breast fed and 1/125 was exclusively formula fed.	Maternal 25OHD was known in 41 (32.8%) cases of whom 10 (8%) had levels < 25nmol/L. Nearly 20% of the total reported cases (n=319) were excluded due to lack of complete data. Risk of under-reporting due to omission of cases in primary care and the community.

Retrospective post-mortem studies					
Scheimberg et al 2014 [47]	Review the role of low vitamin D in paediatric morbidity and mortality	Retrospective review of post-mortem cases (July 2009 and April 2012) where 25OHD levels were available at The Royal London Hospital in North East London, UK	Total 52 cases 41% (n=21) white, 23% (n=12) Asian and 36% (n=19) black Age: 2 days to 10 years.	**Main findings:** 17/52 (33%) had VDD (<25 nmol/L) and 24/52 (46%) had insufficiency (25–49 nmol/L). Hypocalcaemia due to VDD was considered accountable for death in 3/52 BAME children (2=DCM, 1=hypocalcaemic seizures) and they all showed radiological and histological rickets. **Histology:** Growth plate histology was reported in 35 of the 52 cases. Abnormal histological was present in 10/17 (59%) with VDD and 8/24 (33%) with vitamin D insufficiency. **Radiology:** 3/17 (18%) with deficiency had radiological evidence of rickets. None with insufficiency or sufficiency had rickets. **Supplement use:** Not reported **Feeding mode:** Only available for 5 cases	A higher proportion of deficient children had histological changes compared to radiological evidence of rickets. The growth plate histological changes were not quantified and extent of osteomalacia was not studied.
Cohen et al 2013 [48]	Determine if there is an increased	Retrospective review of post-mortem cases (2009	Total 41 cases	**Main findings:**	Predominantly white population

	prevalence of VDD in cases of sudden death in infancy and childhood; and establish whether there is a link between VDD and infection	and 2010) where vitamin D levels were measured at the Sheffield Children's Hospital, Sheffield, UK	90% (n=37) White 7% (n=3) Asian 3% (n=1) Black Age range: 9 hours to 14 years, 24/41 (58.5%) < 6 months	10/41 (24.5%) had 25OHD < 25 nmol/L and 16/41 (39%) had 25OHD 25-50nmol/L. All 4 non-white cases had 25OHD < 50 nmol/L **Histology**: was reported in 16 of the 41 cases. 11/16 (69%) had abnormal histology with 25OHD < 75nmol/L **Radiology:** 3/41 were thought to likely have rickets **Supplement use:** Not reported **Feeding mode:** Not reported	There was lack of agreement between the two radiologists on the presence of rickets (3/3 cases). Growth plate histological changes were not quantified and the extent of osteomalacia was not studied.

The British Paediatric Surveillance Unit (BPSU) survey of the incidence of hypocalcaemic seizures identified an overall annual incidence of 3.49 per million children (0-15 years) and 26.04 per million children in South Asians and 20.70 per million children in Blacks [5]. The BPSU survey of the incidence of nutritional rickets reported an overall incidence of 0.48/100,000 for all children (<16 years), 0.03 for whites, 2.75 for South Asians and 5 for Blacks [9]. During the 2-year BPSU survey period between 2015 to 2017, four cases of dilated cardiomyopathy were identified with 2 deaths [9]. The cardiac consequences and morbidity have been highlighted in a previous case series (n=16) where 3 infants died, 2 each required mechanical ventilation, circulatory support and referral for cardiac transplantation [6]. The uptake of vitamin D supplements, where reported, is consistently low despite robust campaigning [46]. Retrospective post mortem studies report a high proportion of histological rickets in infants [47,48].

3.2.4 Discussion

The risk factors for nutritional rickets such as dark skin and covered clothing are uniformly highlighted in all the studies detailed above. Two studies reported on the 25OHD status of the mother [5,6] given its direct bearing on the infant who is breast-fed. None of the studies to date have explored the morbidity in siblings, of affected individuals, who share the common risk factors. The work described in **Chapter 4** will systematically explore this hidden burden.

Studies which report on supplement use demonstrate that its uptake in the UK infants is < 20% at best, even after robust campaigning [46]. In the study of infants with dilated hypocalcaemic cardiomyopathy, none of the mothers received antenatal vitamin D supplements and none of the infants were on supplements at the time of presentation [6]. To give the infant the best start in life, adequate antenatal supplementation is crucial. The work

described in **Chapter 5** was designed to assess the effectiveness of the UK antenatal supplementation programme in preventing vitamin D deficiency in the newborn.

Nutritional rickets on radiographs is mostly uncovered in children presenting with symptomatic hypocalcaemia. These cases only represent the tip of the iceberg of a public health crisis. Radiological changes are only evident in around 50% of the cases of symptomatic vitamin D deficiency [8] and histological changes occur long before then (**Figure 2.5).** One way to gain insight into the true prevalence of rickets and osteomalacia in the UK is from post-mortem studies. The two retrospective post-mortem studies detailed above [47,48] uncovered hidden histological changes of rickets in the growth plate in nearly 70% of the infants studied. However, the reported histological changes were not quantified in either of the studies and neither of them assessed for the presence of osteomalacia. Moreover, these studies did not provide information on vitamin D supplement use and mode of feeding. An adult post-mortem study in northern Europe identified osteomalacia in nearly 25% of the population studied (n=675) [49]; similar studies in children are non-existent. Therefore, a prospective post-mortem study was designed to quantify the presence of osteomalacia in children which will be discussed in **Chapter 6.**

3.3 A review of factors contributing to disease burden

Following a brief review of the history of rickets and population demographics in the UK, this section will focus on reviewing the current public health policies relating to prevention of vitamin D deficiency, rickets and osteomalacia.

3.3.1 History and politics

In the UK, 'Nutritional' rickets was indeed originally caused by poor nutrition but was always also of 'solar' origin due to the high latitude and high air pollution during the industrial revolution. During, and immediately after World War II, rickets was effectively eradicated in the UK through rationing, food fortification and supplementation of infants and pregnant women with cod-liver oil. In the 1950s, uncontrolled fortification of infant formula and food (4000 IU/day, which is 10 times the current recommended dose of 400 IU/day) was implicated as a cause of infantile hypercalcaemia [50] which led the UK government to withdraw vitamin D food fortification. Following the withdrawal of fortification, cases of rickets began to rise again in the 1960s, and were predominantly seen in the Pakistani immigrant population [51]. This led the UK government to consider rickets as a disease of the immigrant population and referring to it as 'Asian rickets'. Rickets or 'Asian rickets' has enormous political significance and has been the subject of frequent debate in the UK parliament since the 1960s [52] due to the suggestion that resurgence of rickets symbolises socioeconomic inequality [53].

3.3.2 Changing population demographics

The National census data have demonstrated an upward trend in the proportion of the non-white population from 5.9% in 1991 to 14% of the total population in 2011 (**Figure 3.1**) [54]. Concurrently, a downward trend in the proportion of white population from 94.1% in 1991 to 86% in 2011 has been noted (**Figure 3.1**) [54]. Every year, nearly 13% of babies born in England and Wales have been of BAME background since 2014 [54].

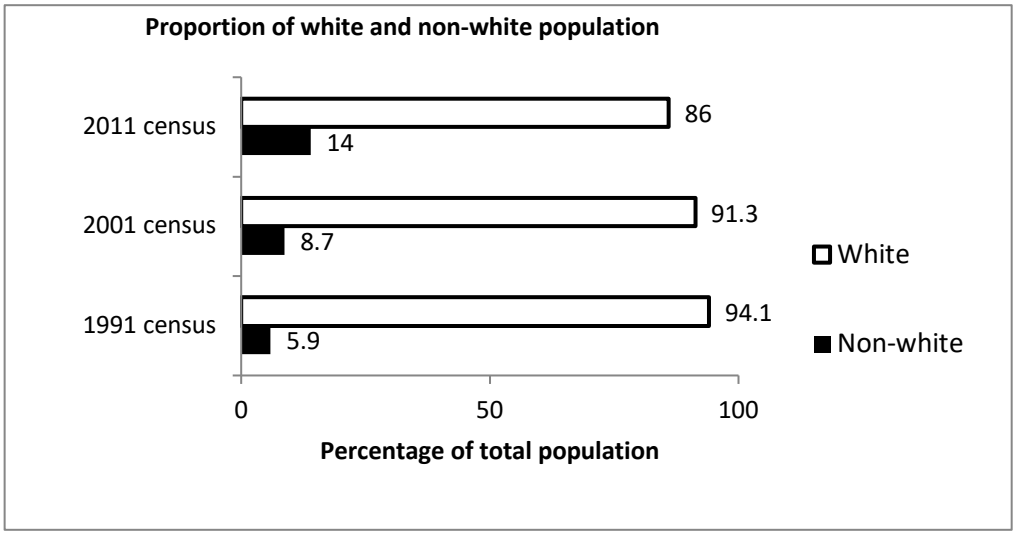


Figure 3.1: Changing population demographics in the UK

In addition to the increased proportion of the resident BAME population, many developed countries, including the UK, have experienced an influx in immigrant and refugee populations [3,38]. The data on annual UK migration over the last decade (2007 to 2016) was reviewed on the Office of National Statistics website [55] to estimate the proportion of BAME migration. The assumption was made that migrants from/to EU and North America are exclusively white, and from/to BAME global regions (Asia, Sub-Saharan & North Africa, Central & South America and Oceania) are exclusively dark skinned. A negative net migration of the White British population and a positive net migration in the BAME population was noted

(Figure 3.2) [54]. It was estimated that roughly 100-150,000 people of BAME background (net) are added to the UK's population through immigration each year **(Figure 3.2**) [54].

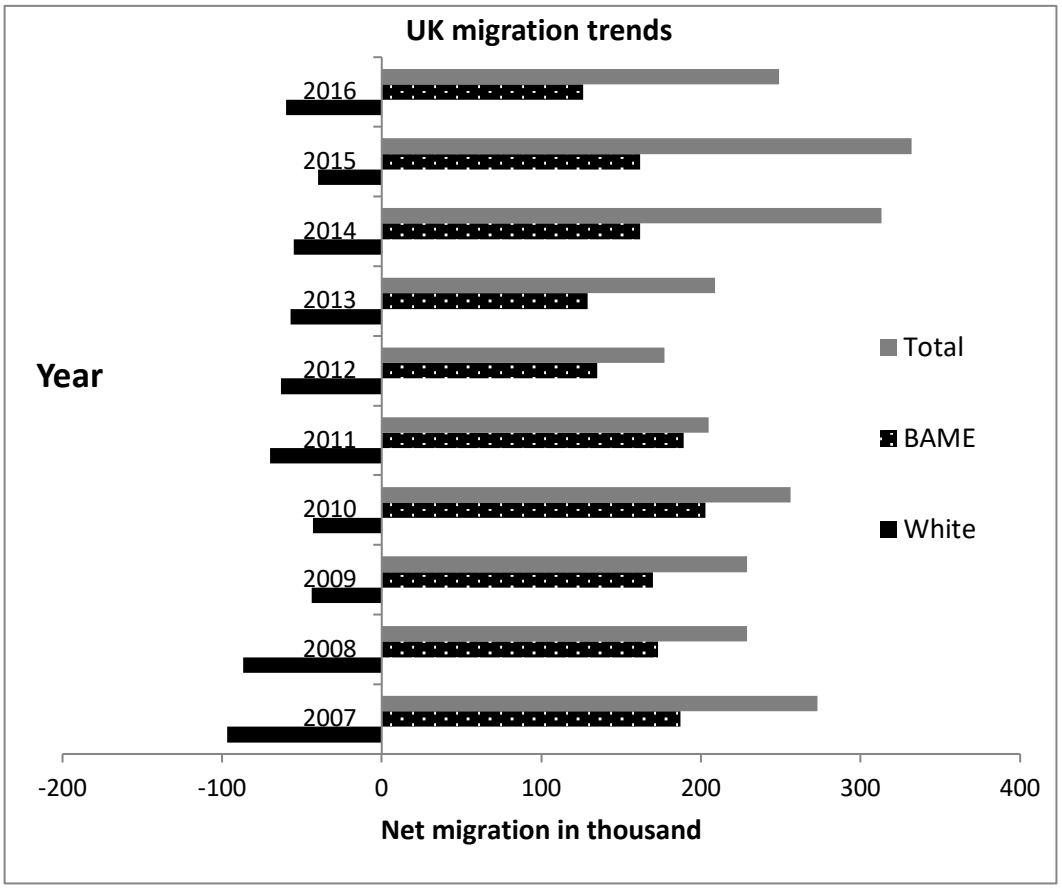


Figure 3.2: Net UK migration

UK net migration data over the last decade demonstrating a negative net migration of the White British population and a positive net migration in the Black, Asian and Minority Ethnic (BAME) population. BAME net migration = net Asian migrants + net Rest of the World migrants [Sub-Saharan & North Africa, Central & South America, Oceania; excluding North American migrants]. Migrants from/to EU and North America were assumed to be exclusively white, and from/to BAME global regions to be exclusively dark skinned. Net total migration (grey bar) equals net BAME (black bar) + net white British (white bar) + net North America + net EU migration.

3.3.3 Review of public health policies

The most commonly adopted strategies worldwide for prevention of vitamin D deficiency are food fortification [56] and/or targeted supplementation of risk groups. Very few countries have mandated fortification, with most countries adopting voluntary fortification, of frequently consumed products such as milk and other dairy products [57,58]. Achieving optimal vitamin D status at a population level requires a combined approach with fortification and targeted supplementation [59]. Fortification of a range of dairy and non-dairy products such as wheat flour [60] may be necessary to achieve sufficiency in the risk groups.

3.3.3.1 Food fortification policy

In the UK, vitamin D fortification of spreadable fats and infant formula [61] is mandated and breakfast cereals are voluntarily fortified. Infant formula milk is fortified with 1(minimum)-2.5 (maximum)µg vitamin D /100ml, which was recently upgraded to 2-3µg/100ml [62]. Similarly, the vitamin D content in follow-on formula milk was upgraded from 1-3µg/100ml to 2-3 µg/100ml [62]. In the 2014/15 – 2015/16 NDNS (National Diet and Nutrition Survey) mean vitamin D intakes from food sources were below the recommendation of 10µg/day in all age groups, with children only achieving around a fifth of the recommendation [63]. Products such as wheat flour, which are frequently consumed by certain high-risk groups, are not fortified in the UK.

3.3.3.2 Vitamin D supplementation policies

The current UK supplementation policies relating to infants, children, pregnant women and high risk groups are detailed in **Table 3.2.**

Table 3.2: The current UK recommendations for vitamin D requirements and supplement use

	Infancy **Vitamin D µg (IU) / day**	**Childhood** **Vitamin D µg (IU) / day**	**Pregnancy** **Vitamin D µg (IU) / day**	**High risk population** **Vitamin D µg (IU) / day**
Chief Medical Officers recommendations 2012 [64]	7-8.5 (280-340) supplements in all infants. Those receiving formula do not need supplements unless taking < 500ml/day. Breastfed infants may need supplements from 1 month onwards if mother has not received antenatal supplements.	7-8.5 (300-340) supplements up to 5 years	10 (400) supplements	10 (400) supplements
Royal College of Obstetricians Gynaecologists 2014 [65]	No recommendations	No recommendations	Supplements 10 (400) in all, 25 (1000) in high risk and 20 (800) + calcium if risk of pre-eclampsia	No recommendations
Scientific Advisory Committee on Nutrition 2016 [66]	8.5- 10 (340-400) Safe intake	10 (400) 1 to < 4 years: Safe intake >4 years: Reference nutrient intake	10 (400) Reference nutrient intake, as per adult guidance	10 (400) Reference nutrient intake as per adult guidance
Public Health England advice 2016 [67]	8.5-10 (340-400) supplements if consuming <500ml/day of formula feed	10 (400) supplements if consuming <500ml formula/day (1-4 years)	No specific reference or recommendation for pregnant women	10 (400) *Consider* taking supplements
Royal College of Paediatrics and Child Health guide 2019 [68]	8.5- 10 (340-400) birth to 1 year unless they are consuming > 500ml formula milk a day	1- 4 years :10 (400) daily supplement. Parents should consider giving the same dose to children over four years, particularly through the winter months.	No recommendations	No recommendations
National Institute for Health and care excellence (NICE) 2014 [69]	NICE guidance on vitamin D supplementation in specific population groups sets out the high risk groups which include infants, children under 4 years, pregnant women and dark-skinned individuals. NICE also makes recommendations to clarify the existing policies and enhance supplement uptake (including Healthy Start).			

Vitamin D conversion factor: 1 IU= 0.025 µg

3.3.3.2.1 Infant supplementation

The evidence-based global consensus for the prevention and management of rickets recommends 400 IU/day vitamin D supplementation of all infants from birth irrespective of whether they are breast or formula fed [29]. In contrast, the UK PHE recommends supplementation of babies consuming <500 ml of formula feed per day [67]. SACN and PHE recommend a safe dose of 340-400 IU/ day in infants.

3.3.3.2.2 Childhood supplementation

The global consensus [29] and Institute Of Medicine (IOM) [70] recommend that all children > 12 months and adults should meet 600 IU (15 μg) per day requirement through diet or supplements. PHE recommends 400 IU/day supplement in children up to the age of 4 years, if consuming < 500 ml formula feed daily. The RCPCH recommends that parents should consider giving the same amount of supplement (400 IU) to children > 4 years.

3.3.3.2.3 Antenatal supplementation

The global consensus regards women as high risk and recommends 600 IU daily supplementation through pregnancy [29]. The UK NICE guidance regards pregnant women as high risk and recommends supplementation [69]. However, the most recent PHE guidance in 2016 [67] which was based on the SACN report [66] did not make any specific vitamin D supplement recommendations for pregnant women or regard them as high risk. The Royal College of Obstetricians and Gynaecologists recommend 400 IU/day supplementation in all pregnant women and a higher dose of 1000 IU (25μg)/day in dark skinned individuals [65].

3.3.3.2.4 Supplementation of specific high-risk groups

The NICE guidance clearly sets out the high risk population and in these groups recommends vitamin D supplementation rather than the use of fortified foods [69]. The reference intake set by SACN for at-risk groups including pregnant women is the same as adult reference intake of 400 IU/day [66]. PHE does not mandate supplement intake in at-risk groups and only recommends that they consider taking it [67].

3.3.4 Discussion

3.3.4.1 Resident and immigrant high risk population

Rickets in the dark-skinned immigrant population in the UK has been a historical concern, which had led to the origin of the term 'Asian rickets'. The continued rise in the proportion of the UK resident BAME population **(Figure 3.1)** and the lack of effective strategies to stop vitamin D deficiency, a preventable condition, [71] have resulted in an increasing incidence of rickets in the UK [7]. Whilst NICE recognises the risk groups and sets out recommendations for prevention of vitamin D deficiency in these specific groups [69], the lack of political and financial support hinders implementation [38,53].

The burden of nutritional rickets has been heightened by recent immigration trends (**Figure 3.2)** and the global refugee crisis [3]. Other developed countries have also seen a similar rise in cases of nutritional rickets which parallel trends in immigration [72-74]. The most affected groups are those who migrate from sunny countries such as Asia, Africa or the Middle East to Europe and retain cultural habits of covered clothing and/or dairy restricted diet [75]. Public health policies should incorporate screening and prevention of vitamin D deficiency and also other micronutrient deficiencies in these population groups [75].

3.3.4.2 Reference intake and sufficiency levels

The SACN sets a vitamin D 'reference nutrient intake' (RNI) for adults and children aged >4 years and 'safe intake' for infants and children aged 1-4 years [66]. The RNI for children >1 year and adults is lower than the IOM recommendation of 600 IU [28]. The SACN does not recommend supplements or food fortification but acknowledges the difficulty in achieving the recommended intakes from natural food sources and advises the government to consider strategies for the UK population to achieve the above intake [66]. The conservative RNI in the UK is not met through diet and supplement uptake is known to be low. The most recent NDNS (2014/15-2015-16), reported a low median intake of vitamin D in children aged 1.5-3 years of 1.8μg (14% RNI) from diet increasing only to 3μg (16% RNI) when supplement use was included [63]. The SACN recommends that serum 25OHD levels should not fall below 25 nmol/L [66], most scientific authorities consider optimal levels to be above 50 nmol/L [76].

3.3.4.3 Fortification

Given the limited sources of fortified food, very small amounts of the daily vitamin D requirement are met through diet in the UK. 'Milk and milk products' are reported to be the major contributor to vitamin D intake in children aged 1.5 to 3 years, yet only provide 24% of the reference intake [77]. Simulation models suggest that fortification of wheat flour will help improve the vitamin D status of the high risk population in the UK [60] and is also cost-saving [78].

Countries such as Finland that have adopted systematic Vitamin D fortification of fluid milk products and fat spreads, since 2003, have succeeded in improving the vitamin D status of the

population [79]. The mean serum 25OHD increased from 48 nmol/L (95% CI: 47, 48 nmol/L, n=6134) in 2000 to 65 nmol/L (95% CI: 65, 66 nmol/L, n=4051) in 2011 ($P < 0.001$). Additionally, 91% of the population who consumed milk achieved optimal vitamin D status (>50 nmol/L).

3.3.4.4 Supplementation policy

Currently, the SACN or PHE do not recommend vitamin D supplements for formula-fed infants unless they are consuming < 500 ml of formula per day. Rickets in formula fed infants has been reported in the literature [5,9,76]. In the BPSU survey of hypocalcaemic seizures, 19% were exclusively on formula feeds and a further 9% were on combined breast and formula feeds [5]. Evidence suggests that a simple message of universal supplementation enhances adherence [42,45,46]. Also, the targeted supplementation approach assumes that risk groups are being informed of their risk and that supplements are easily available. Other evidence-based policy implementation strategies will be presented in the next section.

3.3.5 Conclusion

The current burden of symptomatic vitamin D deficiency and nutritional rickets coupled with changing population demographics, require that the UK policies on food fortification and supplementation are revised. Adopting successful models of supplementation and fortification from other countries is one way forward. In **Section 3.4**, the supplementation policies across Europe are reviewed; policy features and implementation strategies which best relate to enhanced supplement uptake are presented.

3.4 Infant vitamin D supplementation policies across Europe

3.4.1 Background:

Although vitamin D deficiency is reported to be widely prevalent in Europe it is more rampant in some mid-latitude countries including the UK when compared to northerly latitude countries such as Norway, Iceland or Finland even after accounting for ethnicity [80]. The lower prevalence in these northern latitude countries is attributed to better supplementation policies and also food fortification [80]. Some of the factors which have been previously implicated in reduced adherence to infant supplementation include the need for frequent dosing [81] and lack of maternal advice on supplementation [81]. A higher adherence to infant supplementation has been noted with higher gestational age [82], older maternal age [82] and longer maternal education [82]. Specifically in the UK, a qualitative study attributed low maternal and infant adherence to poor accessibility to vitamins, low promotion of Healthy Strat vitamin scheme, lack of awareness among eligible families and low motivation among mothers to take vitamins for themselves during pregnancy or for their children [44]. Low supplement uptake in UK South Asian population [83] is attributed to younger age group and male gender [84] and a higher intake is associated with higher household income [84].

An understanding of the policy features and implementation strategies that enhance adherence was perceived to be beneficial to inform the UK health policies and policy makers. Infant vitamin D supplementation policies across Europe were reviewed to determine the factors that enhance adherence [42].

3.4.2 Methods

A questionnaire with 20 questions was designed pertaining to the content of vitamin D supplementation policies, practices, infant health care and socioeconomic factors. The questionnaire was designed based on expert opinion (from paediatric endocrinology and public health) of factors that were perceived to influence adherence to infant vitamin D supplementation. The questionnaire was completed by representative members of the European Society of Paediatric Endocrinology (ESPE) bone and growth plate working group.

Adherence was reported from national or local audits or as expert opinion. Adherence was classed as good (≥80%), moderate (50-79%) or poor (<50%). Kendall's tau-b correlation coefficient was used to assess the effect of individual factors and the total number of factors (n=11) on adherence rates.

3.4.3 Results

Contacts were established with 30 countries and 97% (n=29) responded. A detailed report of the findings have been published [42]. All countries reported an adherence rate to infant vitamin D supplementation in the first year of life. Denmark, Turkey and Israel (11%) monitored adherence as part of national statistics. Germany, Ireland, Norway, Spain and UK provided evidence from regional studies. Estonia and Italy specified that the estimates were personal opinions and the remaining countries (19/29) did not specify the source of information and were assumed to be subjective estimates.

Features which influence adherence and key differences in the UK policy compared to other European countries with enhanced adherence are discussed below.

3.4.3.1 Factors influencing adherence:

Good, moderate and poor adherence was reported by 61% (17/28), 28% (8/28) and 11% (3/28) of countries respectively with the UK reporting the lowest adherence (**Figure 3.3**).

Specific factors which were significantly associated with good adherence were:

- universal supplementation of both breast and formula fed infants,
- providing information on supplementation at discharge from neonatal units,
- providing financial family support and
- monitoring adherence at child health surveillance visits (**Figure 3.4**).

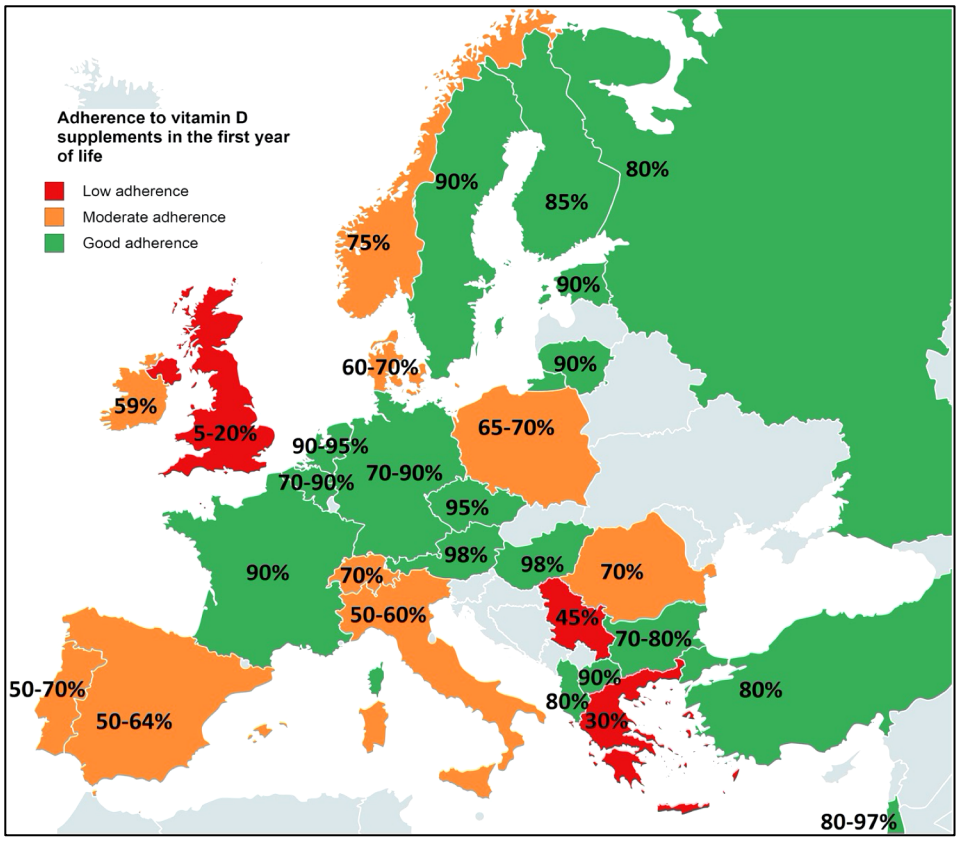


Figure 3.3: Adherence rates for infant vitamin D supplementation in the first year of life

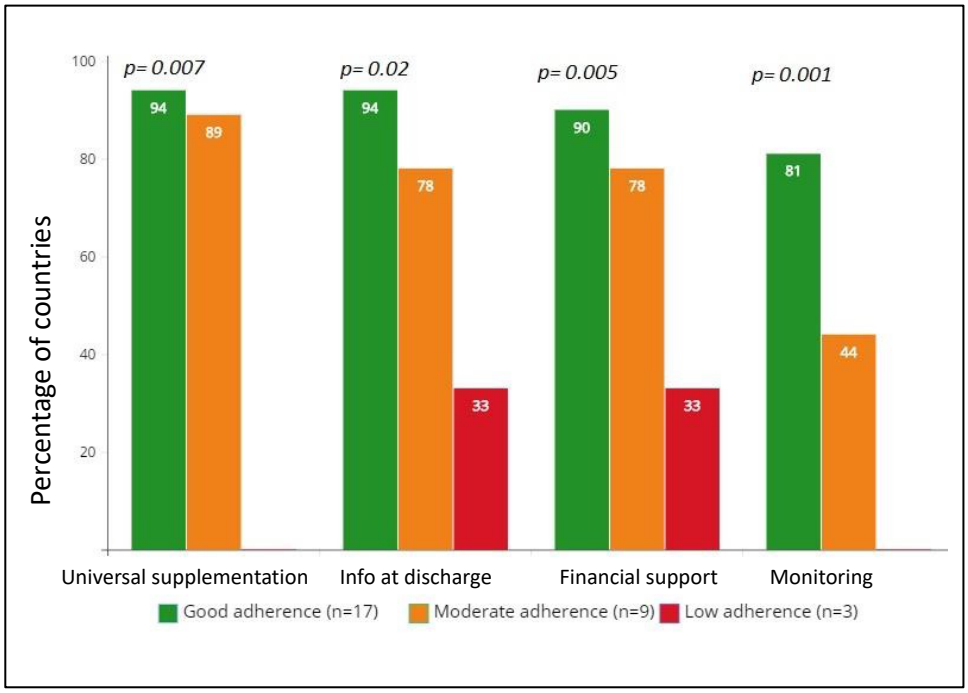


Figure 3.4: Factors influencing adherence to supplements in the first year of life

3.4.3.2 Key differences in UK policies compared to other European countries

A summary of policies in 29 European countries is presented in a table in **Appendix 1**. Some key differences in policy features and implementation strategies between the UK and other European countries are discussed below.

3.4.3.2.1 Supplements based on mode of feeding:

The UK policy suggests that all infants should be exclusively breast fed until 6 months of age; and babies who are exclusively breastfed or consume < 500 ml of formula/day should be given supplements [67]. Most European countries recommend supplementation independent of the mode of feeding (23/29, 79%) and others recommend supplementation of breast-fed infants only (5/29, 17%).

3.4.3.2.2 Commencement and duration of supplementation:

At the time of the survey, the UK was the only country where supplements were not recommended until 6 months of life. A subsequent change in policy recommended supplements for all infants who consume <500 ml of formula/day; without specifying when it should start [67]. Vitamin D supplementation was commenced on day 1-5 of life in 48% (13/27) and day 7-21 in 48% (13/27) of European countries. The recommended duration of supplementation varied widely, ranging from a minimum of 6 months to lifelong supplementation for at risk population; and are summarised in **Table 3.3** below.

Table 3.3: Recommendations on the duration of supplements across Europe

Duration of supplements	**Countries**	**Countries recommending additional lifelong supplementation for at-risk population**
First 6 months	Greece	
First year of life (41%, 12/29)	Poland, Turkey, Germany, Spain, Estonia, Romania, Portugal, Serbia, Albania, Bulgaria, Hungary, Israel	Poland
First 2 years of life (21%, 6/29)	Italy, Ireland, Russia, Denmark, Macedonia, Sweden	Sweden
Autumn born- first year, Winter born first 1½ years	Czech Republic, Austria	
First 1½ years of life	Lithuania	
First 3 years of life	Norway, Switzerland	
First 4 years of life	UK Netherlands (*from birth*)	Netherlands
First 5 years	France	
First 6 years	Belgium	
From 2 weeks to 18 years	Finland	

3.4.3.2.3 Professionals providing primary health care:

The UK, along with Albania and Greece, were the only countries which did not provide information to new parents on the rationale for Vitamin D supplementation before discharge from the neonatal/delivery unit. Of the 29 European countries, only in the UK are baby health checks provided solely by the health visitor. In most other European countries, it is provided by a paediatrician and/or general practitioner or health visitors in conjunction with paediatricians as depicted in the **Figure 3.5**.

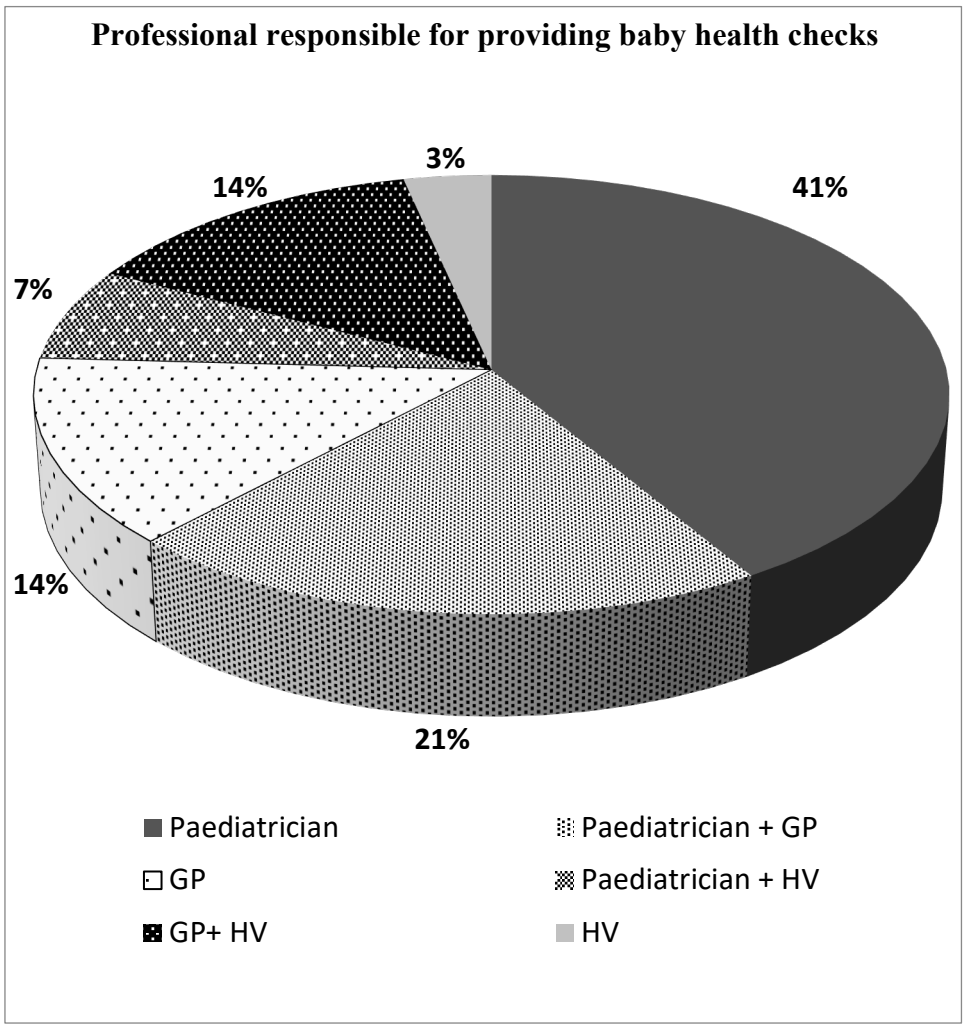


Figure 3.5: Professionals responsible for providing baby health checks across Europe

3.4.3.2.4 Financial family support and child benefits

In the UK, similar to Italy, families on low-income support receive free supplements. In other countries studied families mostly paid for their prescription (18/29, 62%) or it was provided free of charge to everyone by the government (8/29, 28%). In Norway, people from ethnic minority received free supplements whereas others paid.

Financial family support for children was provided by 86% (25/29) of countries, varying from birth to a minimum of 26 weeks to a maximum of 18 years; however only 28% of countries

(7/25) expected a 100% attendance at recommended health care visits to be eligible to claim these benefits.

3.4.3.2.5 Monitoring of adherence

Very few countries monitor adherence to supplementation as part of national statistics (3/29).

A recommended number of childcare visits for on-going assessment of a child's growth and development was practised in 93% (27/29) of European countries and 67% (18/27) of these nations incorporate a check for adherence to vitamin D supplementation.

3.4.3.2.6 Food fortification

Voluntary fortification of foods, other than infant formula, with vitamin D was practised in 50% of countries (14/28). Fortified foods included milk, margarine, yogurt, butter, dairy products, breakfast cereals, baby food and cookies. None of the European countries studied mandated fortification.

3.4.3.3 Limitations

The main study limitation was that information on adherence, feeding practices and non-adherent groups was mainly based on expert opinions. However, in the absence of national data, estimation by experts in the field was the next best alternative. Ideally, adherence should be recorded through parent interviews and assessed longitudinally. Since the publication of our report Hemmingway et al [85] have assessed adherence longitudinally before and after change in vitamin D supplementation policy in Ireland. They report high adherence of 93% at two months with ongoing high compliance of 72% at 12 months. The enhanced adherence is attributed to universal supplementation, robust policy implementation through dissemination

of information to families in pregnancy and at birth by clinical and midwifery staff through written information leaflets which were made available in nine languages [85].

Additional limitations in our work included, some inconsistent responses when there was more than one respondent, which may reflect regional differences within countries. Data from 22 European countries were missing because no contact could be established. Nonetheless, the largest western and central European countries are represented (55%), with different cultural and economic backgrounds.

2.1.1 Conclusions

Based on the findings of the European survey the following evidence-based strategies were proposed for countries, such as the UK, where adherence to vitamin D supplementation is low:

- Monitoring of antenatal supplementation by health care professionals at routine visit.
- Providing specific information on prevention programmes to parents, along with the first bottle of vitamin D supplements at discharge from neonatal units
- Checking for adherence to vitamin D supplementation at registration with General Practice surgeries
- Checking for adherence to supplements at every single visit for new-born checks and also at vaccination time points.
- Inclusion of a monitoring question on adherence to vitamin D supplements in maternity notes and personal child health record (PCHR or red book), at all routine time points, which reminds healthcare professionals and parents.

- Provision of financial remuneration, by the government, to General Practitioners (GPs) delivering this prevention programme in a similar way to that provided to GPs for immunization programmes (prevention of infections).
- Linking financial family support to mandated adherence to all prevention programmes.

Chapter summary

Nutritional rickets burden

- Cases of symptomatic vitamin D deficiency and nutritional rickets may only represent the tip of the iceberg of a public health crisis
- Extent of pathology in affected infants and family members remains unknown

Factors contributing to disease burden

- The proportion of dark-skinned individuals in the UK is on an upward trend
- Clarification and simplification of existing supplementation policies is required to enhance uptake, especially in infants

UK infant policy compared to other European policies

Key differences in policy features between the UK and other European countries with good adherence to supplements were:

- Universal supplementation of breast and formula fed infants
- Monitoring adherence to supplements at child health care visits
- Providing information to parents at discharge from neonatal units
- Family financial support linked to attendance at health care visits

Chapter 4: **Hidden pathology in infants with symptomatic vitamin D deficiency and their family members: a case series**

Parts of this chapter has been published as:

Uday S, Fratzl-Zelman N, Roschger P, Klaushofer K, Chikermane A, Saraff V, Tulchinsky T, Thacher TD, Marton T, Högler W. Cardiac, bone and growth plate manifestations in hypocalcemic infants: Revealing the hidden body of the vitamin D deficiency iceberg. BMC Pediatrics. 2018;18:1-9.

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Roles and responsibilities involved in the work reported in the current chapter:

Suma Uday: Clinician involved in direct clinical care of all patients reported, data gathering, obtained bone biopsy, preparation of manuscript for publication.

Wolfgang Högler: Conceptualised study, clinician involved in care of all patients, obtained consent for biopsy.

Tamas Marton: Pathologist performing post-mortem examination, provided histology images.

Ashish Chikermane: Cardiologist involved in the care of reported patients, provided data on echocardiograph parameters.

Nadja Fratzl-Zelman, Paul Roschger, Klaus Klaushofer: Performed bone histomorphometry and back scattered electron imaging, supported the interpretation and reporting of findings.

4.1 Introduction

Symptomatic vitamin D deficiency is common in the UK BAME infants. The extent of hidden pathology, specifically histological changes of rickets at the growth plate and osteomalacia on bone biopsy in these infants remains unknown. As discussed in **Chapter 3:** , to date, there are no bone biopsy studies in infants presenting with symptomatic disease due to the invasive nature of the investigation. Family members of infants who present with symptomatic vitamin D deficiency often share common risk factors and are also predisposed to deficiency [29] which may go undiagnosed. To the best of our knowledge, no studies to date have systematically explored the extent of biochemical abnormalities indicating undiagnosed rickets/osteomalacia (raised ALP and/or PTH, low calcium and/or phosphate, low 25OHD concentrations, radiological change of rickets in growing children) in asymptomatic family members of affected infants.

4.2 Aims and objectives

4.2.1 Aims

Investigate the hidden pathology in infants presenting with symptomatic vitamin D deficiency and their asymptomatic family members.

4.2.2 Objectives

- Investigate clinical, cardiac, growth plate and bone histomorphometric characteristics, bone matrix mineralization in infants with symptomatic vitamin D deficiency.

- Identify the prevalence of biochemical osteomalacia in asymptomatic family members, of the above cohort, of infants presenting with symptomatic vitamin D deficiency

4.3 Methods

Following informed parental consent, infants presenting with symptomatic vitamin D deficiency were investigated for hidden radiological, cardiac, bone and growth plate abnormalities. Family members of the infants were investigated for biochemical osteomalacia.

4.3.1 Setting and study period

Setting: BWCH provides secondary care service to a catchment population of 450,000 (representing approximately 45% of the population of Birmingham city) and tertiary care to a population of 2,900,000.

Only infants were included because the main focus of the thesis was to explore the burden in infants, the most vulnerable group, and also to ensure all cases were captured. In accordance with the Birmingham Women's and Children's Hospital (BWCH) "Vitamin D deficiency clinical guidelines" all infants presenting with symptomatic vitamin D deficiency are discussed with the endocrine team. Older children without complications may be managed by the general paediatric team.

Study period: Infants (Under 1 year of age) admitted to BWCH, between spring 2017 and 2018, with symptomatic vitamin D deficiency.

4.3.2 Participants

During the study period, four infants were admitted to the study centre with symptomatic vitamin D deficiency (25OHD < 30 nmol/L). Mother and siblings of the index case who shared the same risk factors (skin colour, lack of supplement use, diet lacking in dairy) were also investigated, as per the global consensus recommendation on prevention and management of nutritional rickets [29].

4.3.3 Clinical, cardiac, laboratory and radiological characteristics

Clinical, anthropometric, laboratory, electro- and echocardiography data were extracted from medical notes of index cases. X-rays taken as part of routine clinical care were reviewed for presence of signs of rickets. Blood samples of patients, siblings and parents were analysed for serum calcium, phosphate, ALP, 25OHD and PTH using routine laboratory methods. Specific reference was made to information provided to the family at birth on vitamin D supplementation and adherence to child surveillance visits.

4.3.4 Histology and backscattered electron microscopy

Sadly, one patient died (patient 1). The 7th rib growth plate section taken during routine post-mortem examination was processed using Elastica van Gieson staining. Following parental consent, a trans-iliac bone biopsy sample was taken and histomorphometric analyses were performed following Goldner's Trichrome staining using standard procedures [86] which will be detailed in **Section 6.5.5.** Bone mineralization density distribution (BMDD), reflecting the calcium content of bone matrix, was measured by quantitative backscattered electron

microscopy as described previously [87]. The BMDD curve of patient 1 was compared with a young reference population [87].

4.3.5 Ethics and consent

Ethical approval was not warranted as all investigations were undertaken as part of routine clinical care. Parental consent was sought for publication of findings.

4.4 Clinical case description

All four infants were born in England to mothers of BAME origin. The clinical presentation, investigations, management and outcome for all patients is reported below. **Table 4.1** summarises the characteristics at presentation including results of initial investigations.

4.4.1 Patient 1:

4.4.1.1 Clinical presentation

A 6-month-old exclusively breastfed, African boy presented to the emergency department with an out-of-hospital cardiac arrest. In the weeks prior to presentation, he had had 3 brief episodes of peri-oral cyanosis and pallor and presented twice to the emergency department with increased work of breathing. On initial assessment by paramedics he showed no signs of life and was in asystole. He was resuscitated until spontaneous circulation was restored at 36 minutes.

4.4.1.2 Investigation and management

Investigations revealed low ionised calcium (0.72mmol/L), warranting repeated intravenous calcium boluses followed by continuous infusion. Cefotaxime was commenced for presumed sepsis, and oseltamivir was added after isolating influenza A on a nasal swab. Intravenous fluids and inotropes were administered. An echocardiogram showed severe dilated cardiomyopathy with poor left ventricular ejection fraction with a structurally normal heart (**Table 4.1**). Rickets was confirmed radiographically (**Figure 4.1B**), with elevated serum ALP and PTH concentrations, and low 25OHD <15nmol/L (**Table 4.1**). Cholecalciferol was commenced, and intravenous calcium continued until serum calcium normalised (72 hours). Cardiac failure was managed with diuretics and vasodilators. Brain MRI revealed severe hypoxic-ischaemic encephalopathy.

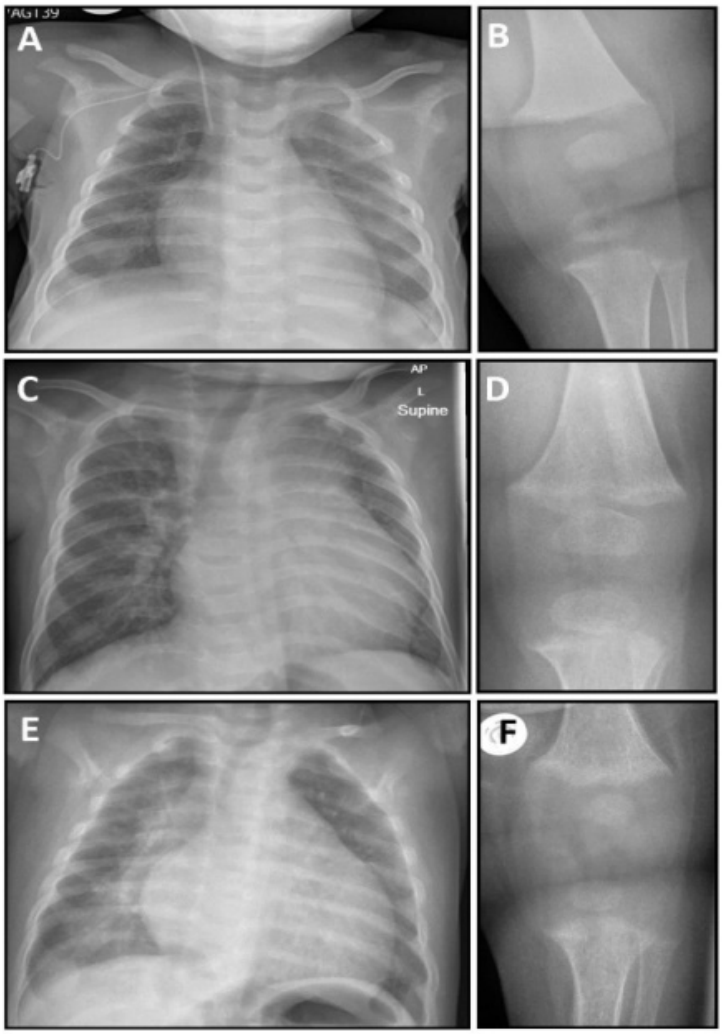


Figure 4.1: Chest and knee radiographs demonstrating cardiomegaly and rickets

Patient 1 (A, B), 2 (C, D) and 3 (E, F)

4.4.1.3 Outcome

The care team and family elected to withdraw life support, and the infant died 6 days after presentation. Post-mortem examination confirmed severe nutritional rickets with rachitic rosary **(Figure 4.2**) [enlarged rib growth plates, craniotabes, soft ribs, dilated cardiomyopathy

(heart weight 71g, >95th centile) with multifocal myocardial necrosis] and massive ischaemic brain injury.



Figure 4.2: Clinical photograph of the rib cage demonstrating rachitic rosary at post-mortem examination of Patient 1.

Histological analysis of the 7th rib growth plate showed signs typical of rickets [32] (**Figure 4.3A**) which included:

1. loss of the columnar arrangement of chondrocytes and disordered proliferation of chondrocytes in the hypertrophic zone secondary to a lack of apoptosis
2. irregularity of the border between the proliferative and hypertrophic zones
3. tongue-like projections of cartilage extend into the spongiosa
4. penetration of blood vessels into the hypertrophic zone
5. Bulbous expansion of the costo-chondral junction

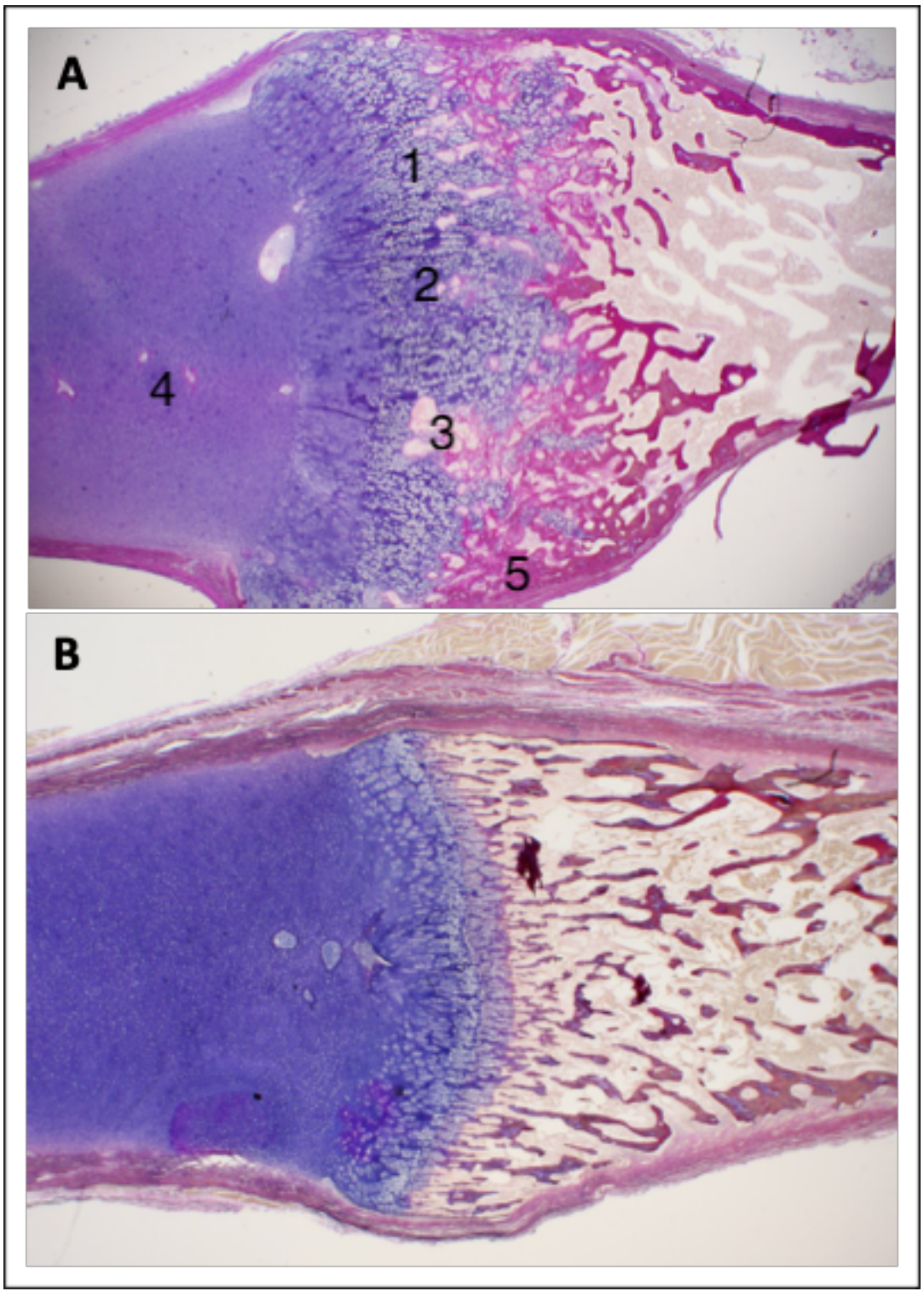


Figure 4.3: Elastica van Gieson stained rib growth plate of an infant with severe 25OHD deficiency. Images demonstrating classic signs of rickets in Patient 1 (A) compared to normal growth plate of control infant with normal serum 25OHD levels (B).

Histomorphometric analysis of a transiliac bone sample identified severe osteomalacia with increased osteoid thickness (23.2µm [normal 6.4 +/- 1.4]), osteoid surface/bone surface (76.3% [normal 24.9 +/- 10]) and osteoid volume/bone volume (40.5% [normal 2.4 +/- 1.22]) when compared to normative data from children aged 1.5-22.9 years [86]. Osteoid volume/bone volume was +1573% of normal reference values, specifically osteoid thickness which has been reported to be constant in children aged 1.5-22.9 years was +262% in our patient confirming osteomalacia [86].

Since Goldner's Trichrome staining does not discriminate well between non-mineralized and poorly mineralized matrix, quantitative backscattered electron imaging was performed, which confirmed the extremely low bone mineralization density **(Figure 4.4)**. The BMDD curve was compared to reference data [88] from children aged 1.5-23 years as no histomorphometry data is available for younger children. The BMDD curve was shifted towards lower mineral content, its width at half-maximum is broader (CaWidth + 55%) due to increased heterogeneity in mineralization, and the fraction of poorly mineralized matrix was markedly increased (CaLow + 640%) [88]. The parameters of the BMDD curve in the reference data remain constant from 1.5 years through to 23 years with no correlation to age [88] which indicates that the shift in curve to the left noted here is significant and corroborates with lower mineralization which is supported by the increased osteoid accumulation.

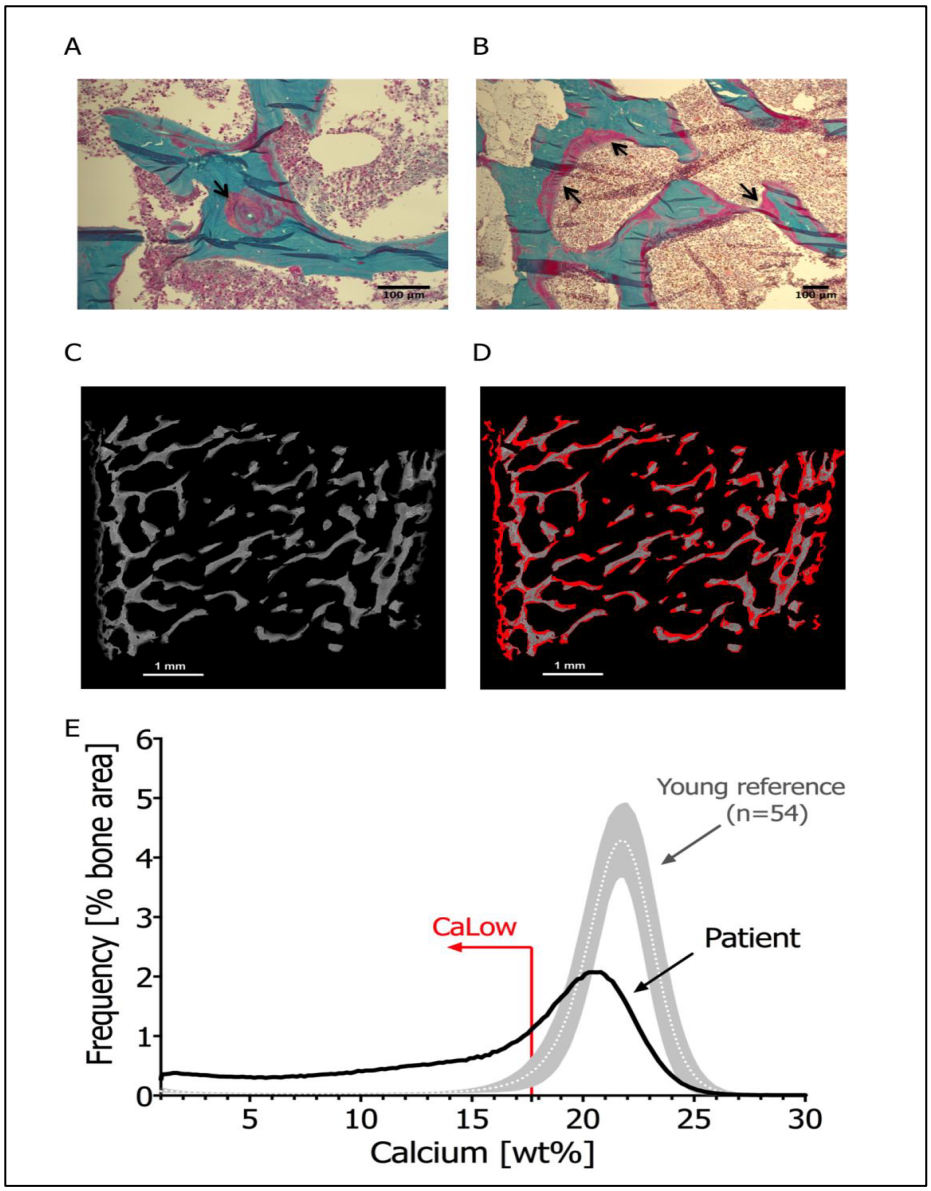


Figure 4.4: Histomorphometric and Quantitative Backscattered Electron Microscopic Analysis of a post-mortem transiliac bone sample

Goldner's Trichrome staining (light microscopy) (A, B) demonstrated broad seams of pink stained areas corresponding to non- or poorly mineralized matrix and regions with blurred pink-green transition (black arrows), next to mineralized matrix (green). Backscattered electron images of the complete bone sample surface (C, D) show low mineral content in dark grey, normal/high mineral content in bright grey and unmineralized matrix appears black (C). Areas highlighted in red (D) represent increased primary mineralization i.e. areas mineralized below 17.68 wt% calcium, corresponding to the 5th percentile of the adult reference range (CaLow) [87]. The BMDD curve is shifted towards lower mineral content.

4.4.2 Patient 2:

4.4.2.1 Clinical presentation

A 6-month-old, partially breastfed and previously well Somali boy presented to the emergency department following respiratory arrest and seizure. He was found pale, floppy and not breathing while held by his sibling. Following emergency telephone advice, his mother, a nurse, commenced CPR at home. Two minutes later he had a 2-minute tonic-clonic seizure. With continued CPR, spontaneous breathing was established at 4 minutes. In the emergency department, he responded to pain, respiratory rate was 40/min, heart rate was 112/min with normal capillary refill. A grade 2/6 systolic ejection murmur was present.

4.4.2.2 Investigations and management

A venous blood gas was normal except for low ionised calcium (0.66mmol/L). A chest radiograph showed cardiomegaly **(Figure 4.1C)**, and echocardiogram demonstrated a structurally normal heart with severely dilated left ventricle with reduced Left Ventricular Ejection Fraction (LVEF), confirming hypocalcaemic dilated cardiomyopathy. Diuretics, ACE inhibitors, oral cholecalciferol and IV calcium therapy were commenced. Nutritional rickets due to vitamin D deficiency was confirmed with knee radiographs **(Figure 4.1D),** elevated serum ALP and PTH, and low 25OHD of <5.2nmol/L (**Table 4.1**). Alfacalcidol (1α-hydroxy cholecalciferol) was temporarily administered to improve calcium absorption.

4.4.2.3 Outcome

On day 3, following a switch from intravenous to oral calcium, he had another seizure with respiratory arrest in hospital, requiring mechanical ventilation and intensive care. Intravenous calcium was recommenced, and a head computed tomography was normal. He was extubated 24 hours later and continued intravenous calcium for 5 more days. He was discharged home on day 17 and three months later showed slow recovery (LVEF 35%; FS 16%; LV diameter 42mm [Z-score +4.7]).

4.4.3 Patient 3:

4.4.3.1 Clinical presentation

A five-month-old British Pakistani girl presented to the emergency department with cough, difficulty in breathing and poor feeding. She was born at 35 weeks with a birth weight of 1.75 Kg (9^{th} centile) and required admission to the neonatal unit for 6 days to establish oral feeding. At presentation, she was found to be pale, irritable, tachypnoeic and tachycardic. She had faltering growth (fall across ≥ 2 weight centiles) with a weight of 4.5 kg (<0.4^{th} centile) and length 58cm (on 0.4^{th} centile). She was diagnosed with bronchiolitis.

4.4.3.2 Investigation and management

Further investigations were triggered by faltering growth which identified hypocalcaemia (Adjusted calcium 1.96 mmol/L). Further evaluation of hypocalcaemia revealed raised ALP and PTH, and low 25OHD of 12.5 nmol/L **(Table 4.1)** and rickets on knee radiograph **(Figure 4.1F).** An echocardiogram performed in view of persistent tachycardia, systolic murmur and cardiomegaly on chest radiograph **(Figure 4.1E)** revealed a structurally normal

heart with a severely dilated left ventricle confirming hypocalcaemic dilated cardiomyopathy. She was commenced on oral calcium supplements and cholecalciferol and transferred to our tertiary centre for specialist cardiology care. She was commenced on diuretics and ACE inhibitors.

4.4.3.3 Outcome

The patient was discharged home 25 days after transfer to our tertiary centre. Six months later, her cardiac dysfunction and dilatation had reversed on echocardiogram. Diuretic treatment was stopped but ACE inhibitors were continued for a further year with six monthly echocardiograms.

4.4.4 Patient 4:

4.4.4.1 Clinical presentation

A six-day old Pakistani boy presented to the emergency department with poor feeding from day 1. He was born by normal vaginal delivery following an uncomplicated pregnancy. He was started on breast milk, switched to formula due to poor feeding but had not gained weight. Physical examination was normal.

4.4.4.2 Investigations and management

The patient was investigated for poor weight gain and found to have hypocalcaemia (Adjusted calcium 1.94 mmol/L) which triggered further investigations. He was found to have a raised ALP and PTH, and low 25OHD <7.4 nmol/L **(Table 4.1).** Knee X-ray and echocardiography

were normal. He was commenced on oral calcium and cholecalciferol. He initially required naso-gastric tube feeds.

4.4.4.3 Outcome

The patient's serum calcium and feeding normalised, gained weight and was discharged home on day 10, with close follow up.

4.4.5 Supplement use

Two of the four mothers had been recommended and consumed vitamin D supplements during pregnancy. None of the mothers or infants were on supplements at the time of presentation. Only patient 2 had received a bottle of supplements at discharge from neonatal unit but were not informed of the need to continue the supplements.

Table 4.1: Clinical characteristics and investigations of the cases at presentation

	Patient 1	**Patient 2**	**Patient 3**	**Patient 4**
Presentation & Demographics				
Age	6 months	6 months	5 months	6 days
Ethnicity	South African-Ghanaian	Somali	British Pakistani	Asian Pakistani
Month of presentation	January	February	February	February
Presenting feature	Cardiac arrest at home; down time of 36 min	Respiratory arrest and seizure; apnoea (~4min)	Cough, difficulty breathing and poor feeding	Poor feeding and static weight
Feeding mode at presentation	Exclusively breastfed	Breastfed (solids 2 weeks before presentation)	Exclusively breastfed	Mixed breast and formula
Birth weight in kg (centile)	3.7 (91^{st})	4.0 (98^{th})	1.75 (9^{th}) 35 weeks	2.9 (2^{nd}-9^{th})
Development	Normal	Normal	Normal	Not applicable
Immunisation	Up to date	Up to date	Up to date	Not due yet
Length in cm (centile)	68 (50^{th})	71 (91^{st})	58 (0.4^{th})	-
Weight in kg (centile)	8 (50^{th})	8.5 (91^{st})	4.5 (< 0.4^{th})	2.9 (2^{nd}- 9^{th})
Investigations				
Adj Ca in mmol/L (2.2-2.7)**	1.60	1.22	1.96	1.94
PO_4 in mmol/L (1.3-2.4)	0.47*	1.95	0.69	2.95
ALP in IU/L (105-420)	802	996	1391	556
PTH in ng/L (12-29)	167	219	482	132
25OHD in nmol/L (> 50)	<15	5.2	12.5	<7.4
X-ray knee	Fraying and splaying of the metaphyses characteristic of rickets	Fraying and splaying of the metaphyses characteristic of rickets	Fraying and splaying of the metaphyses characteristic of rickets	No rickets
ECG-QTc in ms (<450)	547	485	455	Not done
Echocardiography LV dimension in diastole (Z-score) LV EF (55-70%) LV FS (29-40%) Function Mitral regurgitation Structural defects	Dilated CMP +4.6 25-30% 7% Global hypokinesia Severe MR None	Dilated CMP +5.5 29% 7% Global hypokinesia Moderate MR None	Dilated CMP +6.5 25% 15% Global hypokinesia Severe MR None	Normal +0.5 65% 30% Normal No MR None

* Initial PO_4 was 3.51 mmol/L (post cardiac arrest) then continuously dropping to 0.47 mmol/L within 48h. ** Serum calcium is adjusted for albumin by using the formula: Adjusted calcium = measured total calcium + 0.02 * (40 - [albumin in g/L]).

4.4.6 Investigation of family members

Investigation of family members revealed widespread biochemical osteomalacia **(Table 4.2)**. All family members had deficient (8/13, 61.5%) or insufficient (5/13, 38.5%) 25OHD levels. The majority (10/13, 76.9%) of family members also had biochemical evidence of osteomalacia with a raised ALP and/or PTH.

Table 4.2: Investigations of family members of infants with symptomatic vitamin D deficiency

		Age years	**Calcium nmol/L**	**Phosphate mmol/L**	**ALP IU/L**	**25OHD nmol/L**	**PTH ng/L**
Normal range	Adults		2.2 - 2.6	0.8 - 1.5	25 - 105	>50	15 - 65
Normal range	Paediatric		2.2 - 2.7	0.9 - 1.8	105 - 357	>50	13 - 29
Patient 1	Mother#		2.42	1.18	77	**33.1**	54
	Sibling 1	9				**33.0**	
Patient 2	Mother#		2.31	1.29	**161**	**19.8**	**91**
	Sibling 1	9	2.34	1.53	**407**	**19.2**	**60**
	Sibling 2	7	2.33	1.63	353	**35.6**	**48**
	Sibling 3	6	2.42	1.59	**407**	**26.7**	**60**
	Sibling 4	3	2.37	1.75	**371**	**23.7**	**55**
Patient 3	Mother		2.25	1.1	86	**24.0**	**87**
	Sibling 1	3	2.45	1.6	227	**45.5**	28
Patient 4	Mother		2.3	-	**179**	**11.9**	**70**
	Sibling 1	8	2.2	-	354	**29.3**	**45**
	Sibling 2	6	2.5	-	**478**	**22.6**	**43**
	Sibling 3*	4	2.47	-	**372**	**41.8**	**37**

*on multivitamins #on vitamin D supplements only during pregnancy - not done

4.5 Discussion

All infants presented were of BAME background who were unsupplemented. Adherence to supplementation was not monitored at contacts with healthcare professionals, including immunisation visits.

4.5.1 Early recognition and prevention

The clinical symptoms of all four infants discussed above were relatively silent until severe complications of hypocalcaemia manifested. The extent of disease was unveiled by detailed investigations including X-rays, echocardiography, blood tests and post-mortem investigations. Patient 1 presented repeatedly to multiple healthcare professionals with symptomatic heart failure and history suggestive of seizures (pale floppy episodes) which were unrecognised. The subtle signs of rickets in infants such as swelling of wrist/ankle joints or frontal bossing may go easily unrecognised by untrained professionals. In the UK, health visitors and general practitioners provide primary healthcare to infants and children (**Figure 3.5)** who do not receive formal paediatric training [89,90].

Patient 4 presented within the first week of life with early symptoms of poor feeding. The mother was profoundly vitamin D deficient (11.9 nmol/L) and did not receive supplements in pregnancy. Therefore, the deficiency state was passed on to the newborn. The amount of vitamin D in formula feed was not sufficient to prevent symptomatic vitamin D deficiency in the newborn in the absence of supplements starting at birth.

4.5.2 Supplement use and monitoring

Similar to the infants in a previously case series of dilated cardiomyopathy [6], none of the infants reported here were on vitamin D supplements. None of the previous reports on symptomatic vitamin D deficiency **(Table 3.1)** have made specific reference to compliance with other routine healthcare checks. We, therefore, specifically gathered detailed information on attendance at routine healthcare visits and immunisations. All infants presented here had received the recommended heath care checks and attended all immunisation visits. Parents had not received information on the need for supplements at any of these routine healthcare visits. Patient 4 who presented in the early neonatal period had not received information on the need for supplementation at discharge from the neonatal unit. Monitoring supplement use at routine childhood healthcare visits or providing information at discharge from neonatal units is currently not mandatory in the UK, a practice that needs to be reviewed. The maternal antenatal handheld notes do incorporate a check for vitamin D supplements, however compliance is not audited or monitored and is not mandatory.

4.5.3 Mortality and morbidity

The mortality and morbidity noted in our cohort of hypocalcaemic dilated cardiomyopathy is similar to that reported in the hypocalcaemic dilated cardiomyopathy case series (n=16) from London [6]. The extent of osteomalacia at post-mortem however has not been previously reported. Patient 1 presented with hypocalcaemic dilated cardiomyopathy with clinically occult rickets as a cause of heart failure and sudden infant death, despite apparently normal clinical development and growth. Post-mortem examination revealed florid rickets with

typical histological changes at the costo-chondral junction **(Figure 4.3)**. Marked osteomalacia with significantly reduced mineralization was recorded **(Figure 4.4)**.

All the infants investigated in this study had significant morbidity and required extensive investigations to exclude other causes of seizures and dilated cardiomyopathy. Infants with cardiomyopathy required prolonged hospitalisation with intensive care and high dependency unit support. Ongoing regular outpatient follow up and serial scans were needed. Both infants with cardiomyopathy (patient 2 and 3) who survived made a full recovery with return to normal cardiac function over a period of 6 months.

4.5.4 Biochemical osteomalacia in family members

Elevated serum ALP [91] and PTH [30] serve as functional markers of calcium deprivation which precede clinical and radiological signs of rickets and/or osteomalacia [33,34] **(Figure 2.5**). Few studies have assessed maternal 25OHD status in the presence of vitamin D deficiency in the infant **(Table 3.1)**. However, the extent of biochemical osteomalacia in family members of symptomatic infants has never been systematically explored before. We uncovered a high proportion of affected family members who were all asymptomatic. Biochemical osteomalacia often goes undiagnosed [2] despite the presence of clear risk factors and lack of supplement use. All family members reported here shared the common risk factors of dark skin pigmentation and residence in a high latitude country. Of all the family members investigated, only one individual (Patient 4's sibling aged 4 years) was on Healthy Start supplements as recommended by the government. Nonetheless, the sibling still had insufficient levels of 25OHD with elevated ALP and PTH, most likely due to the low vitamin D content in Healthy Start vitamin.

4.6 Limitations and conclusions

The cases reported here were not nationally representative as this was not the aim of the study. However, a national survey of nutritional rickets in a similar time period between 2015- 2017 identified dilated cardiomyopathy in 3.2% (n=4/125) of cases presenting with nutritional rickets, of whom 1.6% (n=2/125) died [9]. Although the proportion of osteomalacia in family members in our small cohort was high, further large studies are warranted to confirm the findings. Additionally, studying the genes involved in vitamin D metabolism may clarify the role of genetic and non-genetic factors contributing to deficiency states in family members. Polymorphisms in genes encoding key enzymes (i.e. CYP3A4, CYP24 and CYP27B1) and proteins that affect the circulating concentrations of vitamin D metabolites have been described [92]. However, a genome wide association study (n=4501) indicated that only less than 5% of the variations in serum 25OHD concentrations are explained by genetic variants [92] with major determinants still being lifestyle factors such as exposure to sunlight and dietary habits.

In conclusion, these case studies highlight the hidden cardiac and growth plate disease caused by vitamin D deficiency in infants and their asymptomatic family members. The devastating health consequences may have been prevented through robust, accountable supplementation programmes, with monitored adherence during routine prenatal and child surveillance visits. The implications of the work described in this chapter, with respect to UK vitamin D supplementation policy is discussed in the final chapter of the thesis.

Chapter summary

- Nutritional rickets and osteomalacia remain relatively silent and unrecognised until presented with hypocalcaemic manifestations

- Children presenting with vitamin D deficiency can have significant underlying hidden pathology including: rickets, cardiac failure, growth plate abnormalities and mineralization defects

- It is possible that a high proportion of family members of infants presenting with vitamin D deficiency have undiagnosed/hidden biochemical osteomalacia which warrants investigation and treatment

- The significant burden of deficiency in the reported cases and family members may have been prevented through robust antenatal and post-natal supplementation and monitoring at immunisation visits

Chapter 5: **Prevalence of vitamin D deficiency in neonates: a newborn blood spot screening study**

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Roles and responsibilities involved in the work reported in the current chapter:

Suma Uday: Led the study as Principal Investigator at Birmingham Women's and Children's Hospital (BWCH)

Study set up, funding acquisition, sought all relevant ethical approval, supervision of students, research governance, data gathering, data interpretation, statistical analysis, report writing, manuscript preparation, publication and presentation of findings.

Wolfgang Högler: Conceptualised the study, funding acquisition, intellectual contribution to data interpretation, critical revision of written report and manuscript.

Sunia Naseem (Medical student) - Assisted in completion of ethics application, blood spot sample collection.

Jamie Large (Medical student): Assisted in drafting funding application, blood spot sample collection.

Russell Denmeade (Lead Biomedical Scientist for Newborn Screening, BWCH): Newborn Screening data anonymisation, student supervision with sample collection.

Philippa Goddard (Consultant Clinical Scientist Newborn Screening, BWCH): Expert assistance in study design and set up.

Mary Anne Preece (Consultant Biochemist and Director of Newborn screening, BWCH): Expert assistance in study design and set up.

Rachel Dunn, William Fraser, Jonathan Tang (Norwich Laboratory): Blood spot vitamin D analysis, data provision, intellectual revision of laboratory methodology reported.

5.1 Introduction

Adequate vitamin D status in pregnancy is essential to maintain optimal skeletal health in the mother and her newborn [66]. Maternal supplementation with vitamin D prevents hypocalcaemia [93], and rickets in the newborn [29], and also optimises the future bone health of the offspring [94].

Due to the high calcium demands during rapid growth in infancy [4], hypocalcaemia can manifest as early as in the first week of life [95-97] if the mother was severely vitamin D deficient and did not receive adequate replacement in pregnancy. Growing evidence suggests that, in addition to the adverse effects on bone health, vitamin D deficiency can result in non-skeletal pregnancy-related adverse health outcomes such as gestational diabetes, pre-eclampsia and small for gestation birth [98,99]. It is therefore imperative to ensure adequate vitamin D status during pregnancy and infancy. To date, the success and effectiveness of the UK antenatal supplementation programme remains unknown.

Vitamin D status in apparently healthy children, particularly infants, is poorly studied due to the practical difficulties in obtaining blood samples [100]. In recent years, measuring 25OHD on dried blood spot (DBS), obtained through minimally invasive techniques [101,102], has evolved significantly enabling reliable prediction of vitamin D status of the population [103]. Measuring neonatal 25OHD on DBS collected as part of the national newborn bloodspot screening (NBS) programme is a good way of assessing the population prevalence of vitamin D deficiency in this most vulnerable group. All babies born in England have a blood spot sample collected on day 5 of life as part of the national NBS programme to screen for a specific set of metabolic diseases. This public health study was designed to assess the

effectiveness of the UK antenatal supplementation programmes in preventing vitamin D deficiency in the newborn and provide direction to infant supplementation programmes.

5.2 Aims and objectives

5.2.1 Aims

To determine the prevalence of vitamin D deficiency at birth in a multi-ethnic UK newborn cohort

5.2.2 Objectives

- To determine the prevalence of 25OHD < 30nmol/L in newborns as a key performance indicator of the antenatal supplementation programme
- To assess seasonal variation in 25OHD in newborns
- To assess ethnic variation in 25OHD in newborns
- To study the determinants of 25OHD in newborns

5.3 Research design and methods

5.3.1 Study Design

To collect a representative population sample in an increasingly ethnically diverse population, 3000 DBS samples sent to Birmingham Women's and Children's Hospital's regional newborn screening laboratory, in the Midlands region of England (Latitude, 52.4751° North), were collected after obtaining ethical and relevant regulatory approvals. Data submitted on the NBS cards was gathered following anonymisation. 25OHD

concentration on the DBS was measured by quantitative liquid chromatography tandem mass spectrometry (LC-MS/MS) and plasma equivalent values derived.

5.3.2 Study population

To assess seasonal effects, all DBS samples received over one week in winter (last week of February 2019) and one week in summer (last week of August 2019) were retrieved after all routine NBS testing had been completed.

5.3.2.1 Exclusion criteria

- Samples marked high risk of infection (e.g. HIV)
- Insufficient samples
- Second tests including neonates >21 days
- Cases with any missing information.

5.3.3 DBS Samples

The sample is collected on a standard Whatman 903 filter paper through a heel prick by an appropriately trained healthcare professional, as per usual practice. Samples were collected within a week of receiving in the laboratory, stored at -20°C and analysed at the completion of recruitment (October 2019 - December 2019).

5.3.4 Source Data

Relevant data submitted alongside the NBS card is routinely entered and stored by the newborn screening laboratory team in an electronic format. Data requested for the purpose of

the study included: birth weight, gestational age, maternal age, ethnic group codes and post codes. For anonymity, Lower-layer Super Output Area (LSOA) and Index of Multiple Deprivation (IMD) were used as proxy for socio-economic status.

5.3.4.1 LSOA and IMD

Post code was replaced with the LSOA that each postcode falls within, and IMD for that LSOA at (http://imd-by- postcode.opendatacommunities.org/) [104].

LSOA is a geographic hierarchy designed to improve the reporting of small area statistics in England and wales. LSOAs are designed to be of a similar population size, with an average of approximately 1,500 residents or 650 households. The Index of Multiple Deprivation (IMD) is obtained by combining seven domains of deprivation: income, employment, education, health, crime, barriers to housing and services and living environment [104]. Each domain and the IMD can be represented in ranks (1 to 32,844) or deciles which are calculated by ranking the 32,844 small areas in England from most deprived to least deprived and dividing them into 10 equal deciles, where 1 is the most deprived and 10 the least deprived [105].

5.3.4.2 Ethnic data

Ethnicity group codes prescribed by the UK Office of National Statistics **(Appendix 2)** were used [106]. The independent ethnic group codes were clubbed and grouped as follows:

Group 1: White British

Group 2: Any other white (white Irish + Any other white)

Group 3: Asian (Indian, Pakistani, Bangladeshi, Chinese and any other Asian background)

Group 4: Black (African, Caribbean, any other black background)

Group 5: Mixed white and black (White and black African and white and black Caribbean)

Group 6: Mixed white and Asian

Group 7: Any other mixed (any other mixed background and any other ethnicity)

5.3.4.3 Anonymisation

Patient data was required to be anonymised in keeping with the code of practice for the retention and storage of residual newborn blood spots [107].

- The data was anonymised by the central laboratory manager by excluding any patient identifiable details such as name, hospital number and NHS number
- The link between the identity of the child and samples/data was permanently removed
- A new unique identifier was created for each dataset
- The link between the old and new identification numbers were permanently deleted by the lab manager so that the samples analysed were completely anonymous and not traceable by the researcher or the newborn screening lab staff
- The sample collected were labelled with the corresponding new identification number
- Norwich research park laboratory, where 25OHD was measured, only receive a study number with no patient details

5.3.5 Analysis of DBS 25OHD and plasma equivalent values

Dried blood spot 25OHD3 and 25OHD2 concentrations were measured at the Bioanalytical facility in The University of East Anglia, Norwich, UK, by quantitative LC-MS/MS

following extraction methods previously detailed [102,108]. In brief, a 6mm sub-punch was made from each blood spot sample using a pneumatic DBS card puncher (Analytical Sales & Services, NJ, USA). Samples were extracted using 300 µL of 50:50 (v/v) isopropanol to water solution containing carbon-13 labelled 25OHD3-$^{13}C_5$ internal standard; in an ultrasonic water bath at 35°C for 30 minutes. The extracts were transferred onto Supported Liquid Extraction (SLE+) plate (Biotage, Uppsala, Sweden) for further clean up, then eluted with 1.5 mL of heptane. The eluent was dried to completeness under nitrogen gas heated at 60°, followed by derivatisation with 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD). The PTAD-derivatised 25OHD3 and 25OHD2 were separated using a C18 2.7µm. 50 x 2.1mm chromatographic column (Restek, PA, USA) and detected using the Micromass Quattro Ultima Pt tandem mass spectrometer (Waters Corp., Milford, MA, USA) according to analyte precursor to product transitions; 607>298 (25OHD3) and 613>298 (25OHD2). Each batch of sample analysis was performed with matrix-matched calibration standards and quality controls prepared in vitamin D-free packed red cells. The inter/intra-assay coefficient of variation (CV) was between 3.9-9.4%, with linearity from the lower limit of quantification (LLoQ) of 1 nmol/L up to 150 nmol/L.

5.3.5.1 Plasma equivalent 25OHD

Data are reported as plasma equivalent total 25OHD, which was calculated using the below formula where 1.2607 is to adjust for the difference in slope between plasma and DBS value [102] and 0.60 is the mean haematocrit (Hct).

$$\text{Plasma equivalent total 25OHD nmol/L} = \frac{[(\text{DBS 25OHD3} + \text{DBS 25OHD2 nmol/L}) - 1.2607]}{(1 - 0 \cdot 60)}$$

Given that 25OHD exists primarily in the extracellular fluid compartment in blood, blood spot measurements for 25OHD must be adjusted for Hct. Owing to the higher Hct in the neonatal period [109] and specifically in capillary samples [110] a value of 0.60 was used.

5.3.6 Ethics and consent:

The study was approved by the UK Health Research Authority (HRA) **(Appendix 3)** and the East Midlands - Leicester South Research Ethics Committee (REC Reference 19/EM/0019) **(Appendix 4)**. The study was also approved by the Antenatal and Newborn Research Advisory Committee of Public Health England, UK **(Appendix 5)**. Parental consent was not required as all data acquired was anonymised and compliance with the code of practice for the retention and storage of residual newborn blood spots was ensured [107].

5.3.7 Statistical analysis:

5.3.7.1 Descriptive statistics

Descriptive statistics are presented as medians with interquartile ranges (IQR) or mean with standard deviation (SD) for continuous variables, and frequencies with percentages for categorical variables. 25OHD concentrations were used both as a continuous variable and categorical variable, defining deficiency as <30 nmol/L, insufficiency as 30-50 nmol/L and sufficiency as >50 nmol/L according to the Institute Of Medicine,[28] and Global consensus recommendation classification.[29]

5.3.7.2 Normality test

25OHD values were positively skewed (0.78) and log transformation resulted in a negative skewness (-1.48). A square root transformation achieved normal distribution (0.02)

(Appendix 6). The results obtained using square root transformed values were similar to the results obtained using the actual 25OHD values. Therefore 25OHD values are reported here.

5.3.7.3 Non-parametric tests

Mann-Whitney U test was used to compare 25OHD concentrations between the summer and winter born groups. The Kruskal-Wallis test was used to compare 25OHD concentrations between the different ethnic groups.

5.3.7.4 Multiple linear regression

The factors which were significant ($p<0.05$) on a bivariate analysis were included in the multiple linear regression model. The significant factors included were season of birth, ethnicity, gestational age, maternal age and IMD decile. Ethnicity, gestational age and IMD deciles (1 to10) were used as categorical variables. Gestational age at birth was categorised as term (>37- 41 weeks), extreme pre-term (<28 weeks), pre-term (28-37 weeks) and post-term (>41 weeks). All analyses were performed using SPSS statistical software v25.0 (IBM, Armonk, NY).

5.4 Results

25OHD measurements were available on 2999 (1580 males; 52.7%) subjects; 1499 were winter born and 1500 summer born. Neonates were between 5 and 19 days old at the time of sample collection. Ninety nine percent of the samples were collected in the first week of life between day 5 and day 7 [93.3% on D5 (n=2800), 5.1% on D6 (n=154) and 0.6% on D7 (n=19)]. The baseline characteristics are presented in **Table 5.1**.

The majority were white British (59.1%) and born at term (mean ± SD gestational age of 38.8 ± 1.8 weeks) with a mean (± SD) birth weight of 3306 (± 565) grams. The IMD decile was 4.04 (± 2.7) and nearly a quarter of the cohort (24%, n= 720) were from the most deprived IMD decile (decile 1). The baseline characteristics did not differ significantly between the winter-born and summer-born groups **(Table 5.1)**.

Table 5.1: Baseline characteristics of the study group

Numbers are reported as mean (± SD) or n (%)

	Whole study group	**Winter-born**	**Summer-born**	**p value**
Numbers	2999	1499 *	1500	
Number of males (% of total)	1580 (52.7%)	771 (51.4%)	809 (53.9%)	0.16
Birth weight in g	3306 (565)	3313 (563)	3299 (566)	0.49
Gestational age in weeks	38.8 (1.8)	38.79 (1.8)	38.83 (1.7)	0.57
Maternal age in years	30.4 (5.5)	30.36 (5.6)	30.46 (5.4)	0.61
Ethnic groups n (%)				
White British	1774 (59.1%)	877 (58.5%)	897 (59.8%)	0.64
Any other white	264 (8.8%)	134 (8.9%)	130 (8.7%)	
Asian	494 (16.5%)	249 (16.6%)	245 (16.4%)	
Black	173 (5.8%)	90 (6.0%)	83 (5.5%)	
Mixed white and black	94 (3.1%)	53 (3.5%)	41 (2.7%)	
Mixed white and Asian	45 (1.5%)	21 (1.4%)	24 (1.6%)	
Any other mixed	156 (5.2%)	76 (5.1%)	80 (5.3%)	
Index of Multiple Deprivation decile (1=most deprived to 10=least deprived)	4.04 (2.7)	4.06 (2.7)	4.02 (2.7)	0.34

* One missing sample

5.4.1 25OHD concentrations

The median (IQR) 25OHD concentration was 37.8 (24.8, 54.8) nmol/L. The mean (± SD) was 41.0 (± 21.9) nmol/L.

5.4.1.1 Seasonal variation in 25OHD concentrations

The mean (± SD) 25OHD level in winter-born and summer-born babies was 31.5 (± 17.1) nmol/L **Figure 5.1** and 50.6 (± 22.0) nmol/L **Figure 5.2** respectively with a wide variation in the summer-born cohort. The median (IQR) 25OHD concentration in winter-born was 29.1 (19.8, 40.6) nmol/L and in summer-born was 49.2 (34.3, 64.8) nmol/L. The 25OHD concentration was significantly lower in winter-born compared to summer-born ($p<0.001$) **Figure 5.3.**

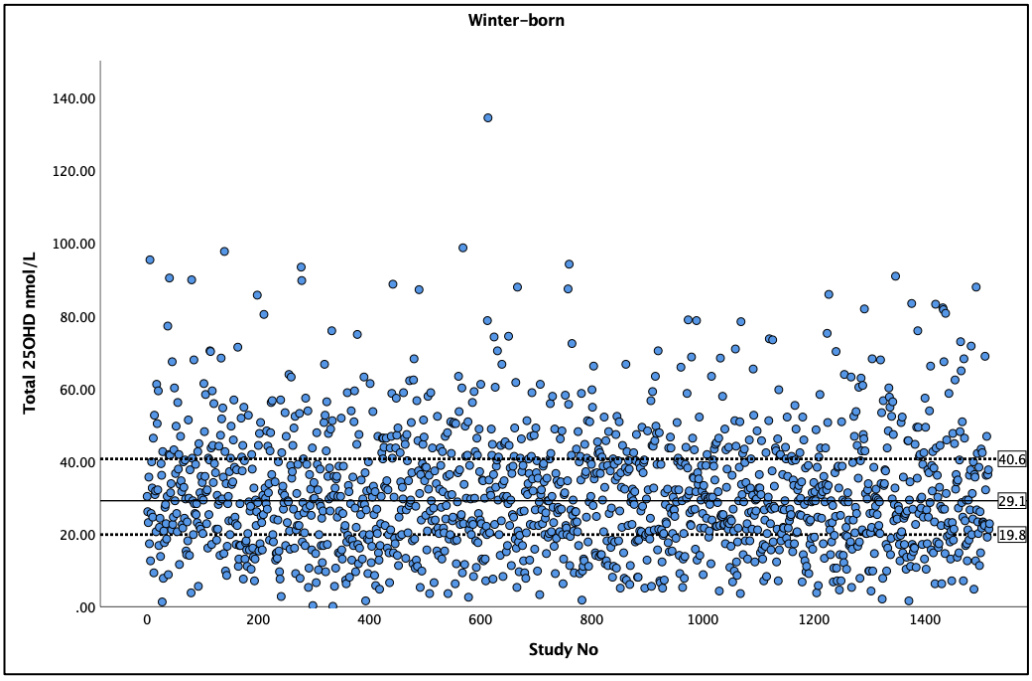


Figure 5.1: Distribution of 25OHD concentrations in winter-born babies

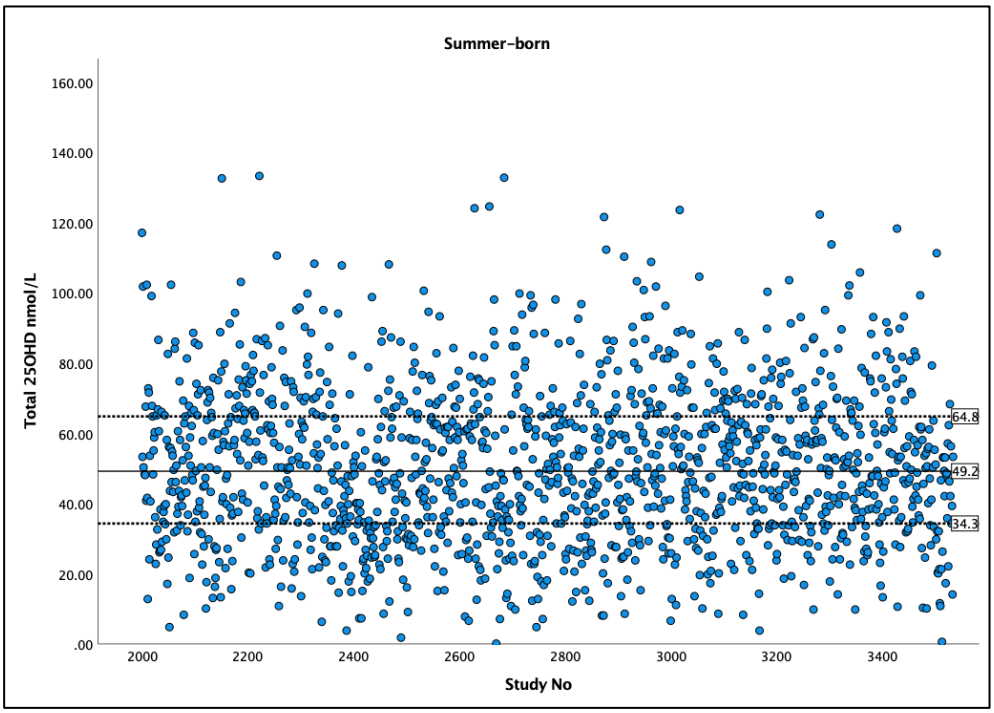


Figure 5.2: Distribution of 25OHD concentrations in summer-born babies

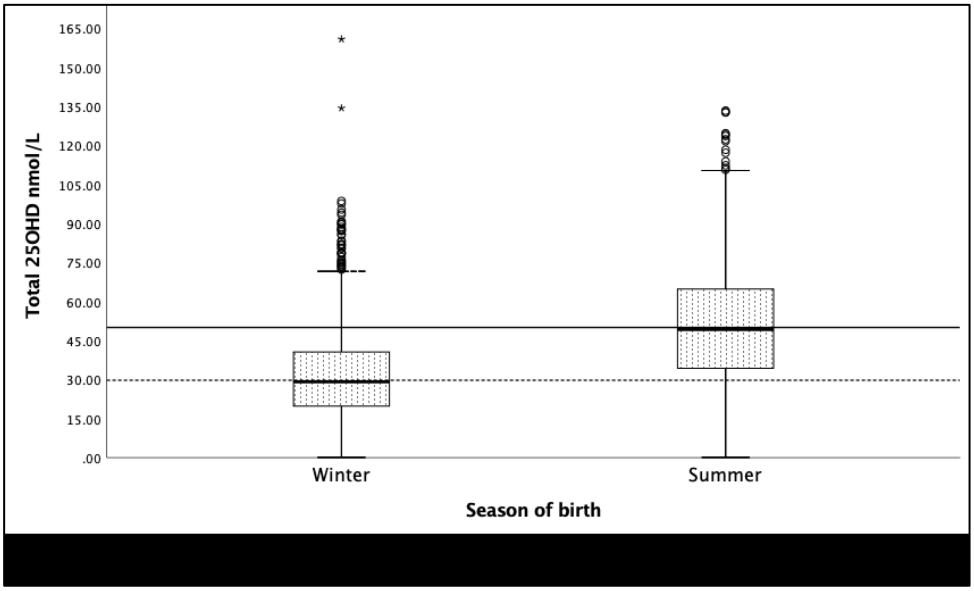


Figure 5.3: Seasonal variation in newborn 25OHD concentrations.

The median 25OHD concentration in winter-born babies was significantly lower that summer-born babies (29.1 vs 49.2 nmol/, $p<0.001$).

5.4.1.2 Ethnic variation in 25OHD concentrations

The median (IQR) 25OHD concentrations differed significantly among ethnic groups, as presented in **Table 5.2** below. Compared to white British, the 25OHD concentrations were significantly lower in babies of black ($p<0.001$), Asian ($p<0.001$), any other mixed ($p<0.001$), mixed white and black ($p<0.05$) and any other white ($p<0.05$) background. Babies of mixed white and Asian background also had lower median 25OHD concentrations compared to babies of white British background, but this was not statistically significant.

5.4.1.3 Seasonal and ethnic variations in 25OHD concentrations

The median (IQR) 25OHD concentrations based on ethnicity and the season of birth are presented in **Table 5.2** and graphically presented in **Figure 5.4**

Table 5.2: Seasonal and ethnic variations in median (IQR) 25OHD concentrations

Ethnic group (n)	**Median (IQR) 25OHD nmol/L**	**Median (IQR) 25OHD nmol/L**	**Median (IQR) 25OHD nmol/L**
	Total	**Summer**	**Winter**
White British (1774)	41.6 (27.6, 59.3)	56.3 (42.1, 69.7)	29.3 (20.8, 40.3)
Mixed white and Asian (45)	39.1 (22.3, 54.9)	52.0 (34.0, 62.8)	25.6 (14.4, 39.6)
Any other white (264)	37.7 (24.7, 53.2)*	48.4 (33.7, 61.1)	30.4 (20.7, 41.0)
Mixed white and black (94)	33.7 (21.6, 50.3)*	45.1 (33.8, 59.9)	25.3 (16.9, 36.4)
Any other mixed (156)	32.9 (20.1, 47.3)**	36.9 (24.7, 51.9)	26.8 (16.8, 44.1)
Asian (493)	31.3 (20.1, 44.0)**	31.8 (23.7, 46.7)	29.9 (18.3, 42.6)
Black (173)	30.3 (19.4, 43.2)**	38.3 (26.3, 49.1)	24.4 (15.0, 34.4)

**$p<0.001$ and *$p<0.05$ when compared to white British

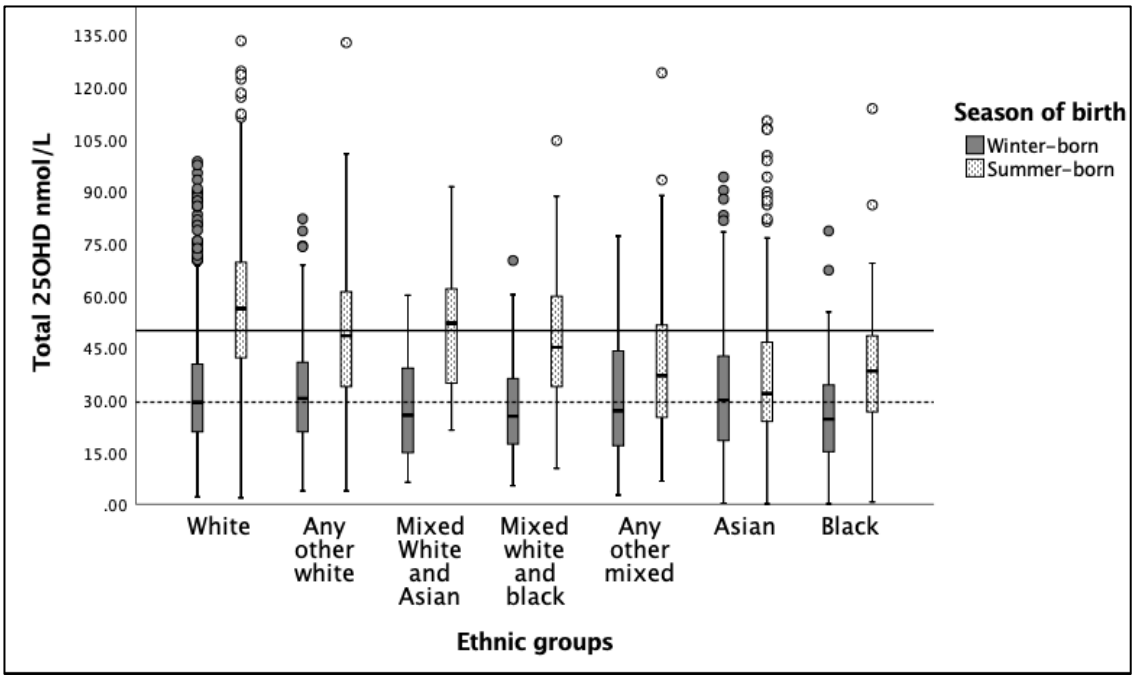


Figure 5.4: Distribution of 25OHD concentrations based on season of birth and ethnicity

5.4.2 Prevalence of Vitamin D deficiency

Vitamin D deficiency (<30 nmol/L) was present in 35.7% (n= 1070) of the cohort **(Figure 5.5)**, of whom 6.2% (n= 186) were severely deficient with concentrations below 12.5 nmol/L (5µg/L). Vitamin D insufficiency (30-50 nmol/L) and sufficiency (>50 nmol/L) were noted in 33.7% (n=1010) and 30.6% (n=919) respectively.

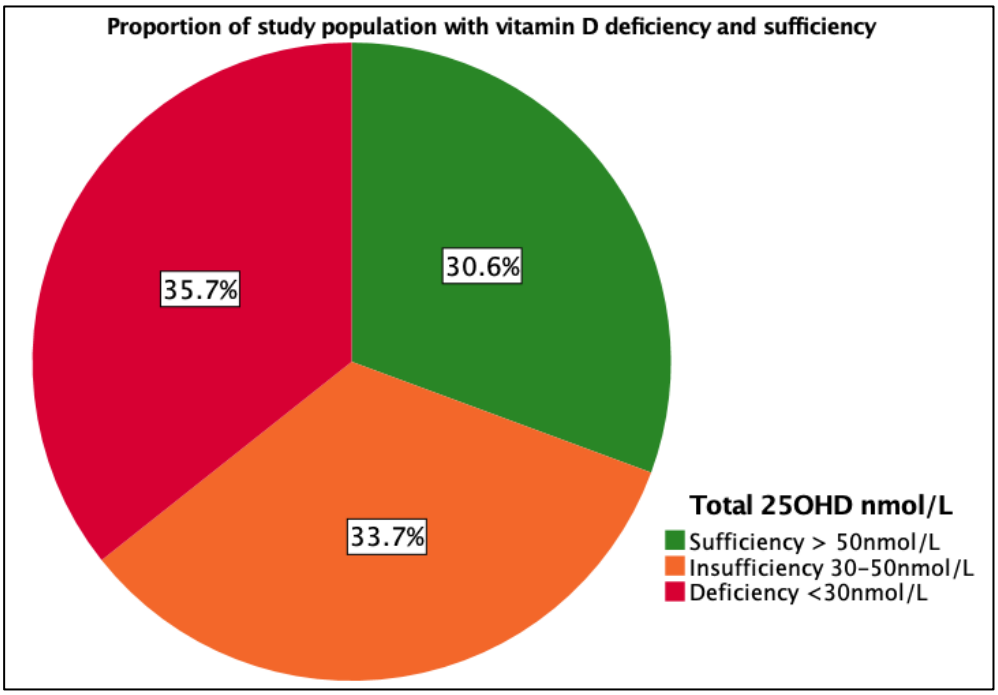


Figure 5.5: Proportion of population with vitamin D deficiency and sufficiency

5.4.2.1 Seasonal variation in prevalence of deficiency

Vitamin D deficiency was more prevalent in the winter-born babies compared to summer born babies [52.9% (n=794) vs 18.4% (n=276) respectively] **(Figure 5.6).**

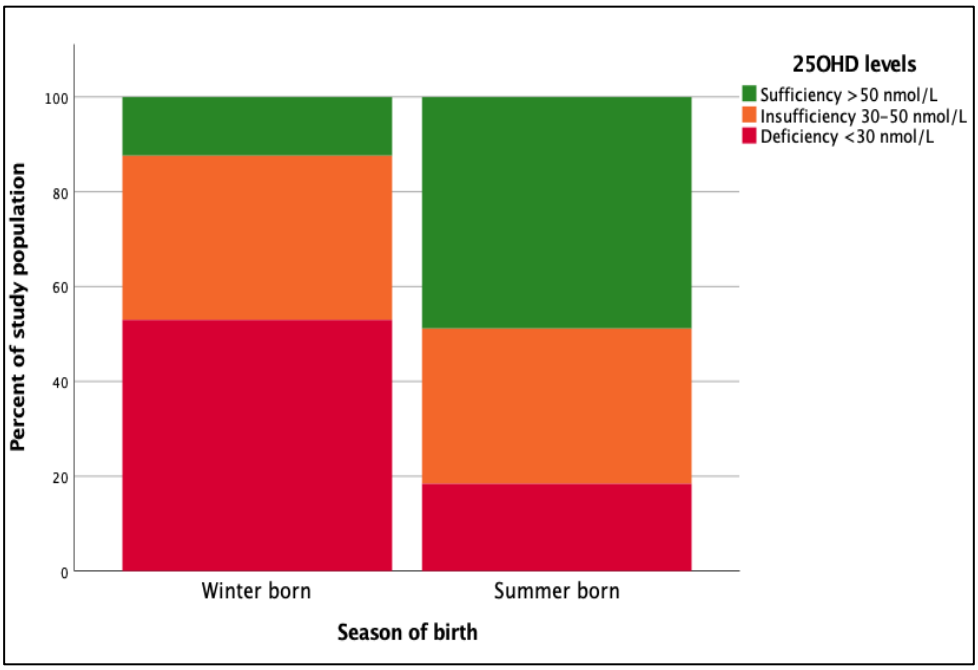


Figure 5.6: Seasonal variation in the prevalence of deficiency

5.4.2.2 Ethnic variation in prevalence of deficiency

The percentage of study group who were vitamin D deficient across both seasons was lowest in white British at 30.27% and highest in babies of Asian and black ethnic background at 47.67% and 47.40%, respectively (**Figure 5.7**).

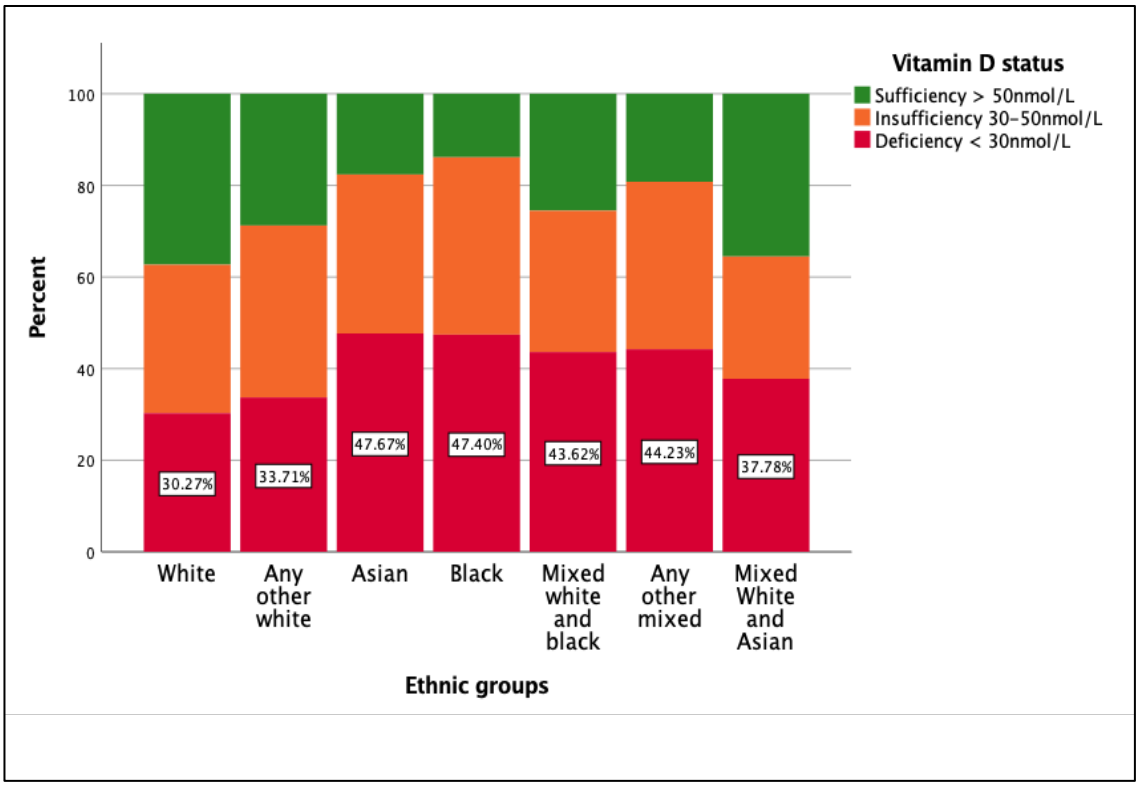


Figure 5.7: Ethnic variation in the prevalence of vitamin D deficiency

5.4.2.3 Seasonal and ethnic variation in prevalence of deficiency

The proportion of winter-born and summer-born babies with vitamin D deficiency, insufficiency and sufficiency are presented in **Figure 5.8** and **Figure 5.9** respectively.

Table 5.3 below, presents the proportion of winter and summer-born babies with 25OHD concentrations below 25 nmol/L (as per UK SACN deficiency definition) [66] and those below 50 nmol/L (as per IOM suboptimal levels definition) [28].

Table 5.3: Seasonal variation in the proportion of deficient population based on ethnicity

Ethnicity	Summer		Winter	
	% <25nmol/L UK/SACN	% ≤50nmol/L IOM	% <25 nmol/L UK/SACN	% ≤50nmol/L IOM
White	3.8	39.2	36.5	86.7
Any other white	14.6	52.3	35.1	89.6
Black	21.7	75.9	53.3	95.6
Asian	30.2	78.4	40.6	86.3
Mixed white and Asian	8.3	41.7	47.6	90.5
Mixed white and Black	17.1	56.1	49.1	88.7
Any other mixed	25	73.8	48.7	88.2

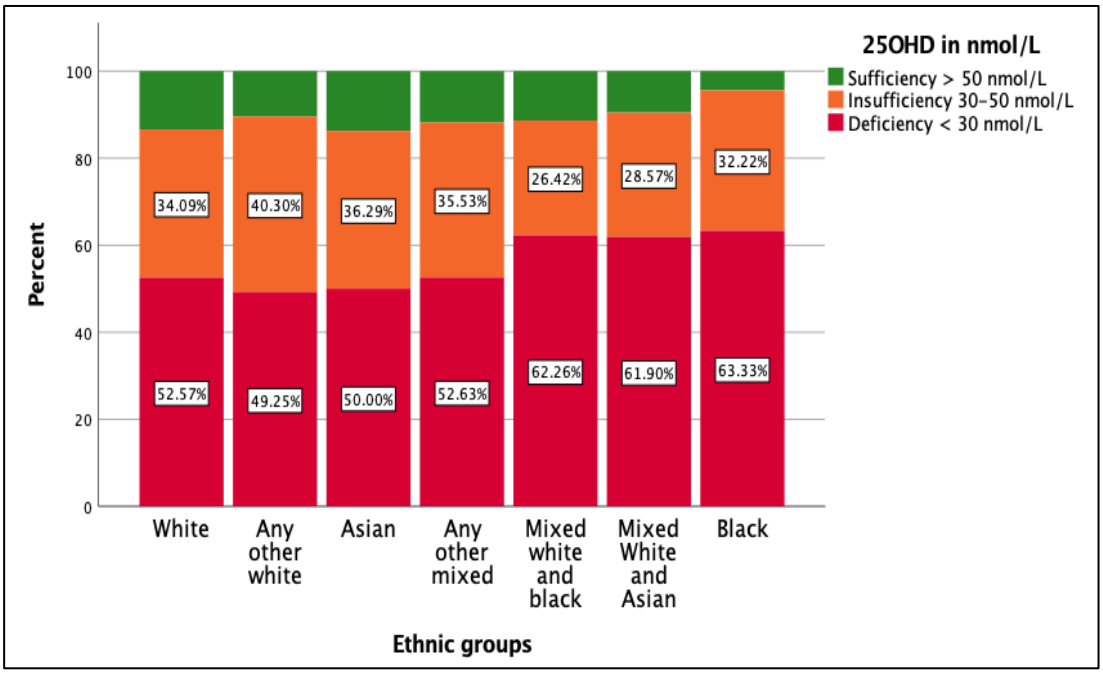


Figure 5.8: Proportion of deficiency, insufficiency and sufficiency based on ethnicity in the winter-born cohort

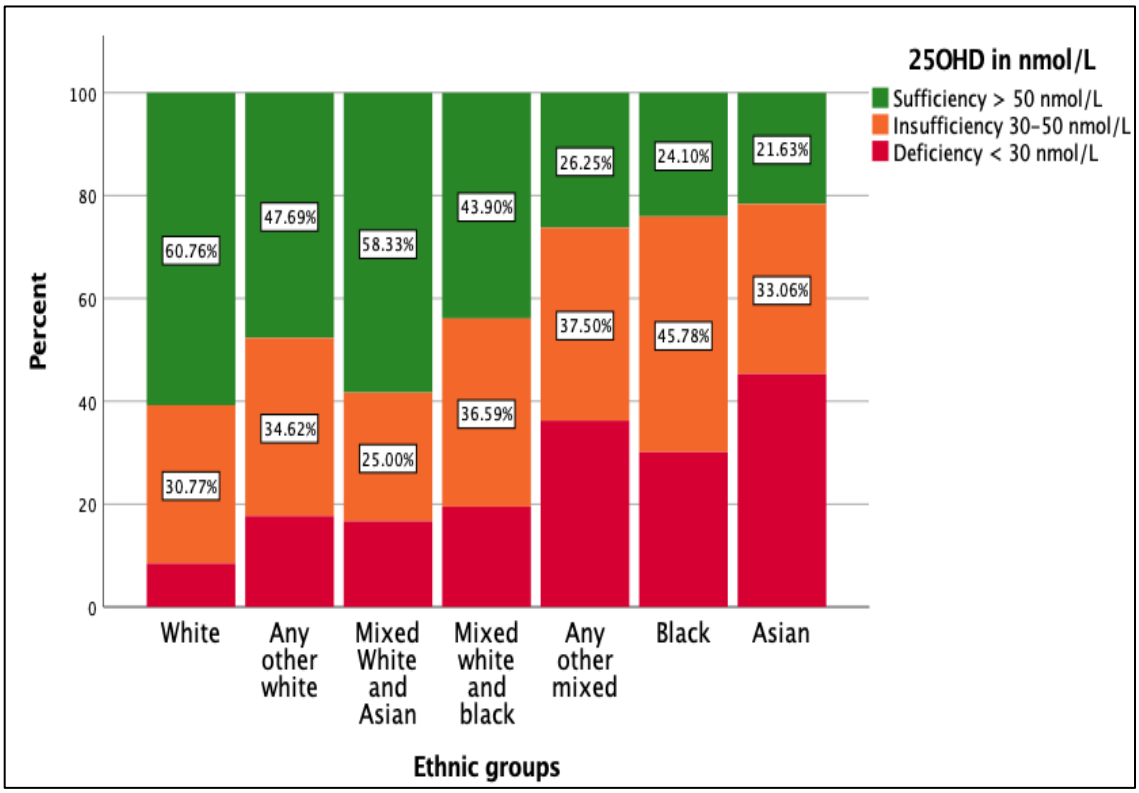


Figure 5.9: Proportion with deficiency, insufficiency and sufficiency based on ethnicity in the summer-born cohort

5.4.3 Determinants of 25OHD concentrations

Parameters which were significant ($p<0.05$) on a bivariate linear regression included in the multiple regression model were season of birth, ethnicity, gestational age, maternal age, and IMD decile. Birth weight and gender were not associated with 25OHD concentrations ($p>0.05$).

The results of the regression model are presented in **Table 5.4**. The statistically significant determinants of 25OHD were season of birth, ethnicity (all ethnic groups except mixed white and Asian compared to white British), gestation (prematurity and extreme prematurity compared to term gestation) and maternal age. Nearly 24% (adjusted R^2 0.242) of the variation in newborn 25OHD was explained by the model used, with season of birth being the major contributor at 19% (adjusted R^2 0.19).

Table 5.4: Determinants of 25OHD concentrations in a linear regression model

Determinant	**β Standardised Co-efficient**	**95% Confidence interval**	**P value**	**R^2 change**
Season (Winter/Summer)	0.43	17.70, 20.46	0.000	0.190
Ethnicity				0.028
White British	Reference			
Asian	-0.17	-12.37, -8.34	0.000	
Black	-0.13	-15.56, -9.35	0.000	
Mixed white and black	-0.05	-11.17, -3.19	0.000	
Any other mixed	-0.09	-12.86, -6.51	0.000	
Any other white	-0.05	-6.54, -1.53	0.002	
Mixed white and Asian	-0.02	-10.60, 0.74	0.089	
Gestation in weeks				0.014
Term >37- 41 (n=2,422)	Reference			
Extreme pre-term <28 (n=7)	0.07	3.13, 8.50	0.000	
Pre-term 28-37 (n=502)	0.05	10.10, 39.43	0.001	
Post-term >41 (n=68)	-0.01	-6.74, 2.49	0.367	
Maternal age	0.06	0.13, 0.38	0.000	0.002
IMD deciles				
Decile 10 (least deprived) (n=136)	Reference			
Decile 1 (n=720)	0.00	-3.55, 3.68	0.972	
Decile 2 (n=501)	0.02	-2.33, 5.07	0.469	
Decile 3 (n=331)	0.00	-3.82, 3.92	0.982	
Decile 4 (n=260)	-0.01	-4.82, 3.19	0.690	
Decile 5 (n=309)	0.00	-3.59, 4.19	0.879	
Decile 6 (n=217)	0.00	-3.90, 4.33	0.919	
Decile 7 (n=201)	0.03	-1.40, 6.96	0.192	
Decile 8 (n=175)	0.00	-3.63, 4.97	0.760	
Decile 9 (n=136)	0.00	-4.49, 4.40	0.985	

5.5 Discussion

Nearly 70% of the babies in this multi-ethnic cohort had deficient or insufficient (<50 nmol/L) 25OHD concentrations. Approximately one third each were vitamin D deficient, insufficient and sufficient. Winter-born babies were at significant risk of deficiency, with only 15% having sufficient 25OHD concentrations. The proportion of deficiency, when compared to white ethnic background (52.5% in winter, 8.4% in summer), was much higher in the Black, Asian and Minority Ethnic (BAME) (63.3%, 30.1%), mixed (62.2%, 36.2%) and other white (49.2%, 17.6%) ethnic groups. Season of birth, ethnicity, gestation at birth and maternal age contributed to 24% of the variation in newborn 25OHD concentrations.

5.5.1 Prevalence of deficiency

The high prevalence of vitamin D deficiency and insufficiency in this multi-ethnic newborn cohort, of 70%, is comparable to the pooled global prevalence of 75% in newborns [111]. The global summary reports a pooled analysis from smaller studies (n=52) which mostly report cord blood 25OHD concentrations except for one study which reported concentrations from archived DBS [111]. The majority of studies from across Europe summarised by Saraf et al consistently report a mean/median 25OHD concentration below 50 nmol/L [111]. However, most of the studies reported here were undertaken at least a decade or two ago [111]. In the more recent years countries such as Finland which have adopted enhanced supplementation and food fortification with vitamin D have reported improved cord blood 25OHD levels in the range of 88 nmol/L and the prevalence of deficiency (<50 nmol/L) in the newborn is reported to be <1% [112]. A randomised controlled trial at a comparable latitude reported mean cord blood 25OHD concentrations of 39 nmol/L in unsupplemented mothers demonstrating

improvement to 50 nmol/L with 20μg/day vitamin D supplement use through pregnancy [113].

5.5.2 Seasonal variation

The well-recognised seasonal variations in 25OHD concentrations in high latitude countries without food fortification [80,114] were confirmed. The prevalence of deficiency (52.9% in winter and 18.4% in summer) was higher compared to the pooled European data (19.7-31.8% in winter and 5.7-15.3% in summer) [80]. The lower prevalence in the European data is due to the underrepresentation of ethnic minority groups and children and also the lack of data on newborns [80].

5.5.3 Ethnic variation

The well recognised ethnic variations in 25OHD levels in the mothers in pregnancy [115,116] was confirmed in the offspring. Babies born to BAME (Black, Asian and Minority Ethnic) mothers had significantly lower concentrations of 25OHD compared to babies born to white British mothers, similar to the reports from pregnant women [115]. Vitamin D status in the UK south Asian community has been widely studied [36,83,116-118] but the black ethnic groups remain under studied, despite the reports of high incidence of rickets in these groups [5,9,119,120]. Significantly lower median 25OHD concentrations in infants of mixed ethnicity, particularly white and black, and other white i.e non-British white groups compared to white British groups was noted. Whilst deficiency in the mixed ethnic groups can be attributed to skin pigmentation [121] the factors contributing to vitamin D deficiency in the other white groups need further investigation. 25OHD concentrations in the white British and mixed white and

Asian groups were comparable, possibly owing to the lighter skin pigmentation or the fewer numbers (n=45 of 2999).

5.5.4 Determinants of newborn 25OHD concentrations

In the chosen model, the significant factors which accounted for 24% of the variations in DBS 25OHD concentrations were season of birth, ethnicity, maternal age and gestation.

5.5.4.1 Season of birth

The season of birth accounted for 19% of variation in our model. Previous studies have reported that 23-24% of the neonatal 25OHD concentrations were dependent on UVB exposure, measured in erythemal dose, in the month before birth [122,123]. The exact erythemal dose exposure preceding birth was not assessed as part of the study but the Ultraviolet Index (UVI) data for the months preceding birth is reviewed below.

The UVI is a measure of solar UV radiation at the Earth's surface that is relevant to effects on the human skin [124]. UVI ranges from zero upward and is classified based on its potential for damage to the skin as low (<2), moderate (3-5), high (6-7), very high (8-10) and extreme (11+) [124]. The maximum UVI in the UK is usually around 8 in the summer months [125]. **Figure 5.10** illustrates the annual Ultra Violet Index (UVI) data for the year of study, obtained from the Department of Environment Food and Rural Affairs [125]. The average UVI exposure in the month before birth was 1 for the winter-born and 7 for the summer-born **(Figure 5.10).**

Additional factors which may have influenced the vitamin D status such as sun exposure behaviour, sunscreen use, and covered clothing habits were not captured in the study. A

previous study reported that maternal sun exposure and clothing choice accounted for 4% on neonatal 25OHD variation [123].

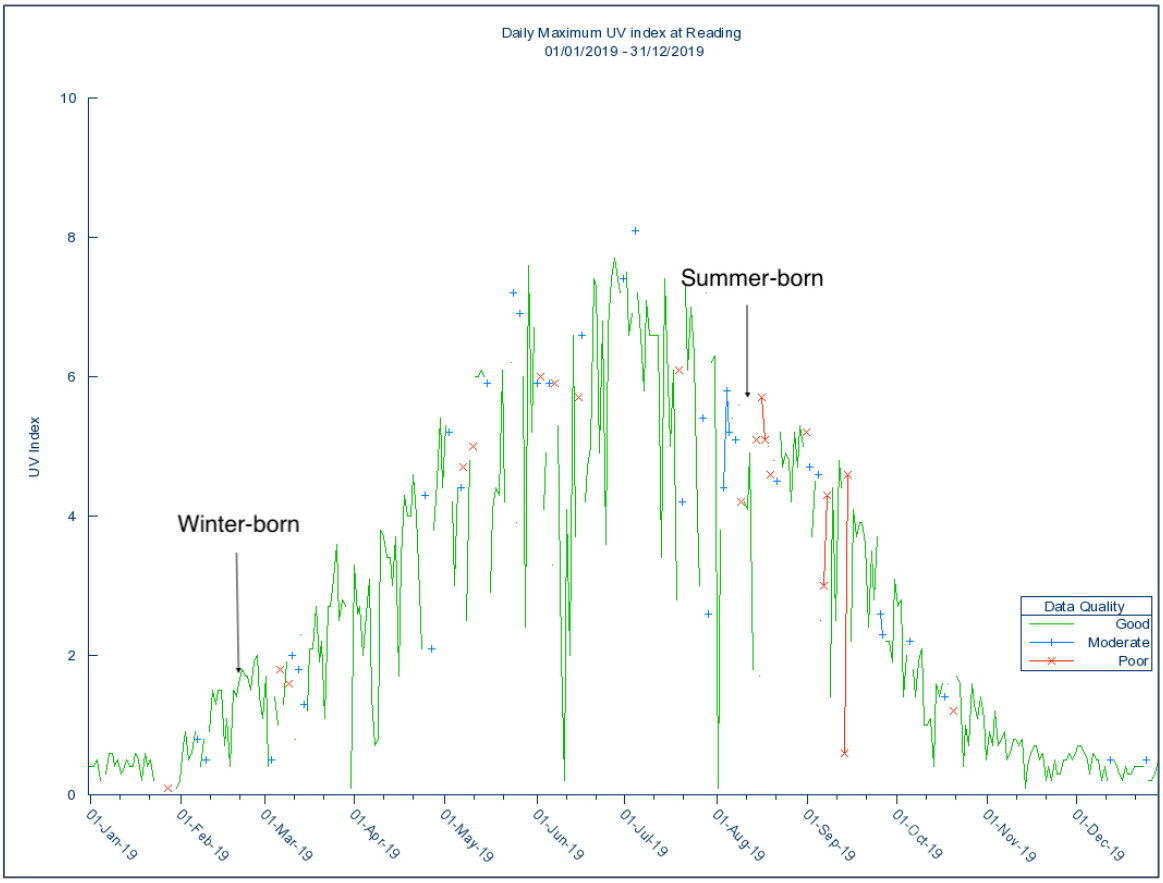


Figure 5.10: Annual solar UV Index graph for the year 2019.

Reproduced in accordance with Open Government License v3.0 from https://uk-air.defra.gov.uk/data/uv-index-graphs

5.5.4.2 Ethnicity

All ethnicities, except white British and mixed white and Asian, contributed to low vitamin D status. In our model, ethnicity accounted for 2.8% of the variation in 25OHD concentrations. Previously, a small Australian study (n=256) of predominantly Caucasian (71%) children aged 0-16 years reported an increase in deseasonalised neonatal DBS 25OHD concentration with lighter skin, hair and eye colour which was consistent with lower 25OHD in people of

Asian and Middle Eastern ethnicities [123]. In the above study, parent reported ethnicity accounted for 1.1% of the variation in DBS 25OHD concentrations and more specifically skin colour recorded by the research nurse at recruitment accounted for 5.2% of the variation [123].

The duration of sun exposure required to synthesise adequate vitamin D varies with ethnicity, and more specifically with skin colour, with dark-skinned individuals requiring longer exposure than those with lighter skin tone [36].

5.5.4.3 Maternal age

A limited number of studies report on the link between maternal age and foetal 25OHD concentrations. A positive association between maternal age and foetal 25OHD concentrations has previously been noted [115]. Similar to our finding, a Danish study reported maternal age to be a determinant of newborn 25OHD concentrations [122]. In adults (≥ 18 years), older age has been linked to higher 25OHD concentrations, possibly owing to higher supplement intake [126].

5.5.4.4 Gestation

When compared to term babies, the pre-term babies had slightly higher 25OHD concentrations. This finding was thought to be secondary to the use of supplements or pre-term formula feeds in most neonatal units. A non-significant trend towards lower 25OHD concentrations was noted in the post-term babies, similar to previous reports [127,128]. Multiple factors have been implicated in the lower 25OHD concentrations in late gestation varying from decrease in decidual transfer of 25OHD3 to altered maternal vitamin D binding protein and reduced sun exposure [122].

5.5.4.5 Socio-economic status

Low socio-economic status has been linked to vitamin D deficiency and insufficiency in children [129,130,131]. Although, socio-economic status, determined by IMD decile, was significantly associated with 25OHD concentrations in our cohort, its contribution in the final model was not significant.

5.5.5 Utility of DBS

The well-established patterns of ethnic and seasonal variations in serum 25OHD concentrations were confirmed on DBS samples. The plasma equivalent 25OHD concentrations derived from DBS reported here are comparable to other reports from DBS [122,123] and also cord serum [132,133]. These agreements across studies confirm the utility of blood spot assays in accurately determining the vitamin D status of the newborn.

5.5.6 Strengths and limitations

This is the first study in the UK to assess newborn vitamin D status in a large, ethnically diverse cohort. Complete data on birth weight, gestation, maternal age and ethnicity were available. Very few studies have assessed 25OHD on archived DBS obtained from NBS [122,127,132] . This is the first study to ascertain prevalence of neonatal vitamin D deficiency in non-archived DBS obtained prospectively, eliminating the concern of 25OHD degradation with prolonged storage [134].

The potential limitation is the lack of data on maternal and early infant supplementation, especially since compliance with national policy on vitamin D supplementation is known to

be poor [65]. In the UK, the uptakes of both infant [42,44] and antenatal vitamin D supplements [94] remain <20%.

5.6 Conclusions

The current UK antenatal supplementation programme fails to protect newborns from vitamin D deficiency, especially those from ethnic risk groups. Nearly 70% of all newborns and 85% of winter-born babies had 25OHD concentrations below 50 nmol/L. Almost 80% of babies of Black or Asian origin have suboptimal vitamin D status at birth with nearly 50% being deficient, which explains their high risk of hypocalcaemic complications and rickets if left unsupplemented.

The implications of the findings detailed in this chapter for future health care and public health policies will be discussed in the final chapter.

Chapter summary

- Nearly 70% of newborn babies in a multi-ethnic cohort in the West Midlands have suboptimal 25OHD concentrations below 50 nmol/L.

- Only 15% of winter-born babies had sufficient 25OHD concentrations and babies of non-white ethnicity had significantly lower 25OHD concentrations when compared to their white counterparts.

- Season of birth, ethnicity, gestation at birth and maternal age contributed to 24% of the variation in newborn 25OHD concentrations.

- Assessing 25OHD concentrations on newborn bloodspots is an elegant way of assessing the effectiveness of antenatal vitamin D supplementation programmes. The high proportion of deficiency in our cohort indicates the ineffectiveness of the current UK programme

Chapter 6: **Prevalence of rickets and osteomalacia in sudden unexpected death in infancy and childhood: An exploratory post-mortem study**

Roles and responsibilities involved in the work reported in the current chapter:

Suma Uday: Co-designed study with supervisor, lead researcher, study set up, funding acquisition as co-applicant, competitive study scholarship acquisition, sought all relevant ethical approval, produced protocol, main study contact for ensuring research governance and audit, parental consent, sample collection and transport to relevant laboratories, data gathering, review of radiology images, statistical analysis, data interpretation, report writing.

Laboratory methodology training:

- Qualitative histology performed independently following training by Dr Marta Cohen (Consultant Pathologist) at Sheffield Children's Hospital, UK

-Quantitative histology performed independently following training by Prof Ola Nilsson at Karolinska University, Stockholm

- Obtained training in histomorphometry and back scattered electron imaging by Nadja Fratzl-Zelman at the Institute of Osteology, Vienna, to enhance understanding of methodology and interpretation of results

-Three-point bending test methodology observed and performed on the first 2 samples under supervision and guidance by Sarah Junaid at the Aston University, Birmingham, to enhance understanding of methodology and interpretation of results.

Wolfgang Högler: Conceptualised the study, funding acquisition, provided supervision, intellectual contribution to data interpretation, critical revision of written report.

Co-supervisors: Martin Hewison (Professor of molecular endocrinology, IMSR, University of Birmingham), Tamas Marton (consultant perinatal pathologist, Birmingham), Marta Cohen (consultant pathologist, Sheffield) and Joanna Garstang (Lead SUDI paediatrician).

Details of other professionals who were involved/consulted in study set up (**Table 6.1** and **Table 6.2**) and performed laboratory assays (**Table 6.3**) are detailed in the methodology section.

6.1 Introduction

Despite its long-standing recognition as a childhood health problem, rickets remains a radiological diagnosis and is only documented in the late stages of the disease. A key feature of this PhD thesis is the central hypothesis that histological and histomorphometric changes associated with rickets appear before radiological changes are evident **(Figure 2.5)** and are likely more prevalent than radiological rickets. Testing this hypothesis required an entirely novel but ethically demanding approach to the evaluation of occult rickets in young children. Specifically, analysis of invasive rib growth plate samples and iliac bone biopsies, obtained at post-mortem examination, would provide a clearer insight into the potential changes in bone histology and histomorphometry, that would clarify the central hypothesis.

To date, two UK paediatric retrospective post-mortem studies have reported on serum 25OHD levels and descriptive changes of rickets at the growth plate. The prevalence of histological changes of rickets at the growth plate in these series ranged from 51% (18/35, total n=52) [47] to 69% (11/16, total n=41) [48]. These studies however, did not quantify the changes of rickets. Given that the underlying pathology in rickets is the lack of apoptosis of mature hypertrophic chondrocytes [17,25] we aimed to quantify these changes by measuring the height of the hypertrophic chondrocyte zone and comparing this to normative reference data [135]. The above retrospective studies [47,48] lacked clinical data on vitamin D supplement use and feeding mode which was also addressed in our prospective post-mortem study.

Static histomorphometry on iliac bone biopsies obtained at post-mortem examination has been reported in one adult study (mean age 58.72 ± 16.99 years, n=675) from Germany [49]. Osteomalacia in the above adult cohort, defined as an osteoid volume per bone volume

(OV/BV) >2% and osteoid surface per bone surface (OS/BS) > 20%, was noted in 25.63% and 36.15% of subjects respectively [49]. To date, there have been no paediatric post-mortem studies exploring the histomorphometry of infants and children with SUDIC. Moreover, there are no histomorphometry data in the literature for infants and children under the age of 1.5 years.

Mechanical testing of bone remains the gold standard for assessing bone strength ex vivo and no studies to date have explored the correlation between histology, histomorphometry and such mechanical strength testing. The aim of the proposed study was to address the above gaps in information using post-mortem samples from subjects of SUDIC.

6.1.1 25OHD and metabolites

Serum 25OHD, the most stable and abundant form of vitamin D, is the most commonly measured vitamin D metabolite. 1,25 dihydroxyvitamin D [1,25$(OH)_2$D] or calcitriol is the active form of vitamin D but is not routinely measured in clinical practice. Its measurement is restricted to certain clinical scenarios such as vitamin D dependent rickets, differentiation of Fibroblast Growth Factor 23 (FGF23) mediated vs non-FGF23 mediated phosphopaenic rickets or granulomatous disorders producing 1,25$(OH)_2$D [136] . 1,25$(OH)_2$D has a short half-life and assays are not widely available in all laboratories. More recently, other vitamin D metabolites are being increasingly measured. **Figure 6.1** illustrates the vitamin D metabolism /catabolism pathway with the key enzymes involved in the process and the metabolites which were of interest and measured in our study are indicated in bold.

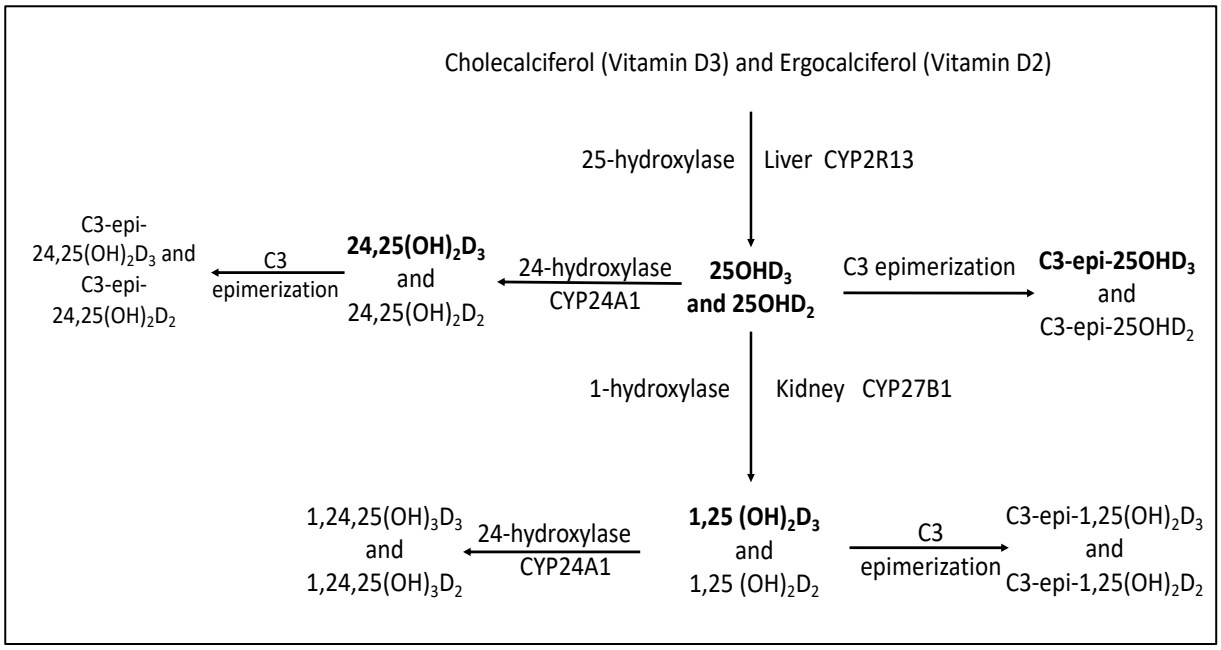


Figure 6.1: Vitamin D metabolism and catabolism pathway with metabolites measured in the study highlighted in bold

Whilst serum 25OHD levels have been reported in post-mortem studies [47-49,137], serum vitamin D metabolites have never been reported. In addition to 25OHD other metabolites were examined in our study. Whilst measuring $1,25(OH)_2D$ was thought to be not feasible due to its short half-life and the time delay in demise and sample collection, nonetheless it was attempted. Additionally, 24,25 dihydroxy vitamin D [$24,25(OH)_2D$] and 3-epi-25 hydroxyvitamin D (3-epi-25OHD) were measured. Only D_3 components of the metabolites were measured due to low levels of $25OHD_2$.

$24,25(OH)_2D$, a biomarker of vitamin D catabolism, is reported to be a better indicator of vitamin D sufficiency than 25OHD alone [136]. The amount of circulating $24,25(OH)_2D$ depends on the availability of substrate (25OHD) and CYP24A1 activity [upregulated by $1,25(OH)_2D$ levels]. Hence, absolute concentrations of $24,25(OH)_2D$ or vitamin D metabolite ratio of $25OHD/24,24(OH)_2D$ are considered to be better markers of sufficiency [136].

Although, 24,25$(OH)_2D_3$, is often considered an inactive form of vitamin D3, some studies have shown that it has a direct role in bone cells, in particular osteoblast maturation and also calcium mineralization [138].

The C-3 epimers of 25OHD, 3-epi-25OHD_2 and 3-epi-25OHD_3, are of particular interest due to the presence of high levels in infants and their structural resemblance to 25OHD_2 and 25OHD_3 respectively that can lead to overestimation of 25OHD levels [139]. In addition to 25OHD, the epimerase enzyme also epimerises 1,25$(OH)_2$D and 24,24$(OH)_2$D as illustrated in **Figure 6.1**. Only C3-epi-25OHD was measured as it is the most abundant epimer found in circulation [140] with levels up to 60% reported in the first year of life [139]. As conventional assays do not distinguish between 25OHD and its epimer forms deficiency states can be misclassified.

6.2 Aims and objectives

6.2.1 Aims

To study the prevalence of vitamin D deficiency, occult rickets and osteomalacia in SUDIC

6.2.2 Objectives

6.2.2.1 Primary objectives

- Assess the prevalence of vitamin D deficiency by determining the status of 25OHD in infants and children with SUDIC
- Report vitamin D metabolites [24,25$(OH)_2D_3$, 3-epi-25OHD_3] and ratios [25OHD_3:24,25$(OH)_2D_3$] in SUDIC subjects

- Determine the proportion of children with SUDIC who have rickets on qualitative (descriptive changes of rickets) and quantitative (height or hypertrophic zone) histology at the rib growth plate
- Compare the clinical (height, weight, age) and biochemical (serum 25OHD and its metabolites) characteristics of subjects with and without histological rickets
- Report the presence of radiological rickets and rickets severity scores and relate these to histological changes of rickets
- Report static bone histomorphometry data (osteoid thickness, osteoid surface and osteoid volume) in infants and young children with SUDIC

6.2.2.2 Exploratory objectives

- Compare static histomorphometry in children with and without histological evidence of rickets
- Report the bone mineral density distribution curve in young children and compare it to reference data from older children
- Report the fracture toughness on three-point bending test of the rib bone

6.3 Setting up a post-mortem study

Approaching bereaving families to consider participating in research is challenging and requires compassion and sensitivity. This section will detail the pre-requisites and steps which were adopted to set up a post-mortem study **(Figure 6.2)**.

Setting up a post-mortem study requires:

- Awareness of the terminologies relating to child death
- An understanding of the procedures and timeline following child death
- Knowledge of various professionals and agencies involved in the investigation of child death
- Awareness of parental views on participation in post-mortem studies

Firstly, the various terminologies relating to unexpected child death were reviewed **(Section 6.3.1)**. Subsequently, the procedures and timeline following child death was reviewed **(Section 6.3.2)** to design the study and plan the recruitment process. The remit of various professionals involved was reviewed to enable consulting the relevant parties on study design and the process of recruitment **(Section 6.3.3).** Professionals involved in clinical care and/or research at the end of life or following bereavement were interviewed on recruitment process, consent and communication skills **(Section 6.3.4).** Patient and public views on post-mortem research and feedback on study design was sought from bereaved parents' and young people actively involved in research **(Section 6.3.5).**

6.3.1 Terminology

Sudden Unexpected Death in Infancy and Childhood (SUDIC)

Sudden unexpected death in infancy (SUDI) or childhood (SUDC) encompasses all cases in which there is death (or collapse leading to death) of a child, which would not have been reasonably expected to occur 24 hours previously and in whom no pre-existing medical cause of death is apparent [141,142]. This is a descriptive term used at the point of presentation and includes those deaths for which a cause is ultimately found ('explained SUDI/SUDC') and those that remain unexplained following investigation.

Sudden Infant Death Syndrome (SIDS)

The majority of SUDI remain unexplained and are labelled as unascertained deaths or Sudden Infant Death Syndrome (SIDS). Although SIDS is common in infants, it can be diagnosed in young children up to the age of 2 years [141].

SUDIC, unexplained

This is the preferred term for use in cases where there is no clear cause of death and there are no features to suggest unnatural death or inflicted injury, but in which the circumstances do not fit the criteria for SIDS [141].

Unascertained

This is a legal term often used by coroners, pathologists and others involved with death investigation, where the medical cause of death has not been determined to the appropriate legal standard, which is usually the balance of probabilities [141]

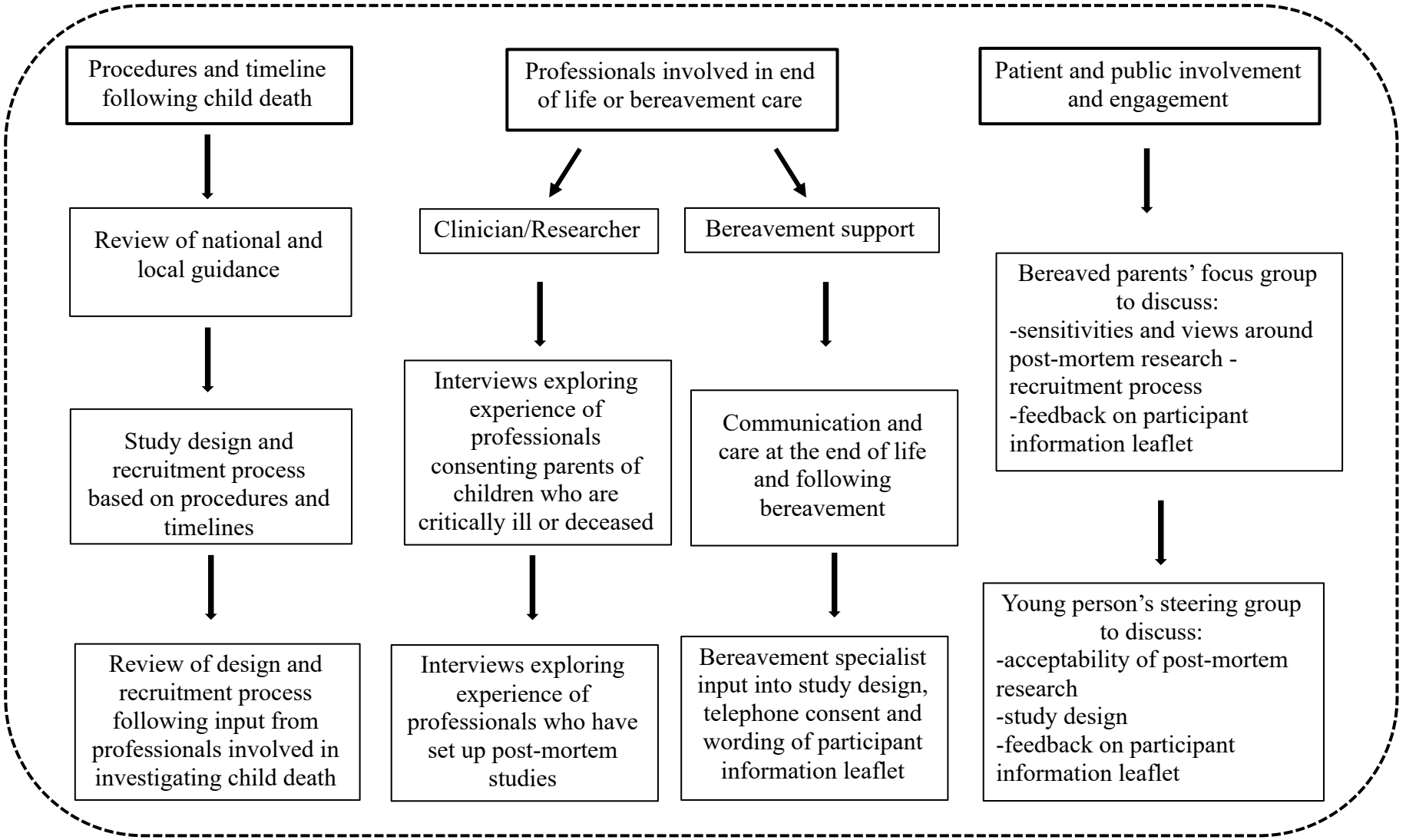


Figure 6.2: Steps taken to set up a post-mortem study

6.3.2 Procedures and timeline following child death

In England, a joint agency approach involving police, health and social care is adopted to investigate unexpected child deaths [143]. The aim of the joint agency approach is to establish the complete cause of death and address the needs of the family.

The events following child death are presented in the flow chart below **(Figure 6.3)** [144]. In brief, the child is initially taken to the nearest emergency department by the ambulance staff. A detailed medical history is taken by the attending consultant paediatrician (or their nominee), along with a police officer from the Child Abuse Investigation Unit where feasible. In addition to physical examination of the child, a joint examination of the scene of death by a senior investigating police officer and the designated SUDI paediatrician is also undertaken within 48 hours. The hospital and police notify Her Majesty's Coroner of any unexpected infant death and the coroner will request the post-mortem examination. Post-mortem examination is usually done by a paediatric pathologist. Routinely, the police investigation is on behalf of the coroner unless it becomes clear that the death is unnatural or suspicious. The investigation of all suspicious deaths is led by the police. The post-mortem examination in suspicious deaths is conducted jointly by a paediatric and forensic pathologist.

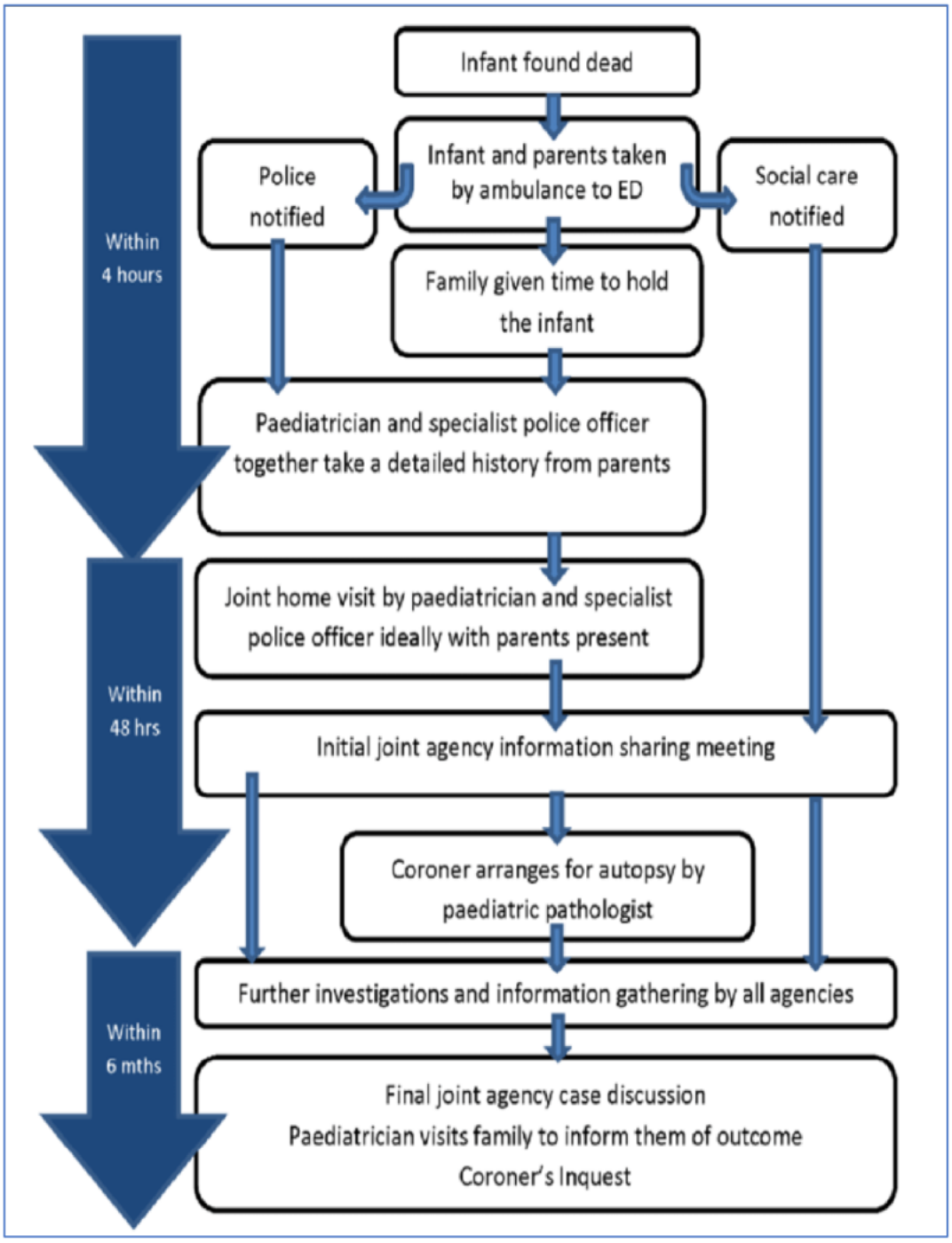


Figure 6.3: Joint agency approach to investigating sudden unexpected child death.

Flowchart reproduced from [144] in accordance with Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).

6.3.3 Inter-disciplinary professional engagement

Key professionals such as the pathologist and the lead SUDI paediatricians who have experience of investigating child death were invited to be part of the research team to help design the study. Her Majesty's Coroner was frequently consulted from the conception of the study. Other relevant professionals were consulted at a later stage to fine-tune the study design, recruitment and consent process. The various professionals consulted, and their areas of input are detailed in **Table 6.1**.

Table 6.1: Consultation of professionals involved in the investigation of child death

Groups	Titles	Area of input
Healthcare professionals involved in SUDIC process	Lead SUDI paediatrician	Recruitment and consent process Guidance on bereaved parent telephone consent process based on previous experience
	Lead SUDI nurse	Recruitment and consent process
	Emergency department consultants attending SUDIC cases	Participant identification process
	Pathologist	Recruitment, consent process, participant information leaflets and study methodology
	Mortuary manager	Recruitment and consent process
	Research and Development at Participant Identification Centre and study site	Participant identification, consent and telephone consent
Her Majesty's Coroner	Her Majesty's Coroner	Study design, recruitment, consent process and protocol for exclusion of suspicious cases
	Coroner's investigator	Recruitment, consent form and participant information leaflets
Police involved in SUDIC process	Detective Superintendent	Protocol for exclusion of suspicious cases
Other relevant professionals	Human Tissue Act (HTA) Lawyer	Ensuring study meets HTA 2004 regulations

6.3.4 Consulting professionals involved in clinical care and/or research at the end of life or following bereavement

The following steps were taken to ensure that bereaving families were at the core of the study design:

- I attended a training course on supporting parents with end-of-life care and following bereavement
- I worked closely with charities supporting bereaved parents
- I interviewed professionals providing end of life care
- Input from professionals involved in post-mortem research was sought

Various professional groups, involved in clinical care and/or research at the end of life or following bereavement, were consulted on different aspects of the study (**Table 6.2)**.

I attended a training course provided by 'Child Bereavement UK' which specifically focused on training professionals supporting parents in end of life care and following bereavement **(Appendix 7)**[145]. The course, delivered by skilled facilitators working with bereaved families, provided a sound theoretical knowledge of the bereavement process and the evidence-base that underpins effective and sensitive communication with bereaving families.

I optimised the study design and consent process following communication with specialists from agencies supporting bereaved parents such as 'Child Bereavement UK'[145] and the 'Lullaby Trust'[146]. Specialists from above agencies with extensive experience of working with bereaved families and delivering training to other professionals provided relevant feedback on study design and participant information leaflets.

I held personal interviews with professionals providing end of life care with experience of consenting parents of critically ill children for clinical studies or organ donation. The timing of consent along with the sensitivity surrounding post-mortem research and parental views on the same were discussed.

Table 6.2: Consultation of professionals involved in end-of-life care and related research

Groups	Titles	Area of input
Professionals involved in end of life care	Intensive care clinicians	-Consenting parents of a dying child for clinical studies -Consenting parents for organ donation -Participant information leaflet
	Intensive care nurses	As above
Bereavement support	Child Bereavement UK team	-Communicating with families during end of life care and following bereavement training -Support with focus groups
	Lullaby Trust members	Review of study design
	Bereavement psychologist	-Information leaflets -Content of telephone consent conversation -Facilitate focus groups
	Bereavement counsellors	-Information leaflets -Content of telephone consent conversation
Researchers	Chief Investigators of previous post-mortem studies involving telephone consent	-Insights from personal experience of research with bereaved parent -Experience of telephone consent process
	Lead researchers of post-mortem studies involving telephone interviews	As above
	Specialist bone researcher involved in post-mortem study where bone samples were collected	Study design and methodology
	Pathologists involved in other post-mortem studies	-Consent process -Consent pathway -Human Tissue Act requirements

6.3.5 Patient or Participant and public involvement and engagement (PPIE)

Requesting parents to consider participating in a research study at a difficult time is a sensitive matter. Understanding the acceptability of post-mortem research to bereaving parents and the general public is essential in planning such studies. Hence, bereaved parent and young person's focus groups were held for feedback.

6.3.5.1 Bereaved parents' focus group

I held a bereaved parents' focus group with the help and support of specialists from 'Child Bereavement UK'. The planning and execution of the focus group was overseen by an experienced bereavement psychologist from 'Child Bereavement UK'.

Invitations **(Appendix 8)** were sent to 10 bereaved families. A brief overview of the study (Lay Summary) and planned recruitment process along with participant information leaflets were sent to the five families that accepted the invitation. All relevant information was sent 4 weeks in advance of the meeting. The bereavement psychologist telephoned families two weeks after they received the information to discuss any issues and to confirm their continued interest in attending the meeting. All five families attended the meeting. Specific feedback was sought on the acceptability of post-mortem research and approach by researchers requesting additional samples at a difficult time. Pros and cons including acceptability of both face to face and telephone consent were discussed. Feedback on the readability and wording of the participant information leaflets was requested.

Post-mortem research and being approached for additional samples during bereavement was acceptable to all parents if it benefitted the child directly and it was still acceptable to most

parents even if there were no direct benefits to their deceased child. Whilst most parents preferred a face-to-face consent process, telephone consent was deemed acceptable.

6.3.5.2 Young Person's Steering Group (YPSG)

I sought feedback on the study design and consent at the NIHR Clinical Research Network: West Midlands YPSG meeting [147] co-ordinated by the NIHR PPIE officer. The meeting was facilitated by the West Midlands Clinical Research Network (WM-CRN) Research Portfolio Manager and User Involvement Coordinator.

The West Midlands YPSG, established in 2009 as part of the Generation R or generation research initiative, is a group of young people striving to improve research [147]. The dynamic group of young people, aged 12- 20 years, who have all accessed healthcare with some of them having mild or chronic healthcare conditions help design, develop and deliver research.

Fourteen young people of various ethnic backgrounds aged 12 to 16 years attended the group session. Initially, verbal feedback was sought on the study type, rationale and methodology. Subsequently, comments on the participant information leaflets were requested.

All young people present participated actively in discussions and vocalised the acceptability of post-mortem research. The multi-ethnic group contributed to the discussions on variations in cultural beliefs and its effect on post-mortem research. The group facilitated simplification of the language in the participant information leaflets and advocated producing a shorter version of the information leaflet. To avoid overloading parents with information, using the short version of the leaflet as the primary source of information was suggested by the group and subsequently upheld by the research ethics committee.

6.4 Research design and recruitment

6.4.1 Study design and participants

This was a prospective post-mortem study of children under the age of 2 years undergoing routine, non-forensic Coroner's post-mortem examination to ascertain the cause of death.

6.4.2 Inclusion and exclusion criteria

The following inclusion and exclusion criteria were used:

6.4.2.1 Inclusion criteria

Cases of SUDIC (< 2 years of age) referred to Birmingham Women's and Children's Hospital and Sheffield Children's Hospital for post-mortem examination by participating Her Majesty's Coroners.

6.4.2.2 Exclusion criteria

- Suspected cases of child abuse
- Children with conditions known to affect the quality of bone such as:
    - long standing immobility
    - malabsorptive conditions
    - prematurity (< 32 weeks gestation)
    - pre-diagnosed, heritable metabolic bone conditions such as osteogenesis imperfecta, hypophosphataemic rickets
- Children on medications which are known to affect bone health such as:

- systemic steroids
- anti-epileptics
- chemotherapy
- bisphosphonates

6.4.3 Ethics approval

The study was approved by the East Midlands – Nottingham 1 Research Ethics Committee (REC Reference: 17/EM/0061, IRAS project ID: 207267) **(Appendix 9)** and the Health Research Authority **(Appendix 10).**

6.4.4 Identification of cases

The participating Coroner's office identified any eligible cases and informed the lead researcher via a secure e-mail address. To ensure no eligible cases were missed, the participating centres and the concerned PIs were telephoned weekly. In the event of any planned post-mortem examinations, in order to maintain confidentiality, the Coroner's office was contacted to ascertain eligibility.

6.4.5 Eligibility ascertainment

Eligibility was ascertained based on inclusion exclusion criteria stated above. Additionally, parental suitability from an emotional and psychological perspective was determined by the corner's officer liaising with the family. Any uncertainties were discussed with Her Majesty's Coroner.

If the Coroner and/or the Coroner's officer felt it was inappropriate to ask the parents due to mental health concerns, fragile parental relationships, domestic violence or any other reasons then these families were excluded. If deemed eligible, the Coroner's officer contacted parents to seek permission to be contacted by the research team.

6.4.6 Recruitment and consent

6.4.6.1 Recruitment process

An overview of the recruitment process is illustrated in the flow chart below **(Figure 6.4)**. Approval for recruitment, by the coroner and police, was received only for non-suspicious cases due to the contentions surrounding vitamin D deficiency and child abuse fractures [148-150]. If there was no suspicion of child abuse on clinical examination and skeletal survey, then study eligibility was ascertained jointly by the lead researcher and the Coroner's office as detailed above.

If deemed eligible the coroner's officers contacted families to request permission to be telephoned by the lead researcher. If families agreed to be contacted, the full **(Appendix 11)** and brief **(Appendix 12)** versions of the participant information leaflets were sent by post or e-mail as appropriate. The study was discussed with families over the telephone by the lead researcher or the lead SUDI Paediatrician or face to face by the SUDI nurse where feasible. Due to the known difficulties in receiving signed consent forms back from families indicated by the Coroner's Offices, a telephone consent approach was adopted. Each telephone call was recorded with parental permission and retained as proof of consent. A copy of the consent form signed by the person obtaining consent was posted to families for confirmation of agreed consent. As the hip bone sample collected did not form part of the Coroner's routine post-

mortem examination samples, specific consent for the fate of the sample was obtained from parents and indicated on the consent form. The options provided were similar to that provided by the Coroner's office for the rest of the samples as detailed on the consent form **(Appendix 13)**.

6.4.6.2 Consent process

It was not possible to proceed with the initially proposed plan of face to face consent by SUDI paediatrician or the community paediatrician undertaking the home visit as the paediatricians did not feel comfortable approaching families about research. Moreover, some paediatricians undertook early home visit within 1-2 hours of death which they felt was too early to approach for research. It was therefore agreed that the proposed plan B of telephone consent would be the primary approach for consent. All necessary regulatory approvals were sought.

Telephone calls were recorded, with parental permission, which was saved as proof of consent. A signed consent form **(Appendix 13)** confirming telephone consent was then sent to parents with the details of the lead researcher for future contact in case of any further clarifications, discrepancies in consent or withdrawal of consent.

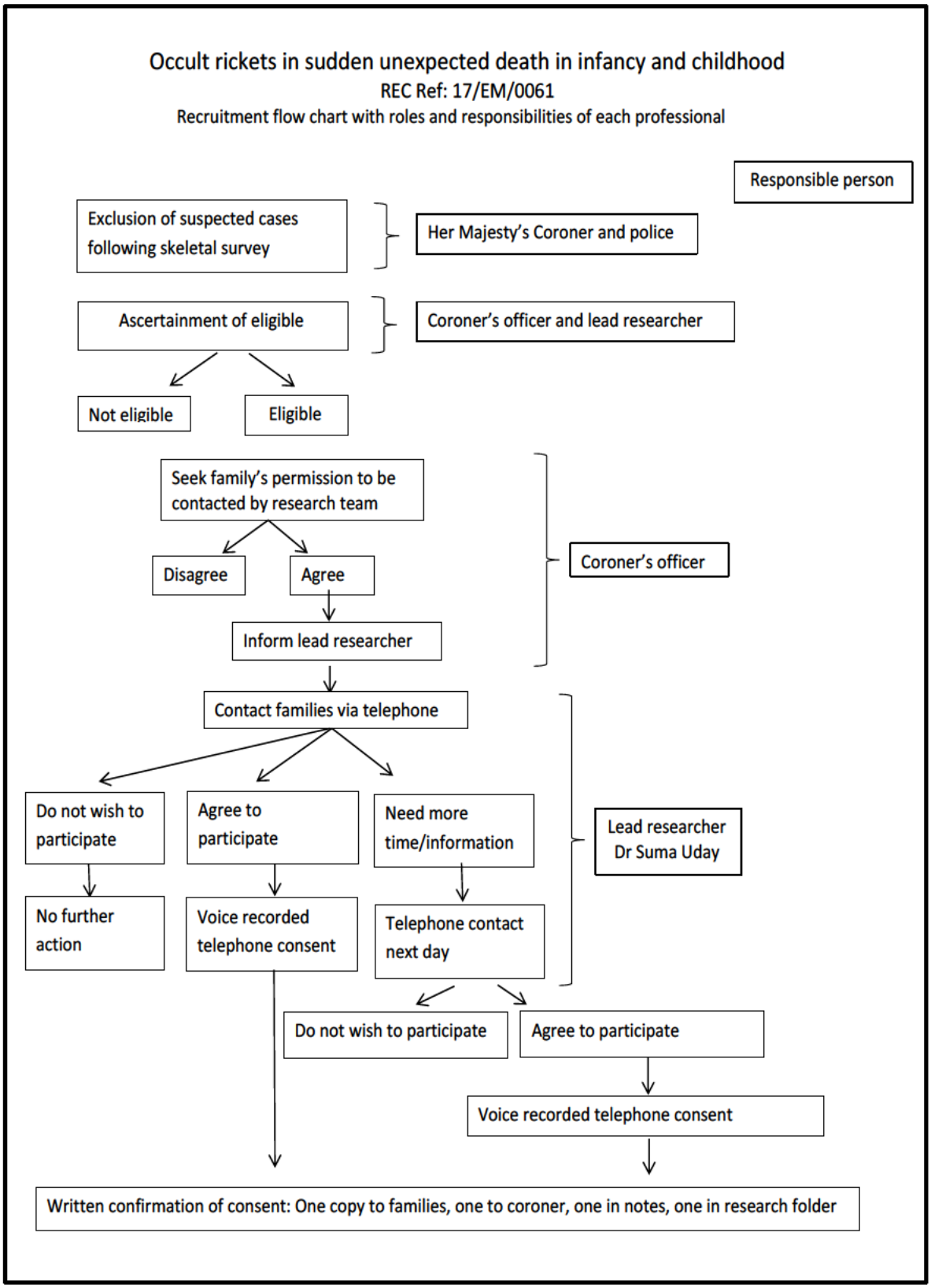


Figure 6.4: An overview of eligibility ascertainment and recruitment process

6.5 Research methodology

Data collection

Details of data collected are as indicated in the proforma shown below **(Figure 6.5).** Relevant data were collected from two main sources:

1) The paediatrician's SUDIC report that is provided to the pathologist which included detailed birth, past and family history, development history, immunisation and medication history, feeding mode and frequency, circumstances surrounding the death of the child.

2) The post-mortem report produced by the pathologist which included details of physical examination; and histological, genetic, and biochemical evaluation.

Additional history on feeding and vitamin D supplement use was obtained from parents at the time of consent if it was not already recorded in the paediatrician's report. Given the interplay between calcium and vitamin D and its role in nutritional rickets, dietary calcium intake was evaluated. Accurate assessment was only possible for formula fed subjects and not breast or mixed breast and formula fed babies. Where the information on the type of formula was available, calcium content as per manufacturer's nutrition information was used **(Appendix 14)**. Where the information on formula type was not available, the European Food Safety Authority recommended minimum calcium of 50 mg/100 kcal or 34 mg/100 ml for infant formula and follow-on milk was used [151]. A detailed dietary history in infants who were weaned was not available as this information was not routinely gathered by the paediatrician and collecting detailed information at the time of consent was not feasible.

Internal organ weights are routinely recorded at post-mortem examination. We specifically gathered data on the presence of dilated cardiomyopathy and heart weight in comparison to

normative data [152] due to its relevance to dilated cardiomyopathy in infants with nutritional rickets [6,119]. A skeletal survey is routinely undertaken prior to post-mortem examination and the details of this report were gathered in addition to reviewing the radiographs particularly for evidence of rickets.

Data collection proforma

Demographics

PM number: Study number:

Date of birth: Date of death: Age at death:

Gender: Ethnicity:

Address (including post code):

Details of SUDIC

Circumstances of death:

Preceding illness:

Past medical history

Gestation at birth: Birth weight:

Co-morbidities:

Medications (including multivitamins with vitamin D):

Feeds: Breast formula mixed weaned other

Type of formula: Volume/frequency of feed:

Any other relevant information:

Post-mortem examination:

Cardiac weight (g): Normal If abnormal- expected reference weight

Skeletal survey

Findings:

Rib fractures reported: Yes/No If yes site:

Radiographic signs of rickets: Yes/No

Rickets severity score

Any other significant findings at PM including biochemistry/toxicology: Yes/No

If yes- detail

Microarray results:

Cause of death:

Figure 6.5: Study data collection proforma

6.5.1 Overview of sample collection and analysis

All samples were collected on the first day of routine post-mortem examination following parental consent. An overview of all routine and additional samples collected and the teams involved in laboratory analysis / reporting are detailed in **Table 6.3**. A part of the blood sample routinely collected at post-mortem examination was used for assay of 25OHD and its metabolites. The rib cost-chondral junction (4^{th}, 5^{th} or 6^{th}) is routinely processed and stained with Haematoxylin and Eosin (H&E) for growth plate histology which was specifically studied for features of rickets using qualitative and quantitative histology. The distal part (distal to the costo-chondral junction) of the rib collected for histology was used to assess mechanical strength using a three-point bending test. Skeletal survey routinely performed prior to post-mortem examination was studied in detail for changes of rickets. An additional iliac bone biopsy was stained to study static histomorphometry and Bone Mineral Density Distribution (BMDD) was assessed on quantitative Backscattered Electron Imaging (qBEI).

Table 6.3: Sample collection, laboratory analysis/ expert review and teams involved

Sample	**Analysis**	**Laboratory**	**Team**
Blood	25OHD and metabolites	Institute of Metabolism and Systems Research, University of Birmingham, Birmingham, UK	M Hewison, C Jenkinson and F Shaheen
Rib (Routinely collected at post-mortem examination)	Growth plate histology as per routine post-mortem examination practice	BWCH and SCH	T Marton and M Cohen
	Quantitative and qualitative evaluation of growth plate histology	University of Birmingham, Birmingham, UK. Sheffield Children's Hospital, Sheffield, UK.	S Uday Independent expert review by Prof Cohen (blinded to 25OHD and radiology)
Radiographs: skeletal survey or babygram	Review for signs of rickets including Rickets Severity Score (RSS) and presence of rib fractures	University of Birmingham, Birmingham, UK. Evelina Children's Hospital, London, UK.	Report from designated radiologist. S Uday Independent review by a radiologist blinded to clinical and laboratory data (Rui Santos)
Iliac bone biopsy	Histomorphometry	Ludwig Boltzmann Institute of Osteology, Vienna, Austria	N Fratzl-Zelman and P Roschger
	Quantitative backscattered electron imaging to assess Bone Mineral Density Distribution (BMDD)	Ludwig Boltzmann Institute of Osteology, Vienna, Austria	N Fratzl-Zelman and P Roschger
Rib	Mechanical testing: three-point bending	Aston University, Birmingham, UK	S Junaid and B McCall

6.5.2 Measurement of 25OHD and other vitamin D metabolites

6.5.2.1 Sample preparation and storage

Blood samples were collected from the heart at post-mortem examination. Whole blood was collected in plain tubes and transported to the processing laboratory within 30 minutes of collection.

Samples were centrifuged in the receiving laboratory at 13,000 revolutions per minute for 40 min at 4 °C. Resulting serum was obtained and stored at −80 °C until subsequent analysis.

6.5.2.2 Sample analysis

Serum concentrations of vitamin D metabolites were analysed by liquid chromatography-tandem mass spectrometry (LC–MS/MS) as described previously [153]. Briefly, 200 µl serum was prepared for subsequent analysis by protein precipitation using a mixture of methanol, isopropanol and water (all LC MS-MS grade, Sigma, Poole, UK). The resulting solution was vortexed for 30 seconds and then centrifuged at 7500g for 5min, with the resulting supernatant used for further analysis. Vitamin D metabolites were extracted from the supernatants by a process known as supportive liquid-liquid extraction (SLE), using 96-well SLE plates (Phenomenex, Macclesfield, UK), after drying these samples using a vacuum extractor, the resulting material was then reconstituted into solution using a mixture of water/methanol (50/50%) (Thermo-Fisher, Poole, UK) and stored at -80°C prior to LC MS-MS analysis.

LC-MS/MS analysis was performed on a Waters Acquity uPLC coupled to a Waters Xevo TQ-XS mass spectrometer as described previously [153] using multiple reaction monitoring

(MRM) for the following vitamin D metabolites: 25-hydroxyvitamin D_3 ($25OHD_3$), 3-epi-25-hydroxyvitamin D_3 (3-epi-$25OHD_3$), 24,25-dihydroxyvitamin D_3 [$24,25(OH)_2D_3$], 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D3$] and 25-hydroxyvitamin D_2 ($25OHD_2$). In addition, the $25OHD_3$/ $24,25(OH)_2D_3$ ratio was calculated as a marker of vitamin D 24-hydroxylase enzyme activity, which reflects the catabolic inactivation of vitamin D. The percentage of $25OHD_3$ measured as 3-epi-$25OHD_3$ was also calculated. The LC–MS/MS method for vitamin D quantification was validated for serum analysis as previously described for accuracy, precision, recovery and matrix effects [153].

6.5.2.3 Classification

Serum concentrations of vitamin D metabolites were reported as nmol/L. Based on the serum concentrations of 25OHD, vitamin D status was defined using the Institute of Medicine [28] and Global consensus recommendation [76] classifications of vitamin D deficiency as serum 25OHD concentrations <30 nmol/L (12 ng/mL), insufficiency as 30-50 nmol/L (12-20 ng/mL) and sufficiency as >50 nmol/L (20 ng/mL). Serum concentrations of 25OHD are known to vary according to the time of the year, reflecting changes in sunlight availability [114]. Therefore, seasonal variations in vitamin D status are also reported. Seasons were classed as winter (December – February), spring (March-May), summer (June-August) and autumn (September- November).

6.5.3 Growth plate histology

6.5.3.1 Sample preparation and storage

The rib growth plate or costochondral junction (4th, 5th or 6th) was collected as part of routine post-mortem examination. Samples were fixed in 10% phosphate-buffered formalin at collection, then decalcified in formic acid and embedded in paraffin. Sagittal 5 μm sections of the growth plates were obtained from the center of the costochondral junction and stained with Haematoxylin and Eosin (H&E). H&E-stained slides were pseudoanonymised and digitised by scanning onto Aperio eSlide manager (Aperio, Vista, CA, USA).

6.5.3.2 Histological analysis

The images were analysed digitally on the WebViewer (Leica Biosystems, UK). All analyses were undertaken by myself and verified by Prof M Cohen.

Qualitative histology:

Qualitative histology provides a descriptive report of the changes in an abnormal growth plate in comparison to a normal growth plate. The growth plate was reviewed for the following well-established descriptive histological features of rickets reported in the literature [32] and numbered as below:

1. Expansion of the hypertrophic zone
2. Loss of columnar arrangement of hypertrophic chondrocytes
3. Loss of distinction between hypertrophic and proliferative zones
4. Presence of penetrating blood vessels in the hypertrophic chondrocyte cartilage zone

5. Presence of tongue-like projections of hypertrophic chondrocytes extending into the primary spongiosa

6. Bulbous appearance of the costo-chondral junction

All changes were recorded on visual inspection only, including expansion of the hypertrophic zone. Qualitative histological changes of rickets at the growth plate were classed as mild-moderate if there were < 3 of the above features. Presence of ≥ 3 features was classed as severe rickets.

A comparison of normal and abnormal growth plate is presented in **Figure 4.3**. A further example from the literature is also provided below **(Figure 6.6)**.

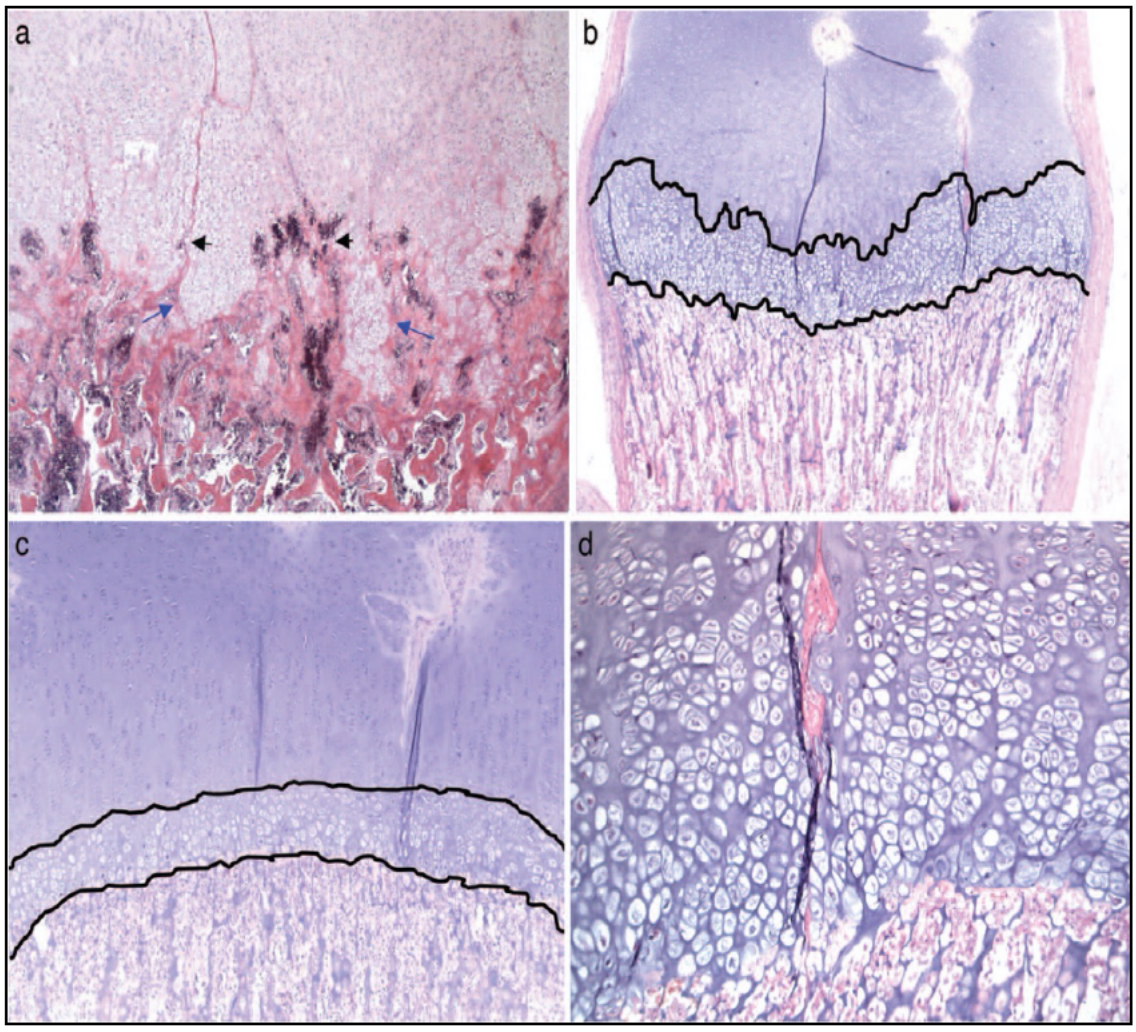


Figure 6.6: Histological section of the growth plate demonstrating abnormalities of rickets

Illustration reproduced from reference [47] in accordance with the terms of the Copyrights Clearance Center. (**a**) Rib growth plate showing typical changes of rickets. Note the enlargement of the hypertrophic chondrocyte layer and the marked disorganization of the growth plate, with irregular "tongues" of hypertrophic chondrocytes (arrows) extending into the diaphysis as well as capillaries within the growth plate (arrow heads). (**b**) Rib growth plate showing milder histological irregularities, such as increased and irregular hypertrophic chondrocyte layer. The changes are less severe than in obvious rickets. The hypertrophic zones delineated. (**c**) Normal rib growth plate with a normal hypertrophic chondrocyte layer with alignment of the chondrocytes in columns and a smooth metaphyseal-diaphyseal edge. The hypertrophic zone is delineated for comparison with **b. (d**) Higher magnification demonstrates increased thickness of the hypertrophic zone and the loss of columnar arrangement.

Quantitative histology

Quantitative histology was used to quantify the changes of rickets at the growth plate. Since rickets predominantly affects the apoptosis of hypertrophic chondrocytes, the height of the hypertrophic zone was measured to quantify the changes, and then compared to normative data **(Figure 6.7)** [135].

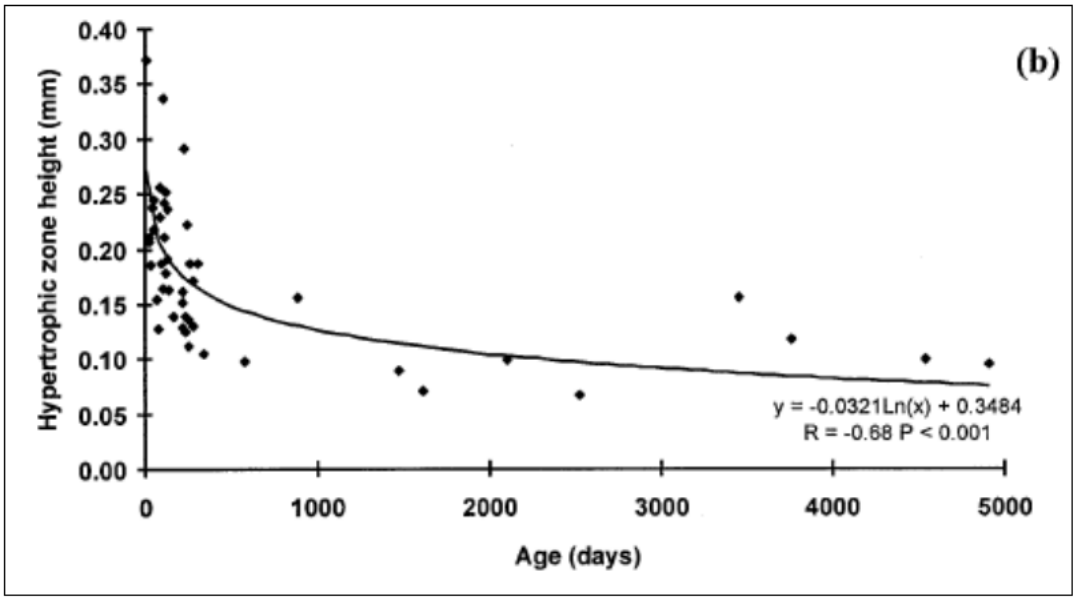


Figure 6.7: Normative data for height of the hypertrophic zone

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Histological measurements were undertaken using methods detailed by Nilsson et al [154] following training from Professor Ola Nilsson at the Karolinska Institute in Stockholm, Sweden. All histological measurements were performed in the central two-thirds of the growth plate sections **(Figure 6.8)** where there is uniform distribution of the growth plate zones. The height of hypertrophic zone was measured parallel to the chondrocyte columns, where these were intact, and parallel to the growth plate **(Figure 6.9)** where there was marked disarray of the chondrocytes as seen in rickets. Growth plate height was assessed in three

separate zones in the middle two-thirds of each growth plate and the results averaged. For this analysis, hypertrophic chondrocytes were operationally defined by a height ≥ 10 μm along the longitudinal axis [155]. The terminal hypertrophic chondrocyte was defined as the cell in the last lacuna which was not invaded by metaphyseal blood vessels. The size of the chondrocyte was measured in x40 magnification and the height of the hypertrophic zone was measured in x20 magnification.

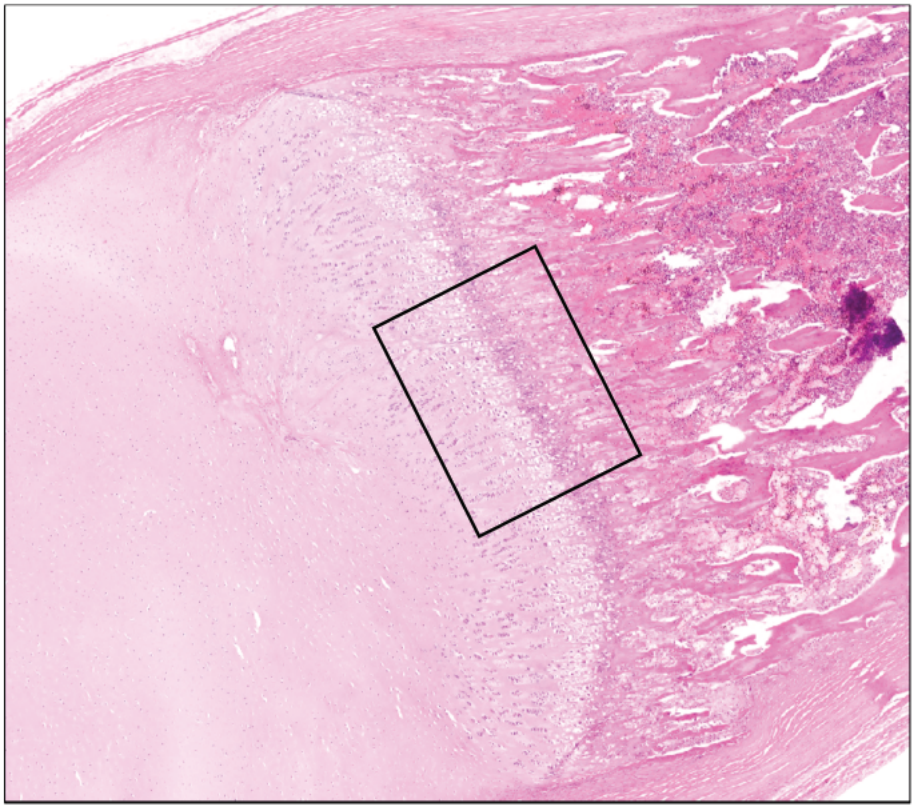


Figure 6.8: Growth plate section (H&E stain, x2 magnification) demonstrating the central two-third area (black square) where all measurement were performed

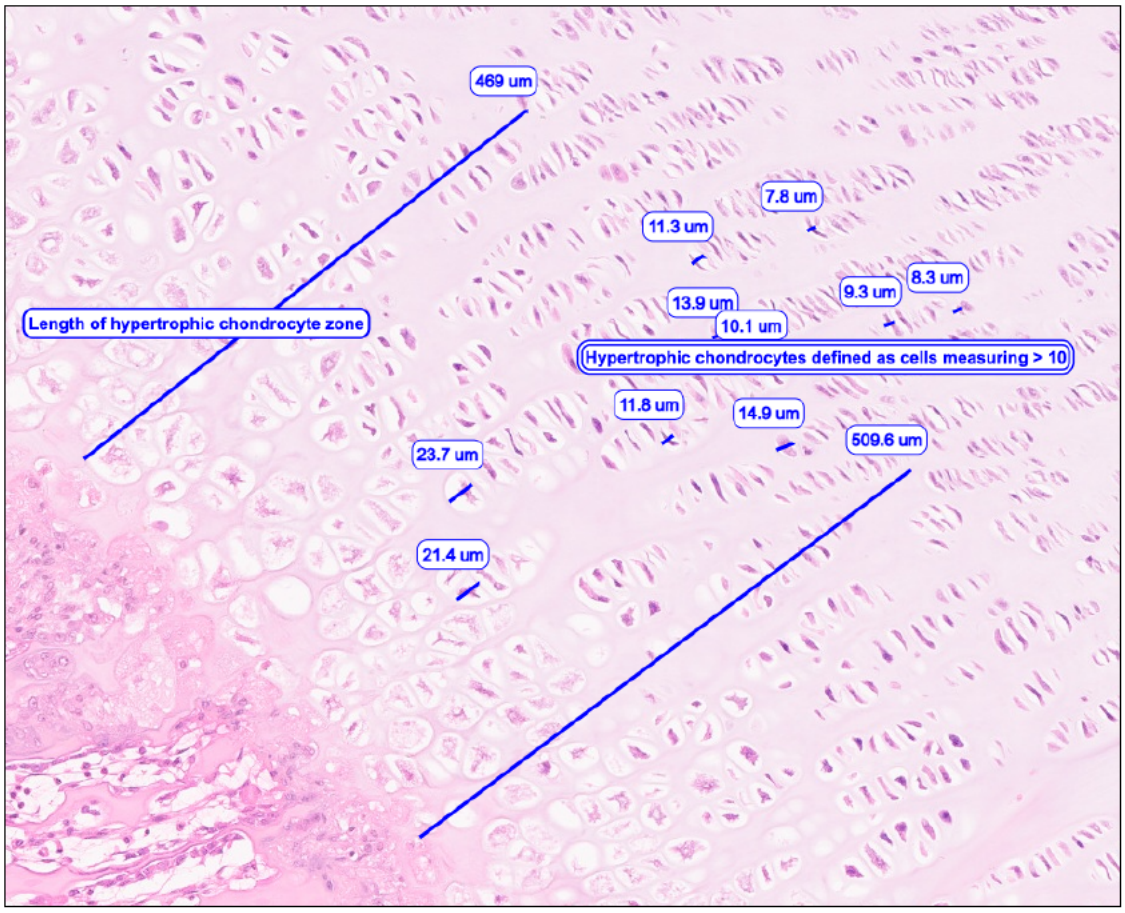


Figure 6.9: Growth plate section (H&E stain, x 10 magnification) demonstrating the measurement of cell size and height of hypertrophic zone parallel to the cell columns.

The height of the hypertrophic zones was assessed in 3 areas of each growth plate (in x10 magnification) and results averaged. The transition from proliferative to hypertrophic chondrocyte zone was determined by measuring cell size in x40 magnification. Cells with a cell height < 10 µm were counted as proliferative chondrocytes, whereas hypertrophic chondrocytes were operationally defined by a cell height ≥ 10 µm [155]. The terminal hypertrophic chondrocyte was defined as the cell in the last lacuna that was not invaded by metaphyseal blood vessel. Cell size was measured parallel to the axis of the growth plate in 25 cells to ascertain the transition.

6.5.4 Radiology

All subjects with SUDIC routinely undergo a full skeletal survey or babygram (whole body x-ray) prior to post-mortem examination. All images are routinely double-reported (two radiologists) by experienced designated consultant paediatric radiologists. If imaging is undertaken at the presenting district general hospital, where local expertise in reporting of post-mortem skeletal surveys is not available, the images are transferred to the tertiary centre (BWCH or SCH) for additional reporting by designated radiologists. Details of the formal radiology report which forms part of the Coroner's post-mortem report were gathered. Individual radiographs were also reviewed by myself for changes of rickets at the growth plate.

An independent review was sought by a radiologist with > 10 years' experience of reporting post-mortem skeletal surveys and experience of reporting rickets severity score (RSS) (R Santos, Evelina Children's Hospital, London). The independent radiologist was blinded to all clinical details and laboratory results and was specifically requested to comment on the presence of any rib fractures and evidence of rickets including RSS, which were evaluated as detailed below in **Section 6.5.4.1**.

Reviewer 1	HM Coroner's designated radiologists reporting post-mortem skeletal surveys. Double reported by two radiologists.
Reviewer 2	Lead researcher (S Uday)
Reviewer 3	Independent reviewer with experience in reporting post-mortem skeletal survey and rickets severity scores.

6.5.4.1 Rickets severity score (RSS)

The wrist was scored from 0-4 and knee from 0-6, with a higher number representing greater severity of rickets. Both knee (0-6) and wrist (0-4) radiographs were scored and the scores added to obtain a total RSS or Thacher score (0-10). A slightly modified version [156] of the original scoring system [157] was used. The modified score (**Table 6.4)** allows for 0.5 point increments at the wrist rather than only 1.0 point increments. And for the knee, the method assigns a multiplier factor to the score at the femur and tibia: 0.5 if one condyle or plateau is affected, and 1.0 if both condyles or plateaus are affected.

Table 6.4: Rickets severity grading scores used to assess the severity of rickets at wrist and knee joints

WRIST grade definitions for radius and ulna						**TOTAL SCORE =** Radius Grade + Ulna Grade
0	Normal growth plate without changes of rickets					
0.5	Lucency of the metaphyseal margin without fraying or irregularity					
1	Widened growth plate, irregularity of the metaphyseal margin without concave cupping					
1.5	Partial metaphyseal concavity or incomplete fraying of metaphyseal margin					
2	Metaphyseal concavity with fraying of margins					
Grade radius	0	0.5	1	1.5	2	
Grade ulna	0	0.5	1	1.5	2	
						Total
Knee grade definitions for femur and tibia						**TOTAL SCORE =** Femur grade x multiplier + Tibia grade x multiplier
0	Normal growth plate without changes of rickets					
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					
Grade femur	0	1	2	3		**Femur Multiplier** One or less condyle or plateau affected = 0.5 Two condyles or plateaus affected = 1
Grade tibia	0	1	2	3		**Tibia Multiplier** One or less condyle or plateau affected= 0.5 Two condyles or plateaus affected = 1
						Total

6.5.5 Histomorphometry and quantitative back-scattered electron imaging

6.5.5.1 Sample preparation and storage

Full-thickness transiliac bone biopsies were obtained with a Bordier trephine (5–7 mm core diameter) from a site located 2 cm below and behind the anterior superior iliac spine. Biopsies were obtained when the iliac bone was exposed for post-mortem examination and were not done trans-cutaneously. Biopsy specimens were fixed in 70% ethanol and were stored and transported at room temperature. In the receiving laboratory, samples were dehydrated in increasing concentrations of ethanol, cleared with xylene, and embedded in poly-methylmethacrylate (PMMA). The undecalcified iliac bone samples embedded in PMMA were hardened for several days at elevated temperatures of about 40 °C. Once the hardening process was completed, the samples were trimmed and cut by a diamond saw (Buehler Isomet 1000, Lake Bluff, IL) in a defined orientation for measurements. Undecalcified 3-4 μm-thick sections were cut with a Polycut E microtome (Reichert-Jung, Heidelberg, Germany), placed on chromium-alum gelatine-coated slides and flattened to dry at 50°C for 18 h. For each specimen, two to five series of consecutive sections were cut at least 150 μm apart. The sections were deplastified with ethylene glycol monoethylacetate to allow for optimal staining. In each series, three consecutive sections were selected. Two were stained with either toluidine blue or Masson Goldner Trichrome, and the third mounted unstained for fluorescence microscopy.

6.5.5.2 Histomorphometry analysis

All parameters were determined in at least two sections of a biopsy to obtain a measurable tissue area of 40–50 mm^2. Cortical width was directly measured at 500 μm intervals as the

distance between the periosteal surface and the endocortical surface of each cortex. For the determination of cancellous parameters, the entire cancellous tissue area was analysed, including the transitional zone. However, the endocortical perimeter was excluded, because, in growing individuals, cellular activity clearly differs between endocortical and cancellous surfaces, due to the presence of modeling drifts [158]. Mineralized bone was defined as a green (with Goldner stain) structure containing osteocytes. Osteoid was counted as red-staining seams of at least 1.5 μm in width at the bone-bone marrow interface. Wall width was measured on Goldner-stained sections under polarized light as the distance from quiescent bone surfaces to the abrupt change in collagen fiber orientation. No specific osteoclast staining was performed. The following primary measures were obtained in cancellous bone: tissue area; bone area; bone perimeter; osteoid area; osteoid perimeter; eroded perimeter; wall width (measured at 50 μm intervals). All other measurements were carried out at a magnification of x 200. Histological analysis was performed according to Parfitt et al [86] on the whole area of the bone sections. A light microscope (Axiophot; Zeiss, Oberkochen, Germany) equipped with a Zeiss AxioCam videocamera was used to obtain digital images of the sections. Images were analysed using standard procedures (NIH Image software, versions 1.62 and 1.63; Wayne Rasband, National Institutes of Health, Bethesda, MD, USA) on a Power Macintosh G4 (Apple, Cupertino, CA, USA). Nomenclature and abbreviations followed the recommendations of the American Society for Bone and Mineral Research [159]. Osteoid thickness, Osteoid surface/ Bone surface (OS/BS) and Osteoid Volume/ Bone Volume (OV/BV) were evaluated using the below formula **(Table 6.5)**.

We did not evaluate bone cells (osteoblasts and osteoclasts) as for such analysis the sample would have to be collected and fixed within 24 hours of death to obtain reliable quantitative information.

Table 6.5: Formula used to calculate histomorphometric values from primary measurements

Parameter	Abbreviation	Unit	Formula
Osteoid thickness	O.Th	μm	(osteoid area/osteoid perimeter) x 2/1.2
Osteoid surface/bone surface	OS/BS	%	(osteoid perimeter/bone perimeter) x 100
Osteoid volume/bone volume	OV/BV	%	(osteoid area/tissue area) x 100

6.5.5.3 Quantitative backscattered electron imaging (qBEI)

The bone mineral density distribution (BMDD) in the hip bone samples was measured by quantitative backscattered electron imaging (qBEI) using a previously detailed method [88] [160]. In brief, the physical principle of the technique is based on quantification of the intensity of electrons backscattered from the surface of a sectioned bone area. The obtained signal is proportional to the weight fraction of calcium (Ca wt%) present locally in the embedded bone tissue.

The samples were prepared as detailed above **(Section 6.5.5.1)** and the residual sample was then ground and polished (Logitech PM5). Finally, the sample was carbon coated (AGAR SEM carbon coater) to achieve a conducting surface. The samples were analysed using a digital scanning electron microscope with a four-quadrant semiconductor backscattered electron detector (DSM 962, Zeiss, Oberkochen, Germany), at a working distance of 15 mm. Probe current was adjusted to 110 ± 0.4 pA and electron beam energy to 20 keV. The pixel resolution of the digital image was 4 microns/pixel at magnification of 50 X, with a resolution of 256 grey levels. For each sample, 5–6 regions of the same size (2 x 2.5 mm wide) were scanned, using a scan speed of 100 s per frame. From the digital images, grey-level histograms were deduced, displaying the percentage of bone area occupied by pixels of a

certain grey level. The parameters characterising the BMDD curve that were obtained are detailed below **(Table 6.6)**. An example of the adult BMDD reference curve in **Figure 6.10** illustrates all the terminologies relating to the calcium parameters used in the curve.

The BMDD curves were evaluated separately for the trabecular compartment and the two cortical compartments. BMDD values obtained from the subjects were compared to those of the reference BMDD of normal healthy children aged 2-20.9 years [161].

Table 6.6: Parameters characterizing the bone mineral density distribution (BMDD) curve

Term	Abbreviation	Description
Calcium Mean	CaMean	The weighted mean calcium concentration of the bone area obtained by the integrated area under the BMDD curve
Calcium Peak	CaPeak	The peak position of the histogram, which indicates the most frequently occurring calcium concentration (calcium value with the highest number of pixels) in the bone area
Calcium Width	CaWidth	The full width at half maximum of the distribution, describing the variation in mineralization density
Calcium Low	CaLow	The percentage of bone area that is mineralized below the 5th percentile of the reference BMDD that is below 17.68 wt.% calcium. This parameter corresponds to the amount of bone area undergoing primary mineralization
Calcium High	CaHigh	The percentage of bone area that is mineralized above the 95th percentile of the reference BMDD that is above 25.30 wt.% calcium. This parameter corresponds to the amount of bone having achieved plateau level of normal mineralization and includes also the contribution of highly mineralized cement lines

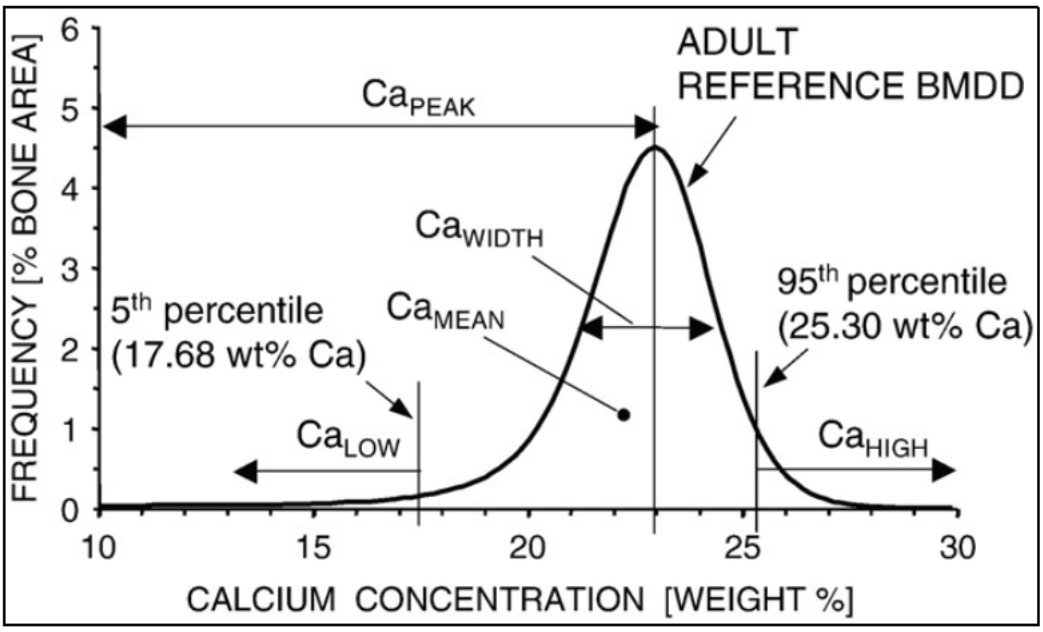


Figure 6.10: The BMDD curve of normal human adult trabecular bone illustrating all parameters relating to calcium used in the report.

Figure reproduced from reference [88]. Copyrights obtained from Elsevier and Copyright Clearance Centre (License number 5123620486593).

The existing BMDD reference curves [88] are derived from a scanning electron microscope (SEM) equipped with a tungsten hairpin cathode (thermionic electron emission), however there has been a recent migration to SEM with a field emission electron source (FE-SEM) in the Institute of Osteology, Vienna. The FE-SEM has a much better spatial resolution based on an electron source size in the order of several 100 nm, corresponding to an about 10^5 to 10^6 times smaller source area compared to thermionic sources. Following the change in equipment the new reference curves for FE-SEM have only recently been reported by Mähr et al [161]. Following change in methodology we have only been able to re-evaluate the trabecular BMDD on a small sub-set of seven samples which will be reported and compared to the above-mentioned reference data. The BMDD for cortical bone have not been re-evaluated and are therefore not reported here.

6.5.6 Three-point bending test

6.5.6.1 Sample storage and preparation

The distal part of the rib collected for histology was used for three-point bending test. Rib samples were covered in gauze soaked in phosphate buffered saline (PBS) and kept in plastic bags in a freezer at -20 °C until tested. Testing was undertaken in batches within 12 months of collection as it is known that storage up to one year does not affect the mechanical properties of the bone [162]. The frozen ribs were thawed to room temperature before testing. The ribs were tested across the depth. By initiating fracture from a pre-existing "worse-case" precrack or a notch, the mechanical role of microstructural defects, specifically microcracks, is minimised [163]. Therefore a notch was created on the anterior surface, for 20 % [164] of the rib depth (average of 0.7 mm, ranging from 0.4 to 1.3 mm) using a 0.25 mm thick microtome blade (MX35 ULTRA, Thermo scientific, Waltham, MA, USA). The blade was mounted on a custom-made mounting device to ensure the depth of the blade is adjustable.

6.5.6.2 Three-point bending test

A controlled fracture test was carried out testing the sample under a three-point bending test using an axial mechanical testing machine [TA Instruments, ElectroForce, DMA3230 (Newcastle, UK)]. The sample was placed with the notch on the under surface and load applied from above as illustrated in **Figure 6.11** and **Figure 6.12**. The sample was pre-loaded to 1-2 newtons (N) compression then loaded under displacement control at a rate of 0.167 mm/min as calculated from ASTM (American Society for Testing and Materials) D790-17 [165]. The sample was tested until failure or until 5 mm depth was achieved. The load and

displacement data were recorded at 20 samples a second using TA Instruments in-house software.

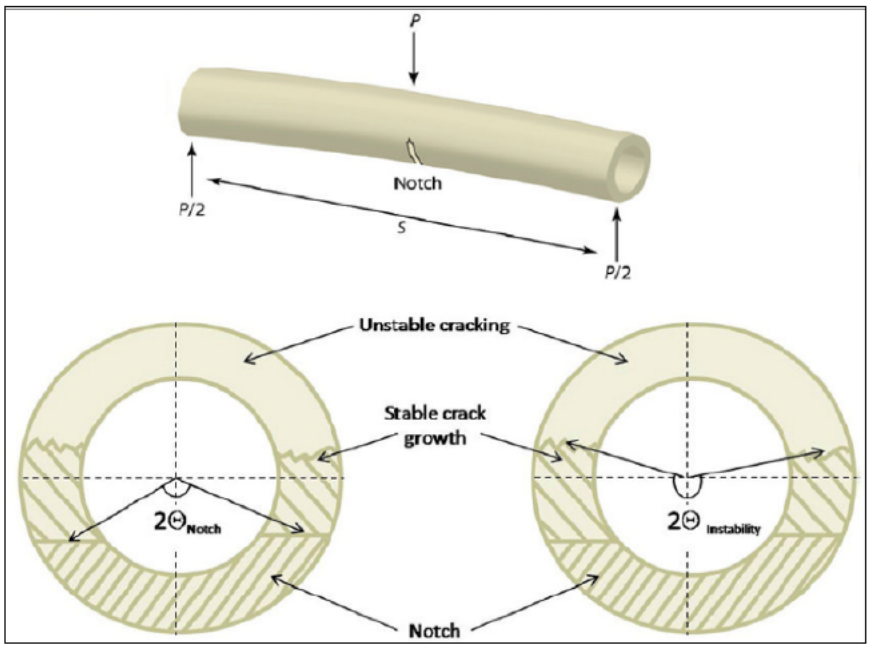


Figure 6.11: A schematic diagram of a notched bone specimen.

S is the distance between the load supports in a three-point bending test and P is the load applied (top). Above is a cross-sectional view of the specimen where 2Θnotch is the angle of the starting notch (left) and 2Θinstability is the angle when unstable cracking begins (right).Figure reproduced from reference [166]. Copyrights obtained from Elsevier and Copyright Clearance Centre (License number 5133720915490)

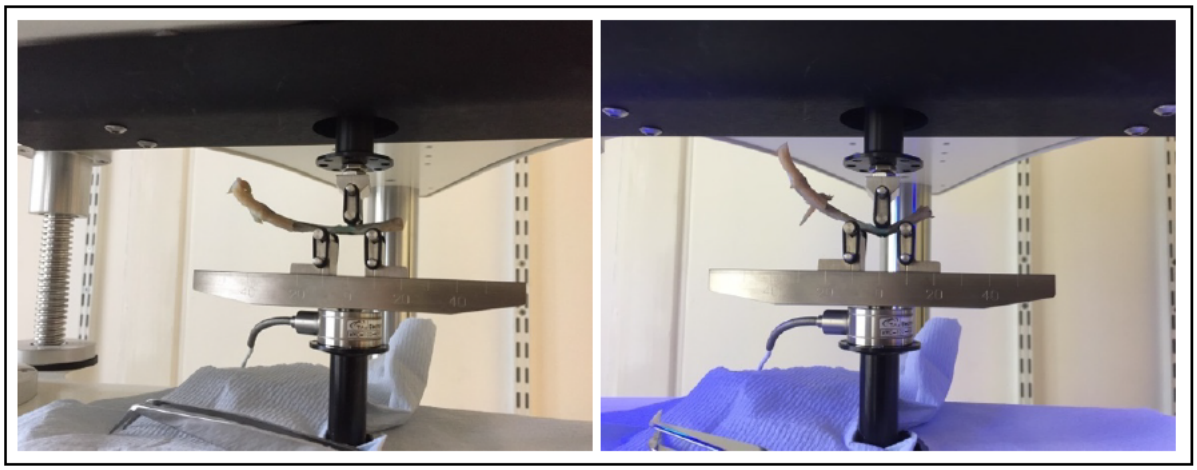


Figure 6.12: Three-point bending test on rib bone.

6.5.6.3 Calculations and analysis of three-point bending test results

The abbreviations and units used in this section are outlined in **Table 6.7** below.

Fracture toughness (Kc), a measure of resistance to fracture, is evaluated at three points on the load-displacement curve **(Figure 6.13)** as detailed by Ritchie et al [166]; at crack initiation (P_Q), maximum load (P_{max}) and crack instability (P_f). Kc at P_f is considered the ideal measurement due to the sudden failure at this point, however we did not note a sudden failure in all of our samples and therefore Kc values for P_Q and P_{max} were evaluated. The equations used to calculate Kc are presented below.

Table 6.7: Abbreviations, formulas and units used in three-point bending test and analysis

	Symbol	Formula	Units
Stiffness	k		N/mm
Peak Load	P		N
Sliding Distance	S		mm
Breadth	B		mm
Width	W		mm
Notch Depth	D		mm
Notch Angle	Θ		mm
Cortical Thickness	t		mm
Bending Moment	M	PS/4	Nm
Outer Radius (Breadth)	R_o	B/2	mm
Outer Radius (Width)	R_{width}	W/2	mm
Cross Section before Notch	A_{bone}	$A=\pi \times R_o \times R_{width}$	mm^2
Cross Section of Notch	A_{notch}		mm^2
Cross Section after Notch	A_{test}	$A_{bone} - A_{notch}$	mm^2
Inner Radius	R_i	$R_i = B - t$	mm
Mean Radius	R_m	$(R_o+R_i)/2$	mm

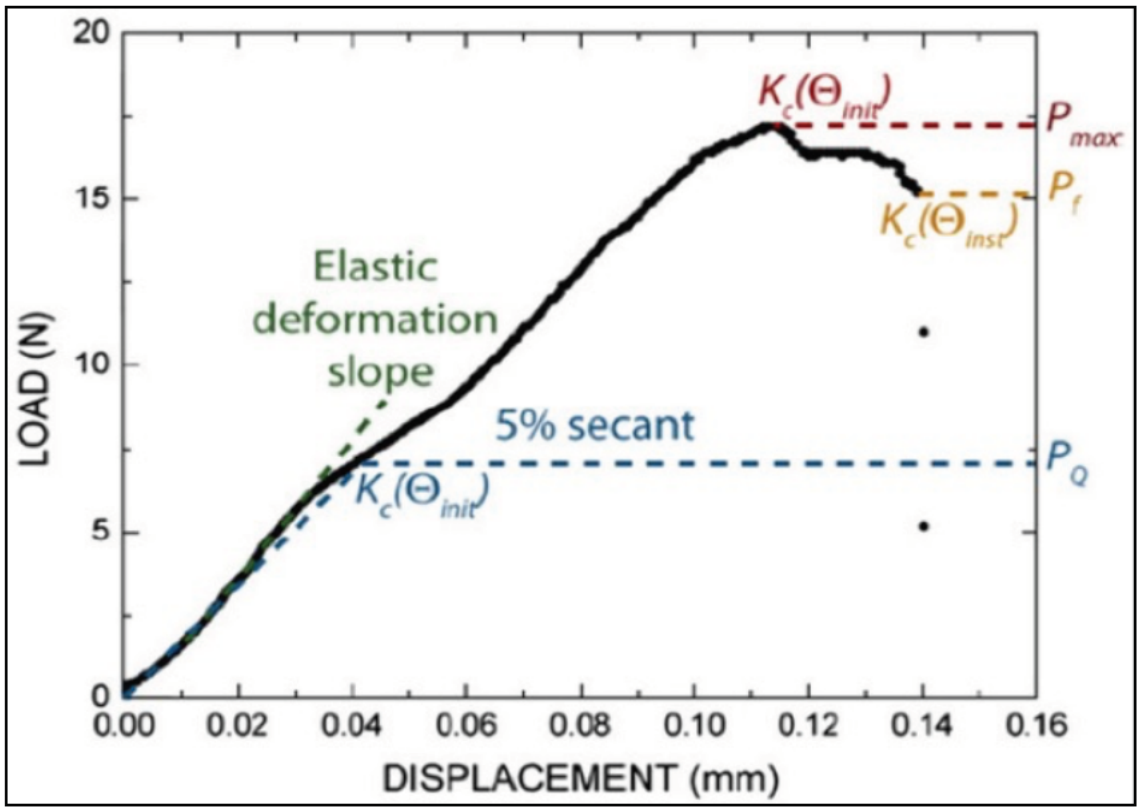


Figure 6.13: Load-displacement curve showing a typical loading behaviour of a sharp-notched bone sample under three-point bending.

Kc (Θ_{init}) at P_Q is the fracture toughness calculated where the 95% elastic stiffness line intersects with the load curve (5 % secant), Kc (Θ_{init}) at P_{max} is the fracture toughness at maximum load and Kc (Θ_{init}) at P_f is the fracture toughness at the unstable fracture point signified by a sudden load drop.

Figure reproduced from reference [166]. Copyrights obtained from Elsevier and Copyright Clearance Centre (License number 5133720915490)

After testing, fracture toughness (Kc) in MPa√m was computed using the below equation:

$$K_c = F_b \frac{PSR_o}{\pi\left({R_o}^4 - {R_i}^4\right)} \sqrt{\pi\left(\frac{R_o + R_i}{2}\right)\Theta_{inst}}$$

Where Fb (in N) is

$$F_b = \left(1 + \frac{t}{2R_m}\right)\left[A_b + B_b\left(\frac{\Theta}{\pi}\right) + C_b\left(\frac{\Theta}{\pi}\right)^2 + D_b\left(\frac{\Theta}{\pi}\right)^3 + E_b\left(\frac{\Theta}{\pi}\right)^4\right]$$

$$A_b = 0.65133 - 0.5774\xi - 0.3427\xi^2 - 0.0681\xi^3$$

$$B_b = 1.879 + 4.795\xi + 2.343\xi^2 - 0.6197\xi^3$$

$$C_b = -9.779 - 38.14\xi - 6.611\xi^2 + 3.972\xi^3$$

$$D_b = 34.56 + 129.9\xi + 50.55\xi^2 + 3.374\xi^3$$

$$E_b = -30.82 - 147.6\xi - 78.38\xi^2 - 15.54\xi^3$$

$$\xi = \log\left(\frac{t}{R_m}\right)$$

6.5.7 Statistical analysis

All analyses were performed using SPSS statistical software v26.0 (IBM, Armonk, NY). Descriptive statistics are presented as mean, standard deviation (SD) or median, interquartile range (IQR) [or median, range in smaller subsets based on ethnicity or season] for continuous variables. Categorical variables are presented as percentages.

Non-parametric tests were used as the data was not normally distributed. Spearman's correlations were used to determine the correlation between continuous variables. Mann-Whitney U test was used to compare groups and one sample T-test was used to compare mean to reference data. Fischer's exact test was used to determine the association between dichotomous variables. A p value less than 0.05 was considered to be statistically significant.

6.6 Results

6.6.1 Recruitment

During the periods when the study was open to recruitment (**Figure 6.14**), a total of 54 non-forensic cases of SUDIC were identified. Seven were deemed not eligible due to prematurity and the Coroner's officers deemed six subjects to be ineligible due to the families being in extreme distress. Of the 41 deemed eligible by study team and the Coroner's officers, four could not be approached due to late notification following post-mortem examination. Thirty seven families were approached and 33 recruited, 3 refused to participate and one was deemed ineligible by the lead researcher as significant barriers in language and understanding were identified despite the use of an interpreter.

A total of 33 subjects have been recruited to date (June 2021), from the two recruitment sites, Birmingham Women's and Children's Hospital, Birmingham, UK (n=24) and Sheffield Children's Hospital, Sheffield, UK (n=9). Due to the Covid-19 pandemic and the resulting severe restrictions in laboratory working conditions, a complete set of results for all recruited subjects was not possible. The study is still ongoing as of July 2021, with a planned recruitment end date of August 2022.

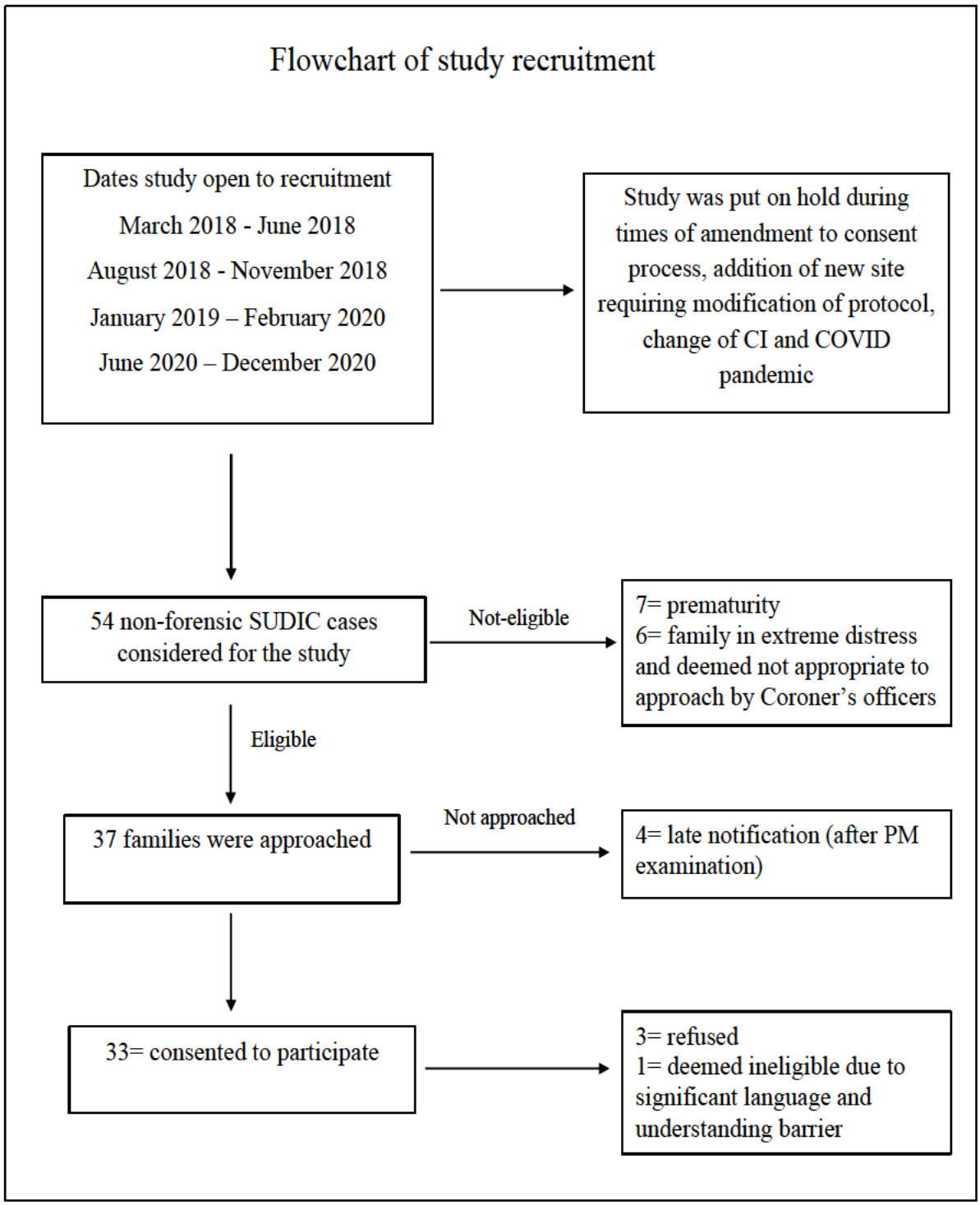


Figure 6.14: Flowchart of study recruitment

6.6.2 Subject demographics

The demographics (age, gender, ethnicity), cause of death as reported in the post-mortem examination report, feeding mode and vitamin D supplement use are detailed in **Table 6.8**.

General demographics:

At the time of death, the median (range) age was 78 (0 – 545) days. The median (range) time from death to sample collection was 7 days (3-10 days). The majority (87.9%, n=29/33) were infants under 1 year of age and 75.8% (n=21/33) were under 6 months of age. 57.6% were male (n=19/33) and 42.4% female (n=14/33). The majority were of white ethnic origin (69.7%, n=23/33), 21.2% (n=7/33) were of south Asian origin, 6.1% (n=2/33) of black ethnic origin and 3% (n=1/33) of mixed white and black heritage.

Most subjects were recruited in winter (December - February: 33.3%, n=11/33), followed by spring (March – May: 27.3%, n=9/33), autumn (September – November: 24.2%, n=8/33) and summer (June – August: 15.2%, n=5/33).

The mean (SD) weight z score and length z score of the cohort were - 0.15 (1.5) and + 0.48 (1.6) respectively. The median (range) weight z score and length z scores were - 0.30 (- 3.0 to + 3.4) and + 0.30 (- 2.50 to + 5.10) respectively.

Table 6.8: Subject demographics, cause of death, pre-existing co-morbidity, serum biochemistry and growth plate histology findings

Number	**Age in days**	**Gender**	**Ethnicity**	**Cause of death** ***Previous co-morbidity**	**Heart weight (expected for age)**	**Feeding modeϕ**	**Vitamin D supplement use**	**Total 25OHD nmol/L**	**24,25(OH)$_2$ D$_3$ nmol/L**	**Histological features of rickets Ψ**	**Height of Hypertrophic Zone mm**
1	68	F	South (S) Asian	SIDS[$]	Normal	F	N	-	-	1,2,3,5,6	1.762
2	135	M	S Asian	Unexplained SUDI[&]	Normal	F	N	44.50	3.17	1,2,5	0.563
3	223	F	White	Unascertained SUDI	Normal	W+F	N	48.35	5.65	Normal	0.342
4	171	M	Mixed	Rhinovirus pneumonia	Normal	F	N	-	-	-	-
5	80	M	S Asian	Post-operative lung haemorrhage *Coronary steal syndrome	Normal	F	N	3.87	0.54	1,3,4,5	0.605
6	545	M	White	Pneumonia *Suspected mitochondrial disorder-Phosphatidylinositol Glycan Anchor Biosynthesis Class A mutation confirmed at post-mortem	Normal	W	N	49.00	6.58	Normal	0.26
7	0	M	White	Unascertained SUDI	Normal	F	N	18.79	1.73	1,2,3	0.89
8	32	F	White	SIDS (mild dysmorphic features)	Normal	F	N	25.40	1.80	1,2,3,4,5,6	1.454
9	5	F	White	Unexplained SUDI	Normal	B+F	N	-	-	1,2,3,4,5,6	1.313

10	35	M	White	Cardiac failure and dilated cardiomyopathy	↑↑ 41.3 g (20 g)	F	N	24.81	2.91	1,2,3,4,5	0.76
11	0	F	White	Unascertained SUDI *Microcephaly	Normal	F	N	31.98	1.88	1,2,3,4,5,6,	0.778
12	18	M	White	SIDS *Low birth weight 2.4 Kg	Normal	F	N	33.60	1.07	1,2,3	0.374
13	489	M	White	Cardiac arrythmia secondary to adenovirus infection	Normal	W	N	31.70	1.97	Normal	0.155
14	318	F	White	Unascertained SUDI	Normal	W	N	-	-	Normal	0.251
15	291	F	S Asian	Pneumonia, staph aureus and RSV isolated	Normal	W	N	-	-	-	-
16	15	M	S Asian	Mitochondrial encephalopathy *Suspected mitochondrial disorder not confirmed	Normal	B	N	5.40	<0.15	1,2,3,4,5	1.023
17	182	M	White European	Unascertained SUDI	Normal	F	Y	34.70	1.87	Normal	0.152
18	41	M	White	Unascertained SUDI	Normal	F	N	22.90	1.01	-	
19	428	M	Black	Unascertained SUDC[ə] *Sickle cell	Normal	W	Y	44.20	3.50	Normal	0.157
20	463	F	White	Myocarditis	Normal	W	N	30.80	2.35	Normal	0.311
21	28	M	S Asian	Unascertained SUDI	Normal	B	N	3.40	<0.15	1,2,3	0.962
22	164	F	White	Cardiomyopathy and endocardial fibroelsatosis	↑↑ 97.4 (29 g)	F	Y	45.80	5.90	Normal	0.199

23	86	F	White	Unascertained SUDI *Very low birth weight 1.5 Kg	Normal	F	N	53.20	2.59	1,2,4,5	0.643
24	241	M	S Asian	SIDS	Normal	W+F	N	42.20	2.15	2	0.34
25	94	M	White	Unascertained SUDI	↑ 35.4 g (23 g)	F	N	21.10	0.67	1,2,4,5	0.423
26	46	M	White	Overlaying	Normal	F	N	-	-	1,2,4,5	0.975
27	32	F	White	SIDS	Normal	F	N	-	-	1,2,4,5	0.36
28	78	F	White	SIDS	Normal	F	N	50.40	2.08	1,4,5	0.34
29	72	F	White	Unascertained SUDI	Normal	F	N	50.10	2.40	1,2,4,5	0.965
30	84	F	Black	Unascertained SUDI	Normal	F	N	21.20	1.58	Normal	0.251
31	21	M	White	SIDS	Normal	F	N	-	-	1,2,4,5	0.649
32	28	M	White	Unascertained SUDI	Normal	F	N	7.00	0.18	1,2,4,5	0.995
33	35	M	White	SIDS	Normal	F	N	56.90	4.07	1,2,5	0.498

[&]SUDI- Sudden Unexplained Death in Infancy [$]SIDS- Sudden Infant Death Syndrome [ɔ]SUDC- Sudden unexplained Death in Childhood ϕ

Feeding modes: F= Formula, W=weaned, B= breast

Ψ 1= Expansion of hypertrophic chondrocyte zone (HZ). 2=Loss of columnar arrangement of hypertrophic chondrocytes.

3=Loss of distinction between hypertrophic and proliferative zones. 4= Penetration of hypertrophic chondrocyte zone with blood vessels

5= Tongues of hypertrophic chondrocytes extending into primary spongiosa. 5= Bulbous expansion of the costo-chondral junction.

Co-morbidities and cause of death (Table 6.8):

Two subjects (subjects 12 and 23) were born with a low birth weight. Five subjects had pre-existing co-morbidities, one infant had a congenital heart defect (subject 5), one child had sickle cell disease (subject 19), one had microcephaly (subject 11), two subjects were suspected to have a mitochondrial disorder (subjects 6 and 16). One child with suspected mitochondrial disorder had genetic confirmation of Phosphatidylinositol Glycan Anchor Biosynthesis Class A (PIGA) deficiency on post-mortem examination (subject 6). Encephalopathy was reported as the cause of death in one child with suspected mitochondrial disorder (subject 16). The child with known congenital heart defect was reported to have died of post-operative lung haemorrhage.

Four subjects with no previous identified co-morbidity were reported to have died of cardiac related events including myocarditis, cardiac arrythmia due to adenovirus infection, dilated cardiomyopathy and cardiomyopathy with endocardial elastofibrosis. Lower respiratory tract infection (pneumonia) was reported as the cause of death in 3 subjects. The cause of death was reported as unascertained/unexplained SUDI/SUDC in 15 subjects, SIDS in eight subjects and overlaying in one subject.

All subjects had microarray as part of post-mortem examination, and none had any significant copy number variants identified. Additional condition specific genetic panels were tested based on post-mortem examination findings and relevant history. Subject number 10 had dilated cardiomyopathy at post-mortem examination and reported a history of poor feeding and sweating, therefore dilated cardiomyopathy genetic panel was undertaken which was negative. Subject number 22 also had a genetic panel for cardiomyopathy which was negative.

Heart weight:

Three subjects (number 10, 22 and 25) had an increased heart weight **(Table 6.8)** compared to reference data [152]. One subject (number 22) was diagnosed with endocardial fibroelastosis and the other two subjects (number 10 and 25) had no cause identified. Subject 10 had a dilated cardiomyopathy genetic panel which did not identify any specific genetic mutations.

Feeding, calcium intake and vitamin D supplement use:

At the time of death, the majority were exclusively formula fed (66.7%, n=22/33) **(Figure 6.15)**. Two subjects were exclusively breastfed (6.1%) and one was on mixed breast and formula feeds (3%). A total of eight subjects were weaned, of whom two were still on formula feeds (6.1%) and six were fully weaned onto solids (18.2%). The two subjects who were still on formula feeds after weaning consumed < 500 ml (380 ml and 460 ml) of formula per day.

Subject 5 was briefly off feeds in the post-operative period. Two subjects (number 10 and 15) reported reduced feeds in the days prior to death. Prolonged history of poor feeding was not recorded in any of the subjects.

In exclusively formula fed infants, the information on the specific formula preparation used was available in 13 of the 22 subjects. The mean (SD) and median (range) calcium intake for the exclusively formula fed cohort was 364.6 (135.3) mg/day and 322.5 (217–657) mg/day. Calcium intake increased with age (r=0.54, p=0.01) **Figure 6.16**.

Three children (9%) were given vitamin D supplements, only one of whom was among the 11 subjects expected to be on supplements as per government guidance [67]. The three children who were on supplements were of white European (subject 17), Black (subject 19) and white British (subject 22) ethnic origin. Subject 17 was on 400 IU daily of cholecalciferol, subject

19 with sickle cell disease was on multivitamins with vitamin D bought over the counter and subject 22 was on Healthy Start vitamins.

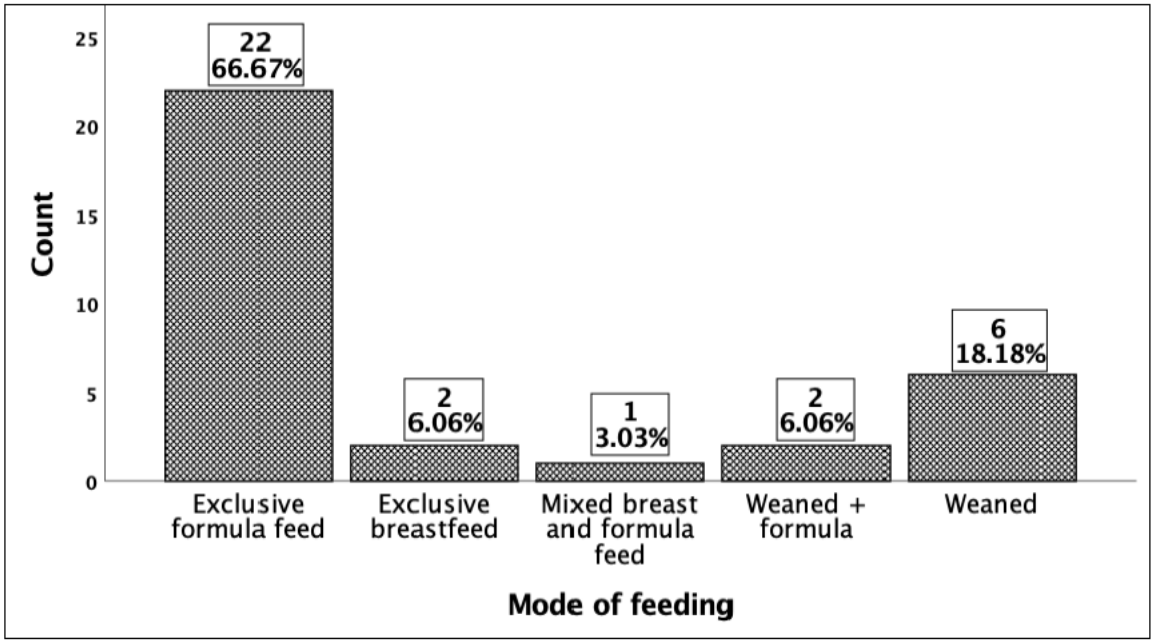


Figure 6.15: Bar chart representing the mode of feeding

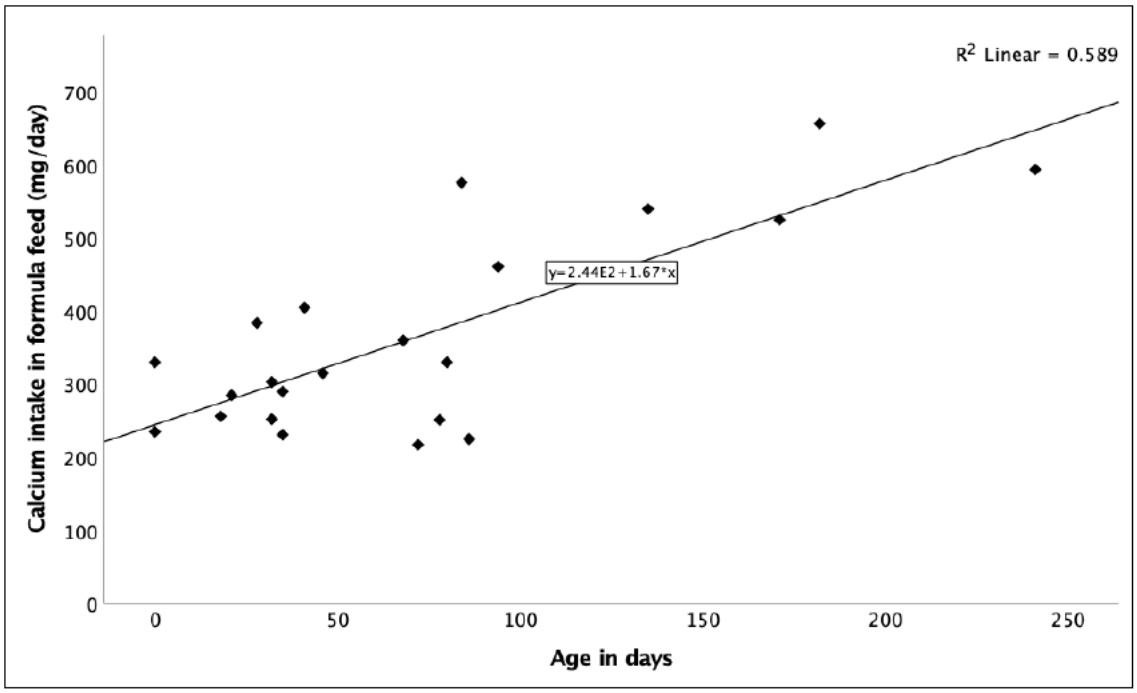


Figure 6.16: Calcium intake (mg/day) in exclusively formula fed infants

6.6.3 25OHD and other vitamin D metabolites

6.6.3.1 Subject demographics

Measurements for serum concentrations of 25OHD and other vitamin D metabolites were carried out for 25 subjects (16 males). Results were not available for eight subjects due to various reasons [insufficient sample for processing (n=1), unable to obtain study sample due to insufficient sample for routine post-mortem tests (n=3), delayed consent resulting in inability to collect fresh sample at the time of post-mortem examination (n=2) and sample frozen before centrifugation in error (n=2)].

The mean (SD) age of the cohort was 144 (165) days and median (range) was 80 (0 – 545) days. The majority (84%, n=21/25) were infants under 1 year of age. Seventy two percent were of white ethnicity (18/25), 20% of south Asian origin (n=5/25) and 8% of black ethnic origin (n=2/25).

Thirty two percent of the samples (n=8/25) were collected in winter (December to February), 28% (n=7/25) in spring (March to May) and 20% (n=5/25) each in summer (June to August) and autumn (September to November), respectively. The feeding modes in the cohort were: exclusive formula feed in 68% (n=17/25), exclusive breastfeeding in 8% (n=2/25), weaned with additional formula in 8% (n=2/25) and completely weaned on to solids in 16% (n=4/25).

6.6.3.2 Descriptive report of 25OHD levels and other vitamin D metabolites

Total 25OHD and $25OHD_3$ levels were detectable in all subjects. $25OHD_2$ levels were detected in eight subjects and the rest were below detectable range (17/25). Serum 1,25 $(OH)_2D_3$ levels were undetectable in the first batch of samples analysed and was therefore not

analysed in the second batch. $24,25(OH)_2D_3$ levels were detectable in the majority (23/25) and below detection levels of <0.15 in 2/25 subjects. 3-epi-$25OHD_3$ was detectable in all subjects (25/25).

Total 25OHD and $24,25(OH)_2D_3$ values for individual subjects are presented in **Table 6.8** above. The mean (± SD) total 25OHD concentration of the cohort was 32.0 (± 16.3) nmol/L and $25OHD_3$ concentration was 31.8 (± 16.4) nmol/L. The mean (± SD) and median (IQR) for $24,25(OH)_2D_3$, 3-epi-$25OHD_3$, percentage of $25OHD_3$ as 3-Epi-$25OHD_3$ and $25OHD_3$:$24,25(OH)_2D_3$ ratio of the cohort are presented in **Table 6.9.**

Table 6.9: Mean (SD) and Median (IQR) for 25OHD and vitamin D metabolites and ratios

Vitamin D and metabolites	**Mean (SD)**	**Median (IQR)**
Total 25OHD nmol/L (n=25/25)	32.0 (16.3)	31.9 (21.1, 47.0)
$25OHD_3$ nmol/L (n=25/25)	31.8 (16.4)	31.9 (21.1, 47.0)
$24,25(OH)_2D_3$ nmol/L (n=23/25)	2.5 (1.7)	2.0 (1.5, 3.1)
3-epi-$25OHD_3$ nmol/L (n=25/25)	4.2 (2.4)	4.1 (2.2, 6.3)
Percentage of $25OHD_3$ as 3-epi-$25OHD_3$ (n=25/25)	14.9 (7.2)	13.9 (9.3, 19.5)
Ratio $25OHD_3$:$24,25(OH)_2D_3$ (n=23/25)	16.9 (8.3)	14.1 (10.9, 20.9)

6.6.3.3 Classification of 25OHD status:

Vitamin D deficiency defined by a total 25OHD concentration below 30 nmol/L was recorded in 40% (n=10/25) of the cohort. Insufficiency was noted in 44% (n=11/25) and sufficiency in 16% (n=4/25) **(Figure 6.17)**.

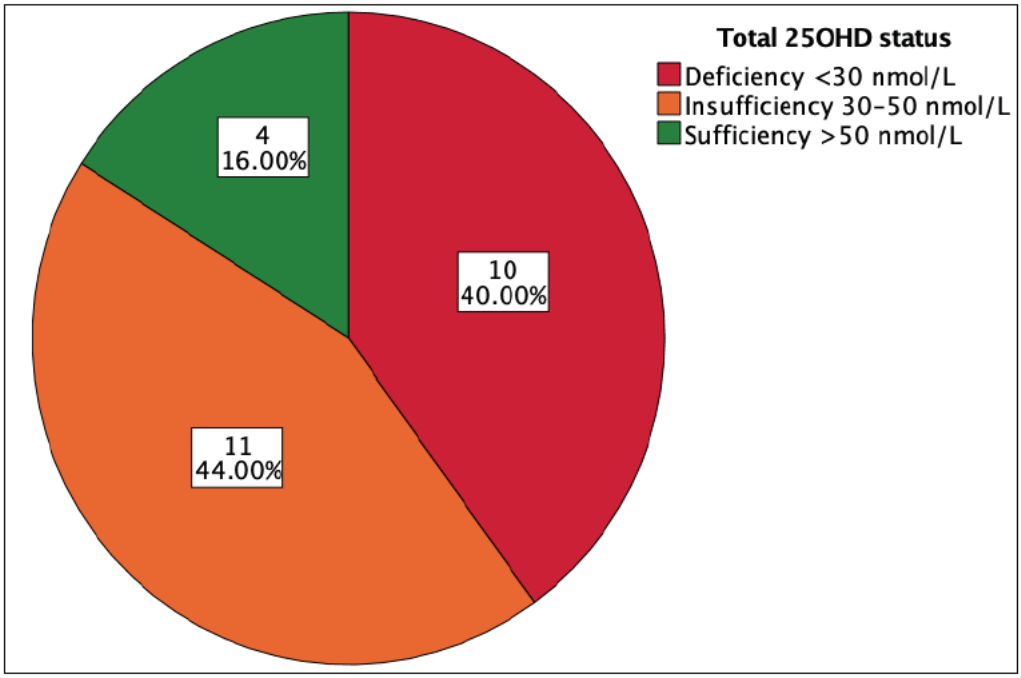


Figure 6.17: Pie chart illustrating vitamin D status based on serum total 25OHD levels

6.6.3.4 Correlations of 25OHD and its metabolites:

Total 25OHD (r=0.42, p=0.04) and $25OHD_3$ (age r=0.41, p=0.04) were positively correlated to age. The percentage of $25OHD_3$ as 3-epi-$25OHD_3$ had a significant negative correlation (**Figure 6.18)** with age (r=-0.60, p=0.001).

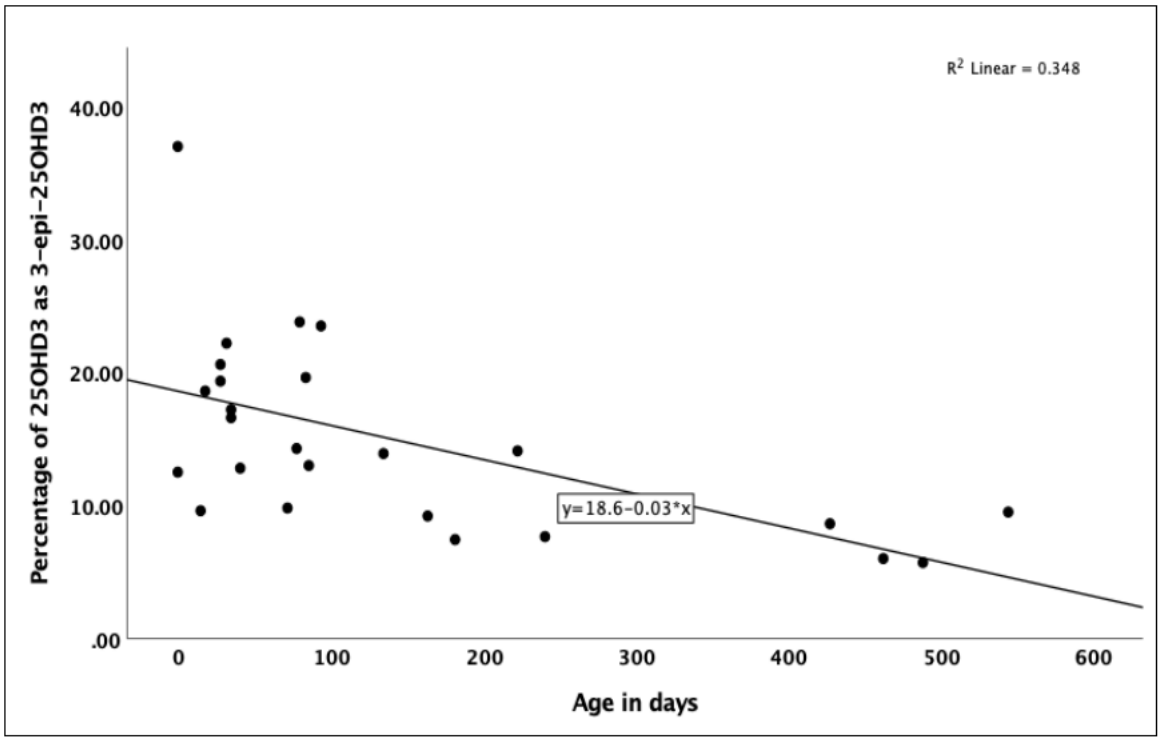


Figure 6.18: Scatterplot demonstrating significant negative correlation between age and percentage of $25OHD_3$ as 3-epi-$25OHD_3$

$25OHD_3$ had a significant positive correlation to $24,25(OH)_2D_3$ ($r=0.83$, $p<0.001$,**Figure 6.19**) and 3-epi-$25OHD_3$ ($r=0.64$, $p<0.001$) and a significant negative correlation to percentage of $25OHD_3$ as 3-epi-$25OHD_3$ ($r=-0.43$, $p=0.03$).

After adjusting for age, $25OHD_3$ was positively correlated with $24,25(OH)_2D_3$ ($r=0.67$, $p<0.001$) and 3-epi-$25OHD_3$ ($r= 0.76$, $p<0.001$) and negatively correlated with percentage of $25OHD_3$ as 3-epi-$25OHD_3$ ($r=0.49$, $p=0.01$).

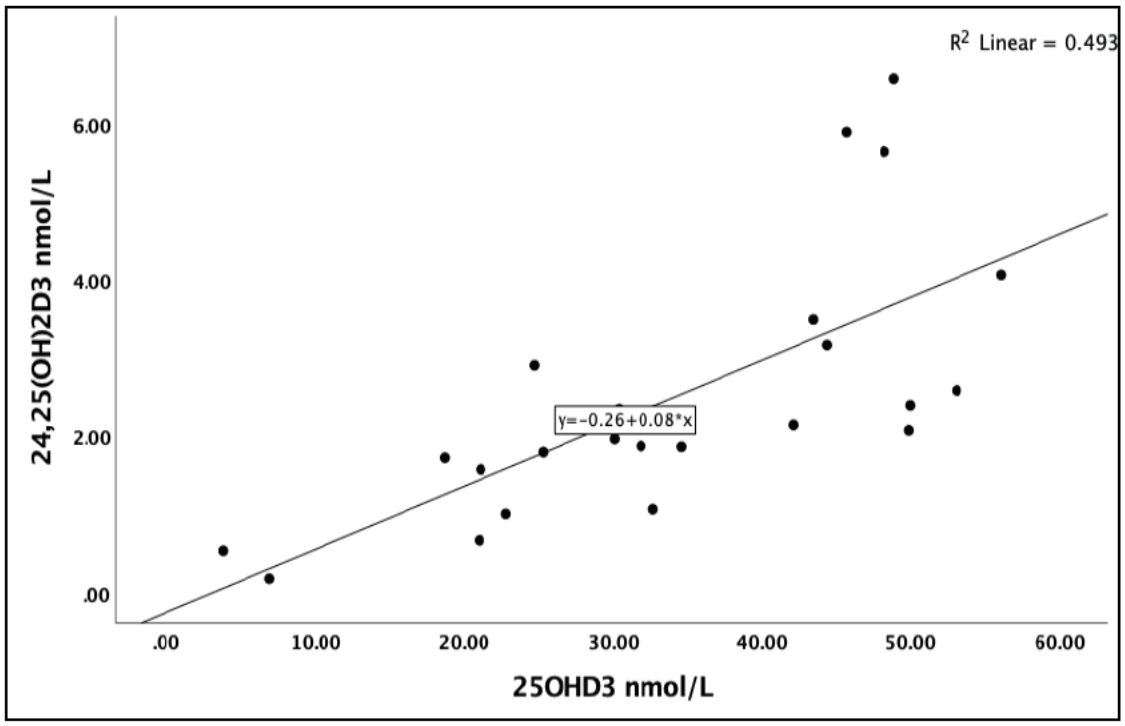


Figure 6.19: Scatterplot demonstrating significant positive correlation between $25OHD_3$ and $24,25(OH)_2D_3$.

6.6.3.5 25OHD and other vitamin D metabolites by season and ethnicity

Season:

The median (range) $25OHD_3$ and $24,25(OH)_2D_3$ are presented in

Figure 6.20 and

Figure 6.21 respectively. The median (range) $25OHD_3$ in nmol/L in winter, spring, summer and autumn were 28.8 (4.6 – 56.2), 32.0 (21.2 – 50.1), 30.5 (3.1 – 48.3) and 32.8 (3.9 – 53.2) respectively. The median (range) $24,25(OH)_2D_3$ in nmol/L in winter, spring, summer and autumn were 1.8 (<0.15 – 6.6), 2.4 (1.6 – 3.5), 2.3 (<0.15 – 5.9) and 2.0 (0.54 – 2.6) respectively. The median (range) 3-epi-$25OHD_3$, percentage of $25OHD_3$ as 3-epi-$25OHD_3$ and $25OHD_3$:$24,25(OH)2D_3$ ratio in winter (n=8) was 4.8 (0.4 – 9.7) nmol/L, 13.5 (7.4 –

37.0) and 18.5 (7.0 – 31.5) respectively, in spring was 4.1 (3.8 – 6.4) nmol/L, 13.9 (8.6 – 22.2) and 14.0 (8.5 – 20.9) respectively, in summer was 1.8 (0.6 – 6.8) nmol/L, 14.1 (6.0 – 20.6) and 10.9 (7.8 – 38.8) respectively and in autumn was 3.2 (0.9 – 6.9) nmol/L, 13.0 (5.7 – 23.8) and 19.6 (7.2 – 30.6) respectively.

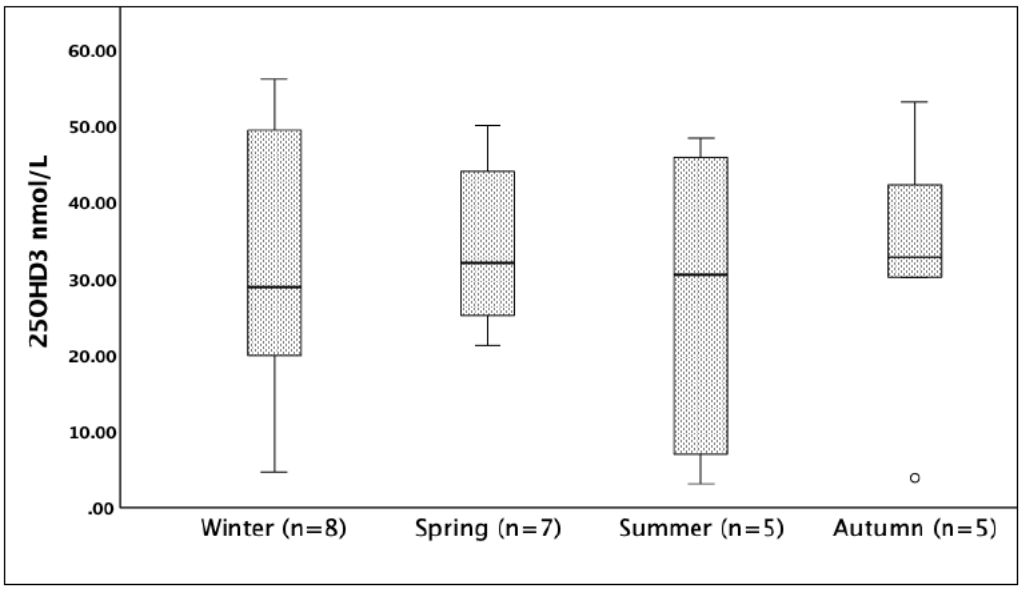


Figure 6.20: Seasonal distribution of serum $25OHD_3$ concentrations

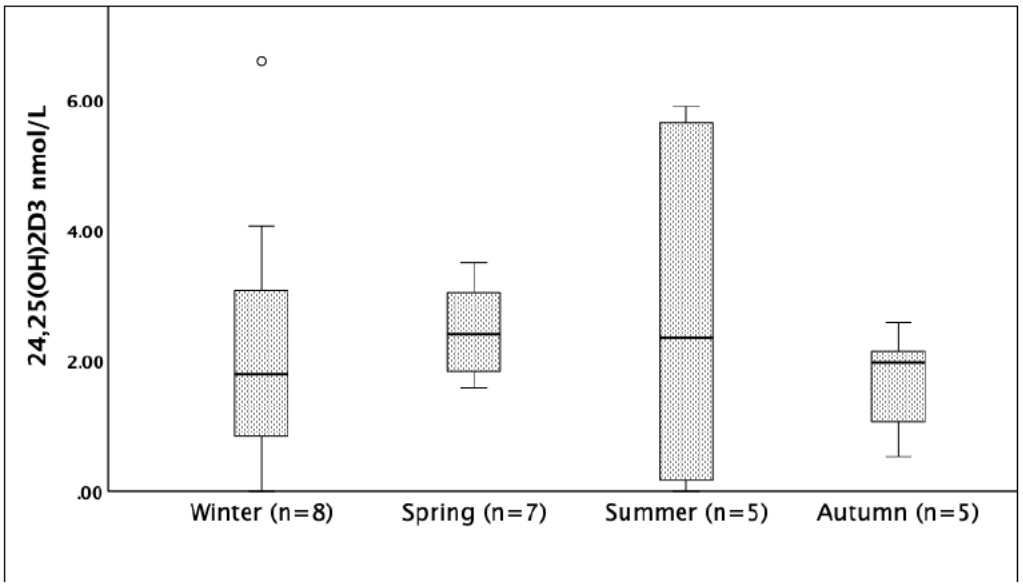


Figure 6.21: Seasonal distribution of serum $24,25(OH)_2D_3$ concentrations

Ethnicity:

The median (range) 25OHD3 in nmol/L in subjects of white, south Asian and black ethnic origin was 32.4 (7.0 – 56.2), 4.6 (3.1 – 44.5) and 32.4 (21.2 – 43.6) respectively **Figure 6.22** . The median (range) 24,25(OH)2D3 in nmol/L in subjects of white, south Asian and black ethnic origin was 2.0 (0.18 – 6.6), 0.5 (<0.15 – 3.2) and 2.5 (1.6 – 3.5) respectively **Figure 6.23.**

The median (range) 3-epi-25OHD3, percentage of 25OHD3 as 3-epi-25OHD3 and 25OHD3:24,25(OH)2D3 ratio in subjects of white ethnic origin was 4.8 (1.4 – 9.7) nmol/L, 13.5 (5.7 – 37.0) and 16.2 (7.0 – 38.8) respectively; south Asian origin was 0.92 (0.4 – 6.4) nmol/L, 13.9 (7.6 – 23.8) and 14.0 (7.2 – 19.6) respectively and black origin was 4.0 (3.8 – 4.2) nmol/L, 14.1 (8.6 – 19.6) and 12.9 (12.4 – 13.4) respectively.

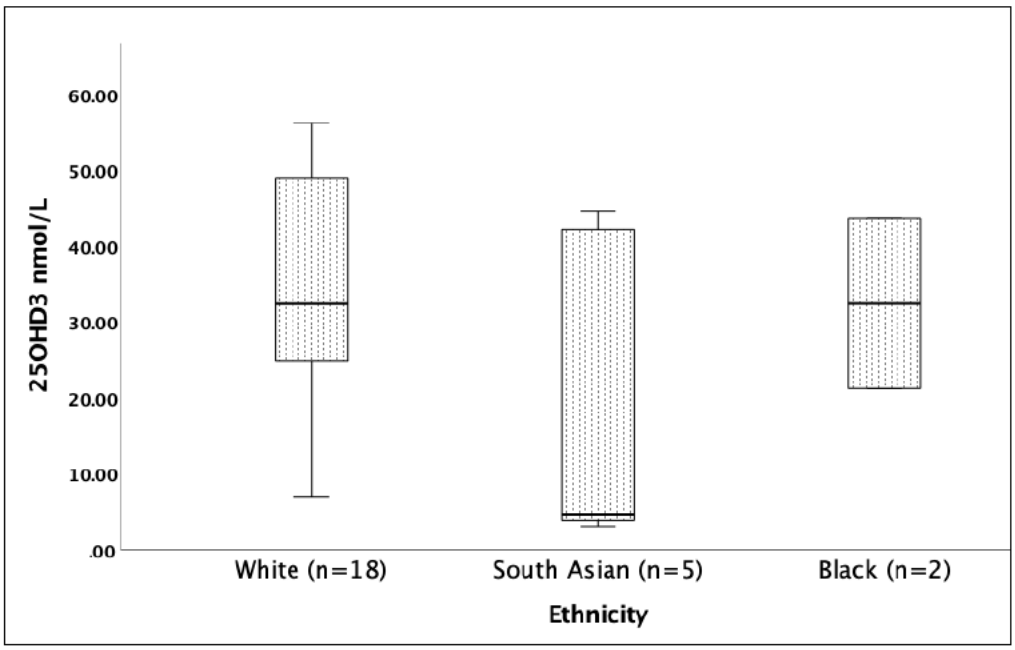


Figure 6.22: Distribution of serum $25OHD_3$ concentration based on ethnicity.

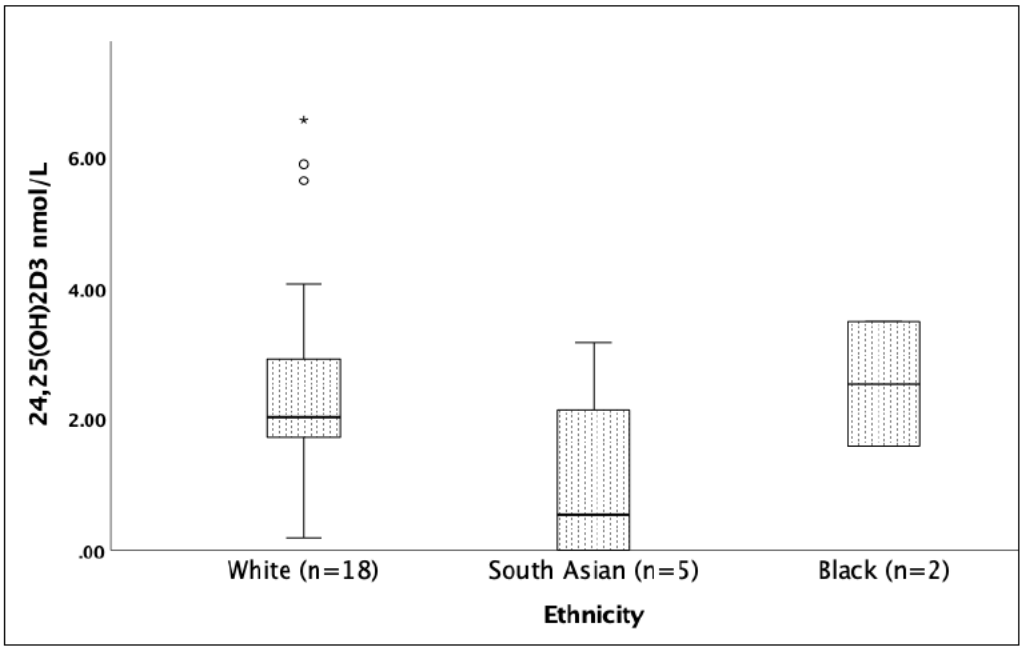


Figure 6.23: Distribution of serum 24,25$(OH)_2D_3$ based on ethnicity.

6.6.4 Growth plate histology

6.6.4.1 Subject demographics

Growth plate histology was available on 30 subjects. The mean (SD) age of the cohort was 135 (159) days and the median (range) was 75 (0-545) days. The proportion of males and females was 56.7% (n=17/30) and 43.3% (n=13/30) respectively. The majority were of white ethnic background 73.3% (n=22/30), 20% (n=6/30) were of south Asian background and 6.7% (n=2/30) of black background.

The mode of feeding was exclusively formula in 66.7% (n=20/30), exclusively breast feed in 6.7% (n=2/30), mixed breast and formula feed in 3.4% (n=1/30). Seven subjects (23.4%) were weaned onto solids, of whom five were fully weaned and two remained on additional formula feeds.

6.6.4.2 Qualitative histology

Histological signs of rickets were noted in 70.0% of the subjects (n=21/30). An example slide from case 20 is presented to demonstrate normal growth plate with distinction between the different zones and columnar arrangement of hypertrophic chondrocytes **(Figure 6.24)**. An example slide from subject 24 demonstrating mild changes of rickets with loss of columnar arrangement of hypertrophic chondrocytes is presented in **Figure 6.25**. The rest of the 20 of 21 subjects had ≥ 3 features associated with rickets at the growth plate as detailed in **Table 6.8**. An example from case 8 is presented to demonstrate all six changes of rickets. **Figure 6.26** in x4 magnification shows the five histological changes at the growth plate and **Figure 6.27** demonstrates the bulbous expansion in x1 magnification. The histological findings of

individual subjects are presented in **Table 6.8**. The proportion of subjects demonstrating changes of rickets based on season and ethnicity are presented in **Figure 6.28 and Figure 6.29** respectively.

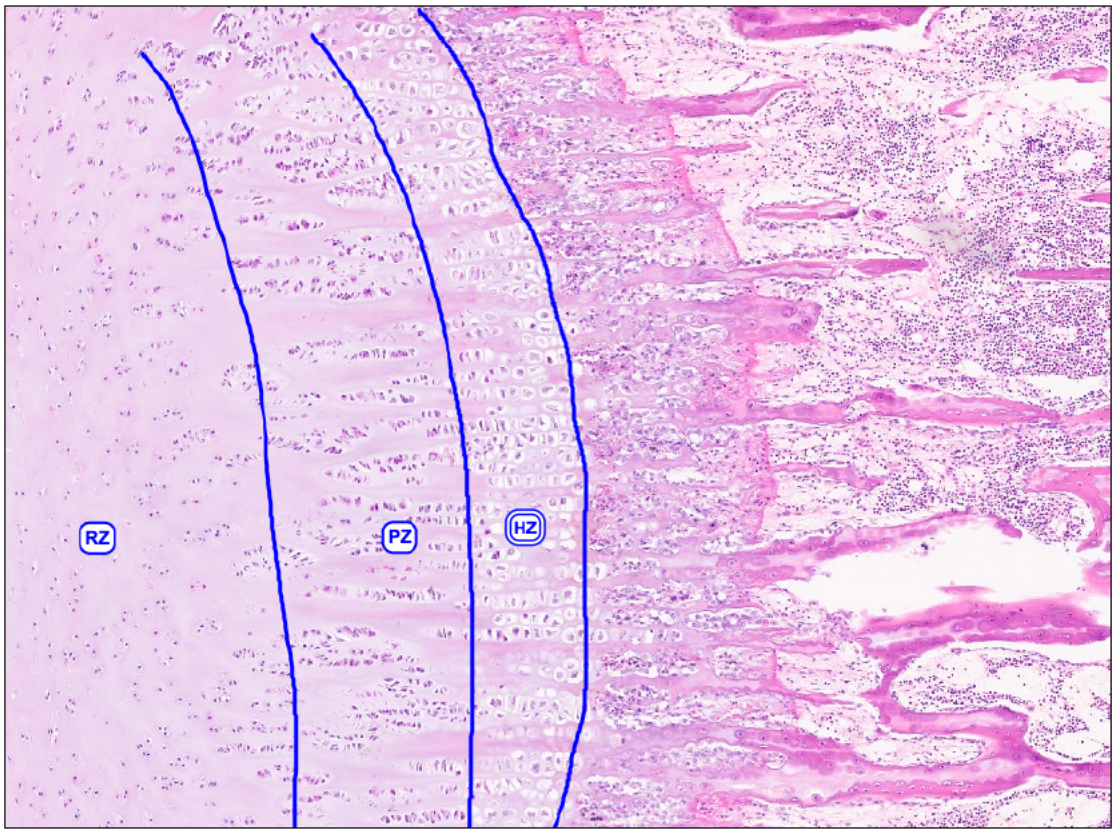


Figure 6.24: Normal growth plate histology

Illustrates H&E stained (x4 magnification) growth plate of subject 20 demonstrating normal growth plate with good distinction between zones such as an imaginary line can be drawn across the three zones, hypertrophic zone (HZ), proliferative zone (PZ) and resting zone (RZ). The hypertrophic chondrocytes are arranged in distinct columns.

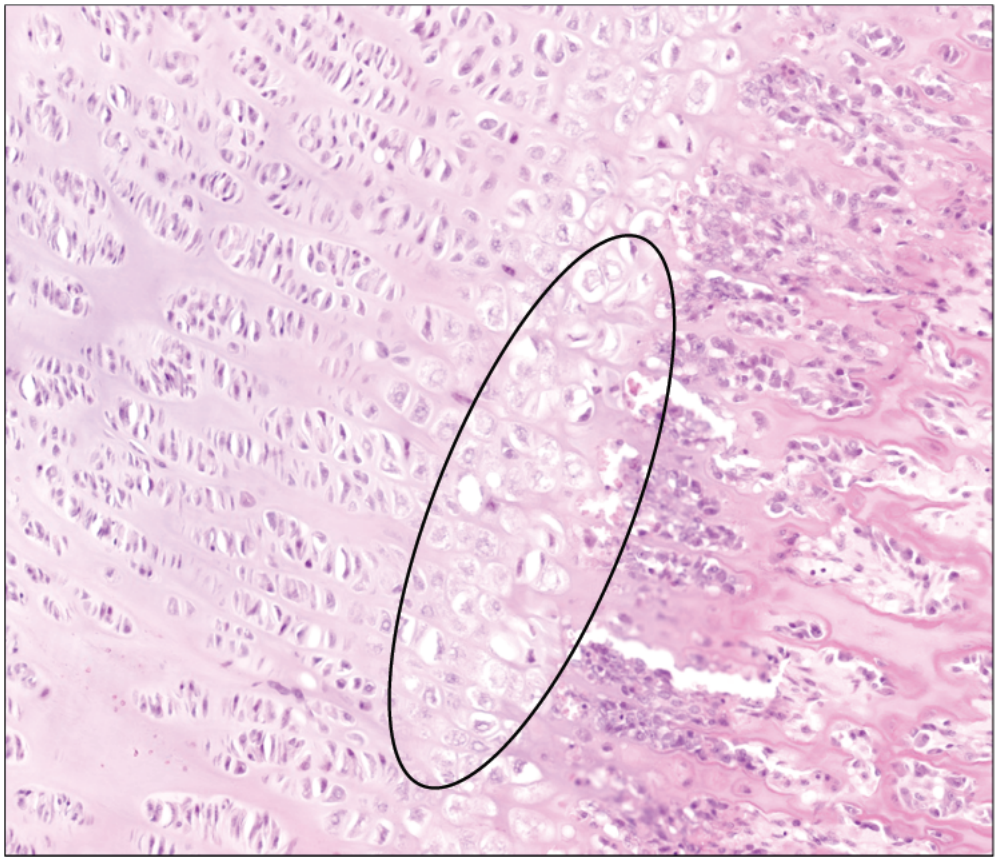


Figure 6.25: Mild-moderate changes of rickets on growth plate histology

Illustrates H&E stained (x10 magnification) growth plate of subject 24 demonstrating loss of columnar arrangement (oval area) of hypertrophic chondrocytes. The distinction between various zones is still maintained.

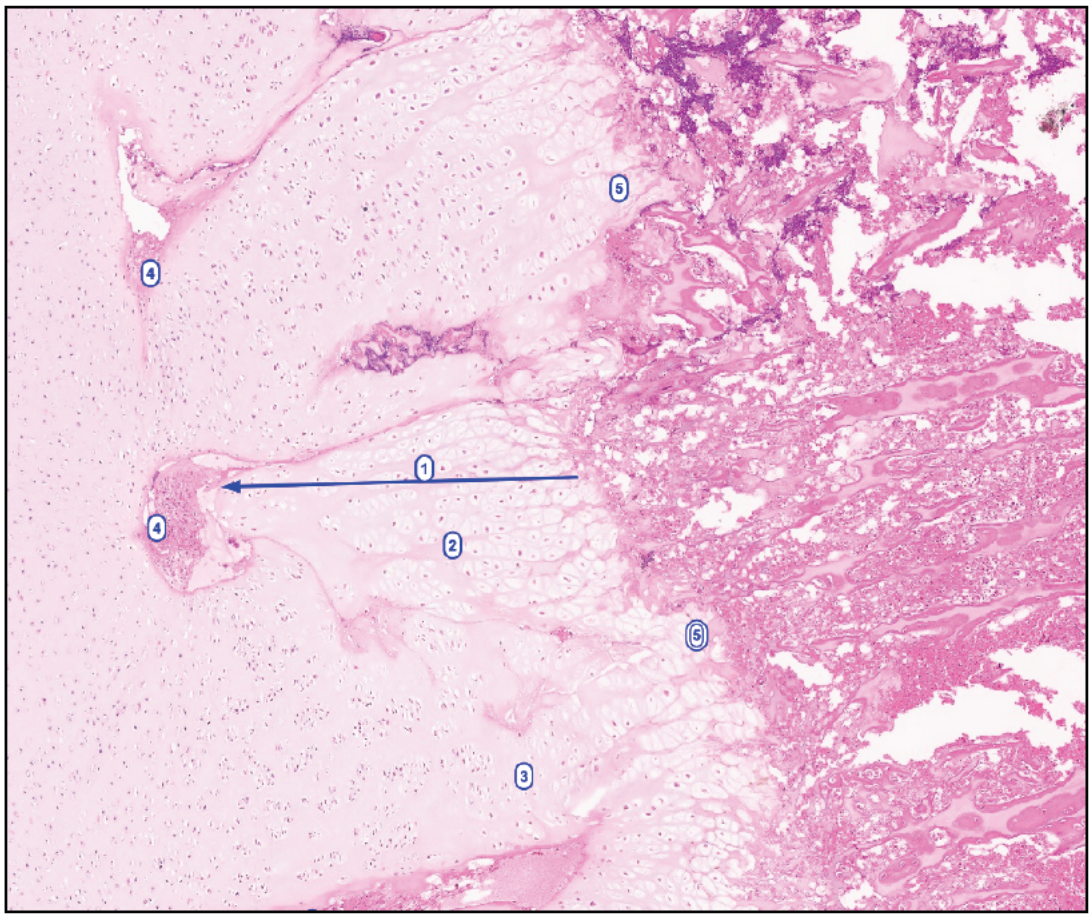


Figure 6.26: Changes of severe rickets on qualitative growth plate histology

Illustrates H&E stained (x4 magnification) growth plate histology of subject 8 demonstrating significant histological changes of rickets. Arrow demonstrates the increased height of hypertrophic zone and 1= expansion of hypertrophic zone, 2= disarrayed hypertrophic chondrocytes, 3= loss of distinction between hypertrophic and proliferative zones, 4= blood vessels penetrating the chondrocytes, 5= tongues of hypertrophic chondrocytes extending into primary spongiosa.

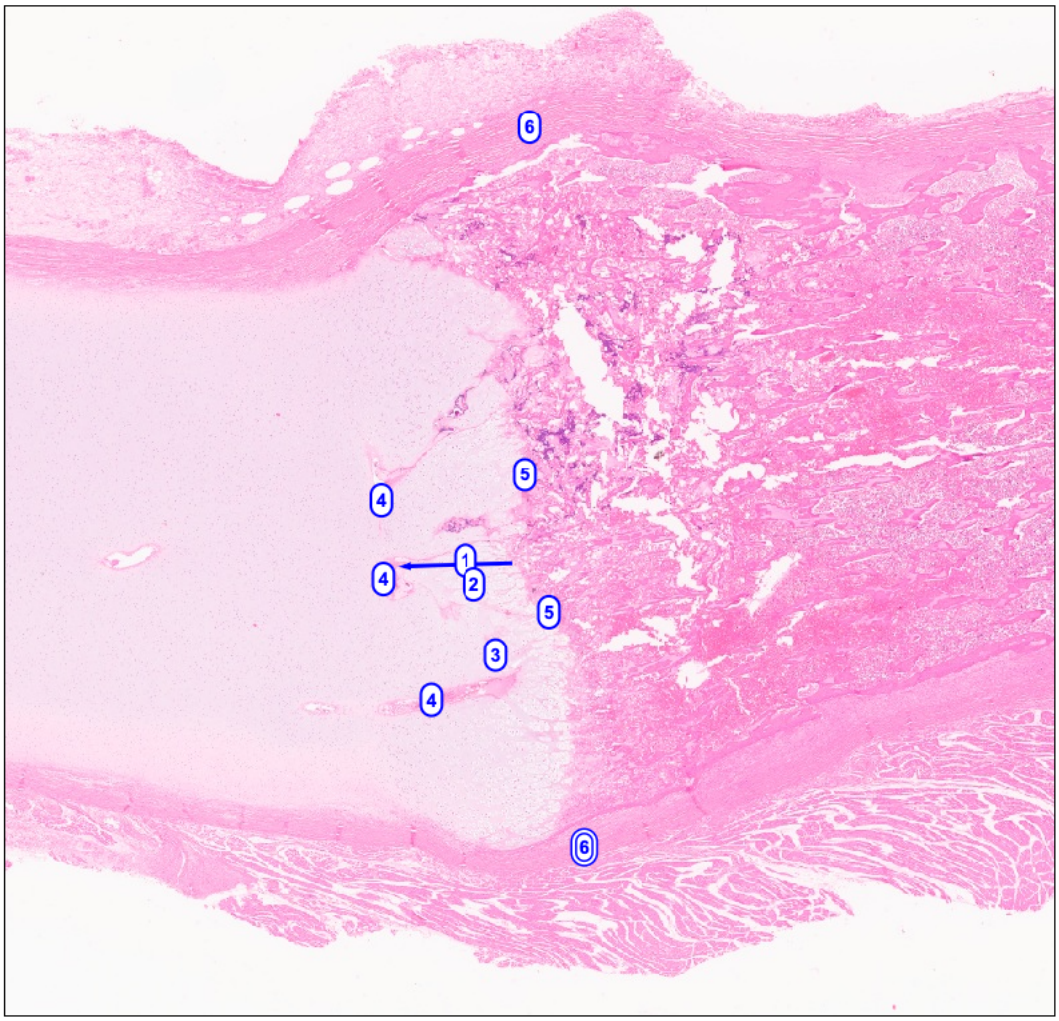


Figure 6.27: Changes of severe rickets on qualitative growth plate histology demonstrating bulbous expansion of the costo-chondral junction

Illustrates H&E stained (x1 magnification) growth plate histology of subject 8 demonstrating significant histological changes of rickets. Arrow demonstrates height of hypertrophic zone and 1= expansion of hypertrophic zone, 2= disarrayed hypertrophic chondrocytes, 3= loss of distinction between hypertrophic and proliferative zones, 4= blood vessels penetrating the chondrocytes, 5= tongues of hypertrophic chondrocytes extending into primary spongiosa and 6= bulbous expansion of the costo-chondral junction.

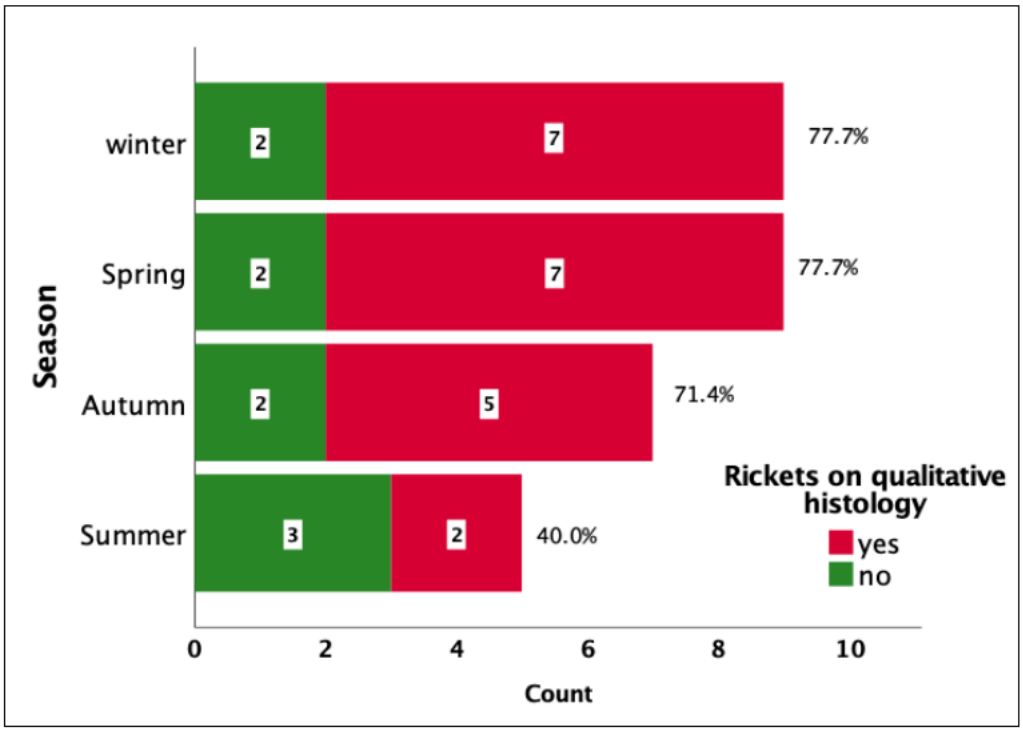


Figure 6.28: Proportion of subjects with and without histological changes of rickets on qualitative histology based on the season of sample collection.

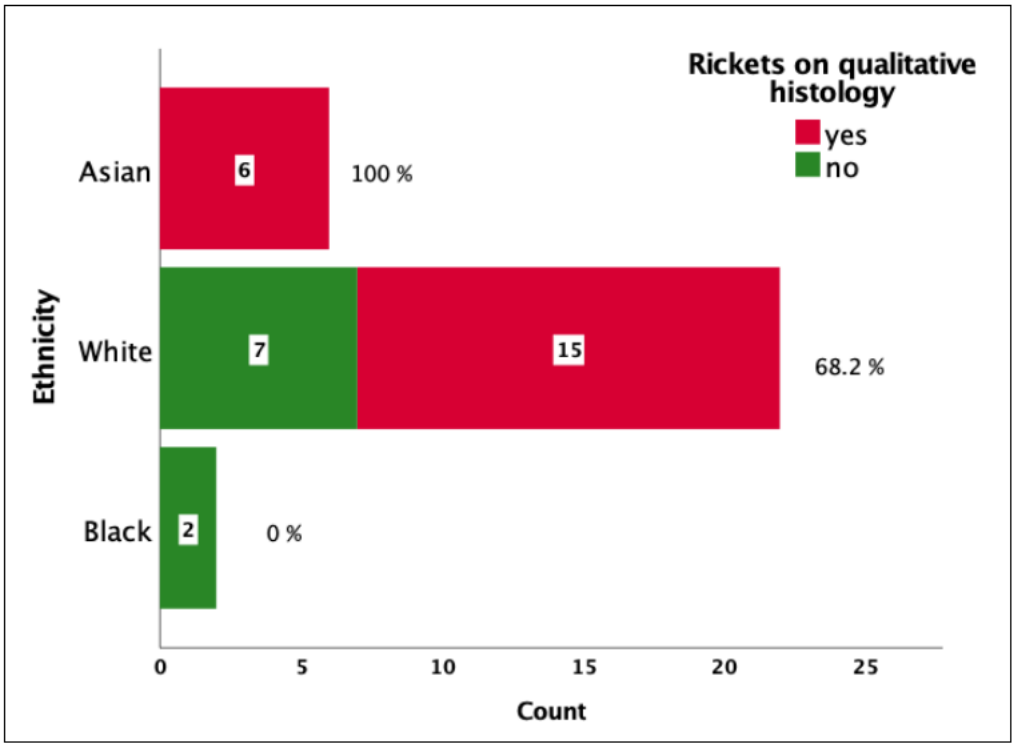


Figure 6.29: Proportion of subjects with and without histological changes of rickets on qualitative histology based on ethnicity.

6.6.4.3 Comparison of subjects with and without qualitative changes of rickets on growth plate histology

Subjects with qualitative changes of rickets were significantly younger than subjects without rickets [mean 54.7 vs 321.8 days, $p<0.001$]. Comparison of the proportion of gender, season of sample collection, co-morbidity and ethnicity between the groups with and without histological rickets are detailed in **Table 6.10** below.

Table 6.10: Characteristics of groups with and without histological evidence of rickets on qualitative histology

	Rickets on qualitative histology (n=21)	**No rickets on qualitative histology (n=9)**	
Age in days Mean, SD Median, range	 54.7, 55.3 35, 0 - 241	 321.8, 165.7 318, 84 - 545	p<0.001
Gender	Male 61.9% (n=13) Female 38.1% (n=8)	Male 44.4% (n=4) Female 55.6% (n=5)	
Season	Spring 33.3% (n=7) Summer 9.5% (n=2) Autumn 23.8% (n=5) Winter 33.3% (n=7)	Spring 22.2% (n=2) Summer 33.3% (n=3) Autumn 22.2% (n=2) Winter 22.2% (n=2)	
Co-morbidities	Yes 23.8% (n=5) No 76.2% (n=16)	Yes 10% (n=1) No 40% (n=4)	
Ethnicity	White 75% (n=15) S Asian 25% (n=5) Black 0% (n=0)	White 77.8% (n=7) S Asian 0% (n=0) Black 22.2% (n=2)	
Length z score (mean, SD)	0.5, 1.8	0.4, 1.1	p>0.05
Weight z score (mean, SD)	-0.2, 1.6	-0.2, 1.2	p>0.05

6.6.4.4 Quantitative histology

The height of the hypertrophic zone for individual subjects are reported in **Table 6.8** above. The mean (SD) height of the hypertrophic zone was 0.625 (0.415) mm and the median (range) was 0.530 (0.152 - 1.762) mm. In subjects with qualitative changes of rickets (n=21/30) the mean (SD) height was 0.794 (0.385) mm and median (range) was 0.760 (0.340 - 1.762) mm. In subjects without qualitative changes of rickets (n=9/30) the mean (SD) height was 0.230 (0.069) mm and median (range) was 0.251 (0.152 - 0.342) mm. The height of the hypertrophic zone was significantly increased in subjects with rickets when compared to those without ($p<0.001$). There was a significant negative correlation between age and height of the hypertrophic zone ($r= - 0.75$, $p<0.001$) **Figure 6.30**.

The normative reference data report a gradual reduction in the height of the hypertrophic zone from a maximum of 0.370 mm at 11 days to 0.104 mm at one year of age as illustrated in **Figure 6.7**, but specific cut off values for different age groups are not reported [135]. Therefore, a height of 0.370 mm for the hypertrophic zone was used as the threshold for all infants and 0.104 for those older than 1 year (365 days). When using a generous cut-off for all infants, no additional subjects would be classed as having rickets **(Figure 6.30)**. Using a cut off height of 0.104 mm for the hypertrophic zone for subjects over one year of age (> 365 days), four additional subjects were classed as having an expansion of the hypertrophic zone (feature 1 of qualitative changes of rickets) as presented in **Figure 6.31**.

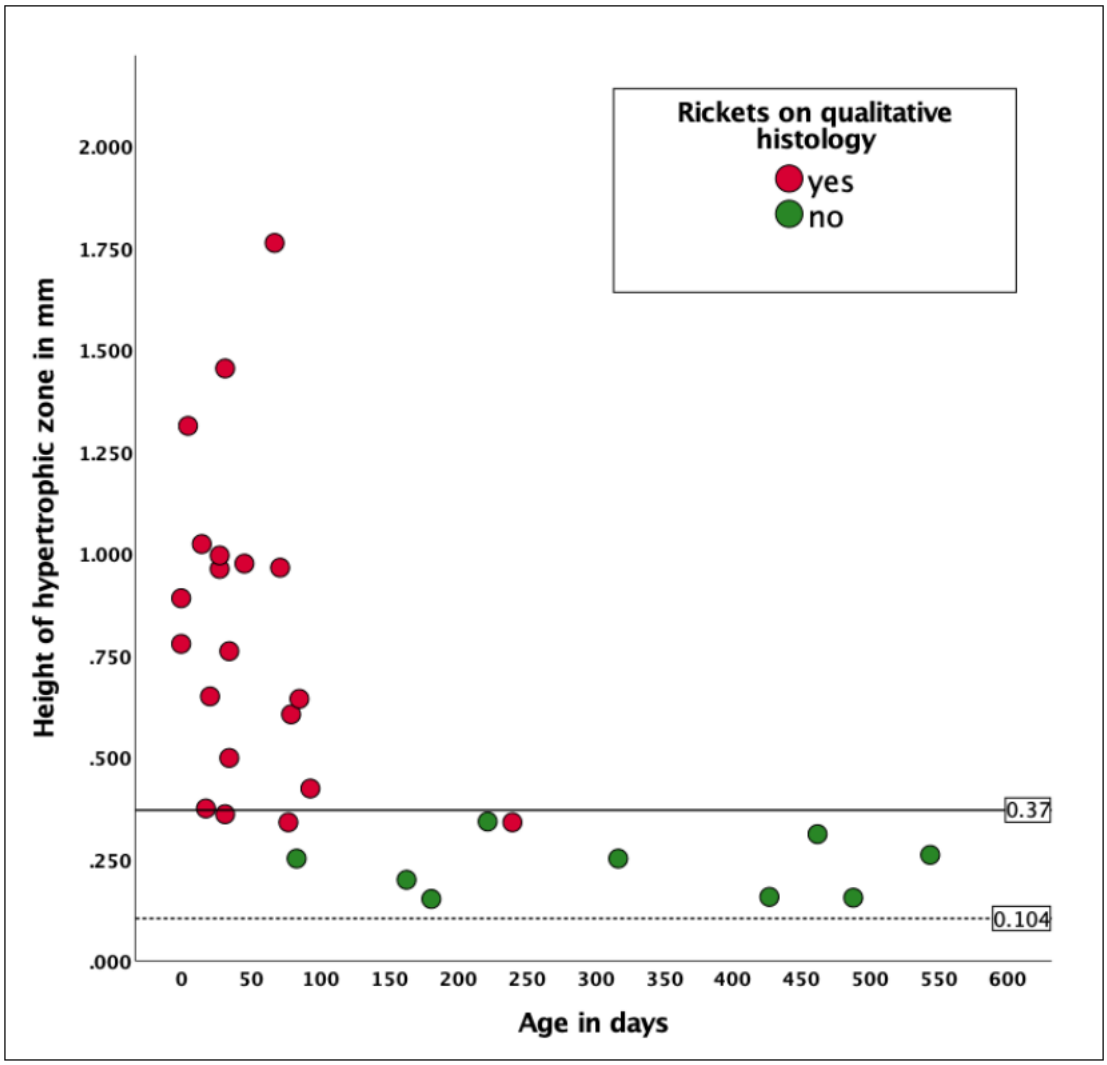


Figure 6.30: Height of the hypertrophic zone vs age in days in subjects with and without qualitative features of rickets at the growth plate

The reference lines indicate cut offs from normative data [135]. The height of the hypertrophic zone reduces with age. The solid line represents the greatest height of the hypertrophic zone recorded at 11 days of age (0.370 mm) and the broken line indicates the height at one year of age (0.104 mm).

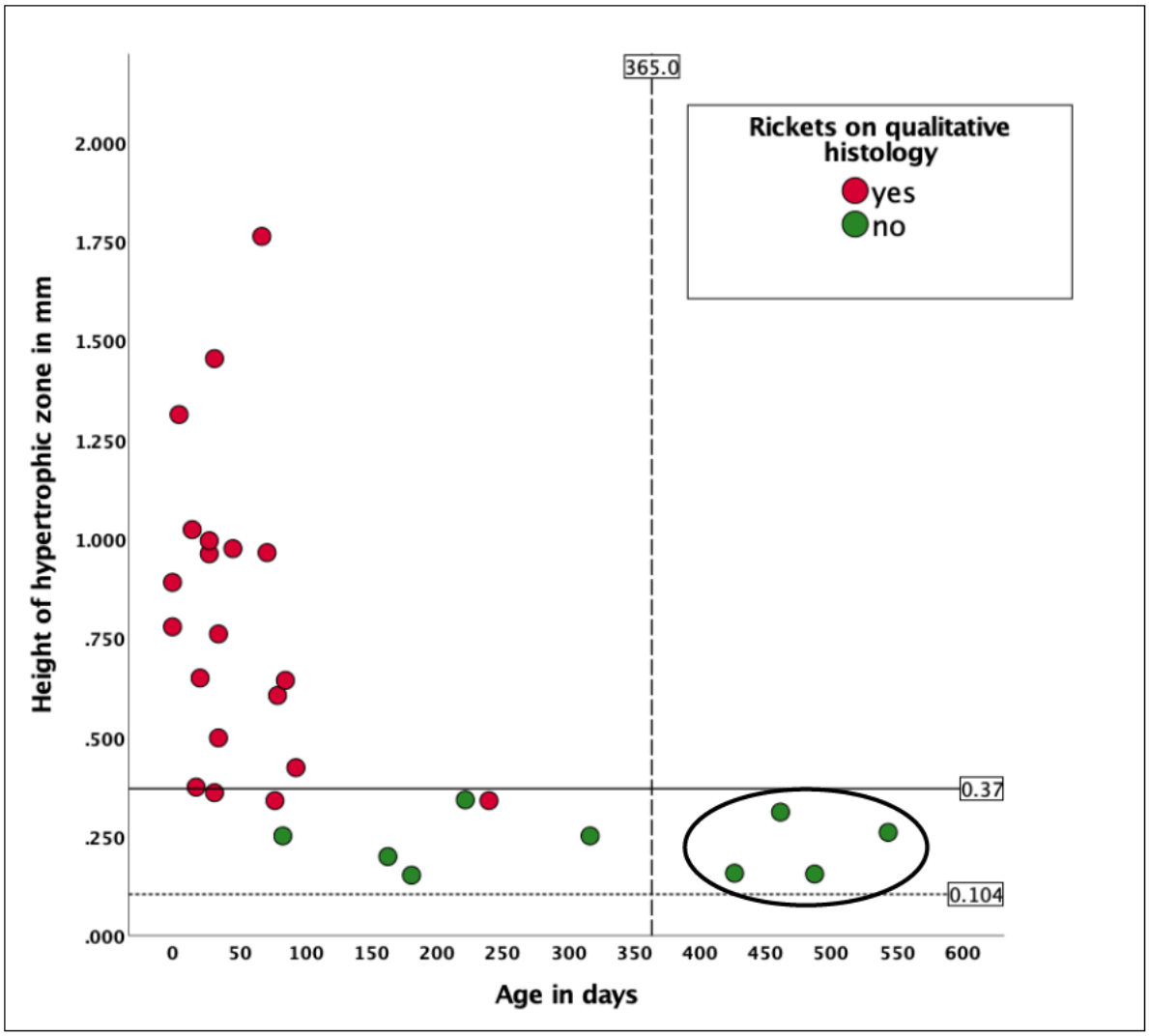


Figure 6.31: Height of the hypertrophic zone vs age in days in subjects with and without qualitative features of rickets at the growth plate

The reference lines indicate cut offs from normative data [135]. The height of the hypertrophic zone reduces with age. The horizontal solid line represents the greatest height of the hypertrophic zone recorded at 11 days of age (0.370 mm) and the horizontal broken line indicates the height at one year of age (0.104 mm). The vertical broken line indicates the age cut off (365 days) beyond which the height of hypertrophic zone > 0.140 mm would be classed as elongation which is a feature of rickets. Four additional subjects (represented by the black oval mark) would be classed as having mild-moderate rickets if the height of hypertrophic zone is used to define rickets.

6.6.4.5 25OHD and metabolites in subjects with and without rickets on qualitative histology

25OHD, its metabolites and ratios in subjects with and without changes of rickets on qualitative histology are presented in the **Table 6.11** below.

Table 6.11: 25OHD and vitamin D metabolites in children with and without qualitative changes of rickets on growth plate histology

	Rickets on qualitative histology (n=16)	**No rickets on qualitative histology (n=8)**	
Total 25OHD nmol/L Mean (SD) Median (range)	 29.5 (18.7) 28.6 (3.4 – 56.9)	 38.2 (10.0) 39.4 (21.2 – 49.0)	 p=0.3
$25OHD_3$ nmol/L Mean (SD) Median (range)	 29.3 (18.7) 28.7 (3.1 – 56.2)	 37.9 (10.2) 39.1 (21.2 – 49.0)	 p=0.3
$24,25(OH)_2D_3$ nmol/L Mean (SD) Median (range)	 1.7 (1.2) 1.8 (<0.15 - 4.0)	 3.6 (2.0) 2.9 (1.6 – 6.6)	 **p=0.04**
3-epi-$25OHD_3$ nmol/L Mean (SD) Median (range)	 4.6 (2.7) 5.0 (0.44 – 9.7)	 3.7 (1.6) 3.9 (1.7 – 6.8)	 p=0.39
Percentage of $25OHD_3$ as 3-epi-$25OHD_3$ Mean (SD) Median (range)	 17.5 (7.2) 16.9 (7.6 – 37.0)	 10.0 (4.7) 8.9 (5.7 – 19.6)	 **p=0.01**
Ratio $25OHD_3$:$24,25(OH)_2D_3$ Mean (SD) Median (range)	 19.4 (9.2) 18.3 (7.2 – 38.8)	 12.0 (4.0) 12.8 (7.0 – 18.5)	 **p=0.03**

A scatterplot demonstrating daily dietary calcium intake and 25OHD$_3$ levels in subjects with and without rickets on qualitative histology is illustrated in **Figure 6.32**. The size of the dots indicates the age (in increasing order) and the colour of the dot indicates the presence (red) or absence (green) of rickets.

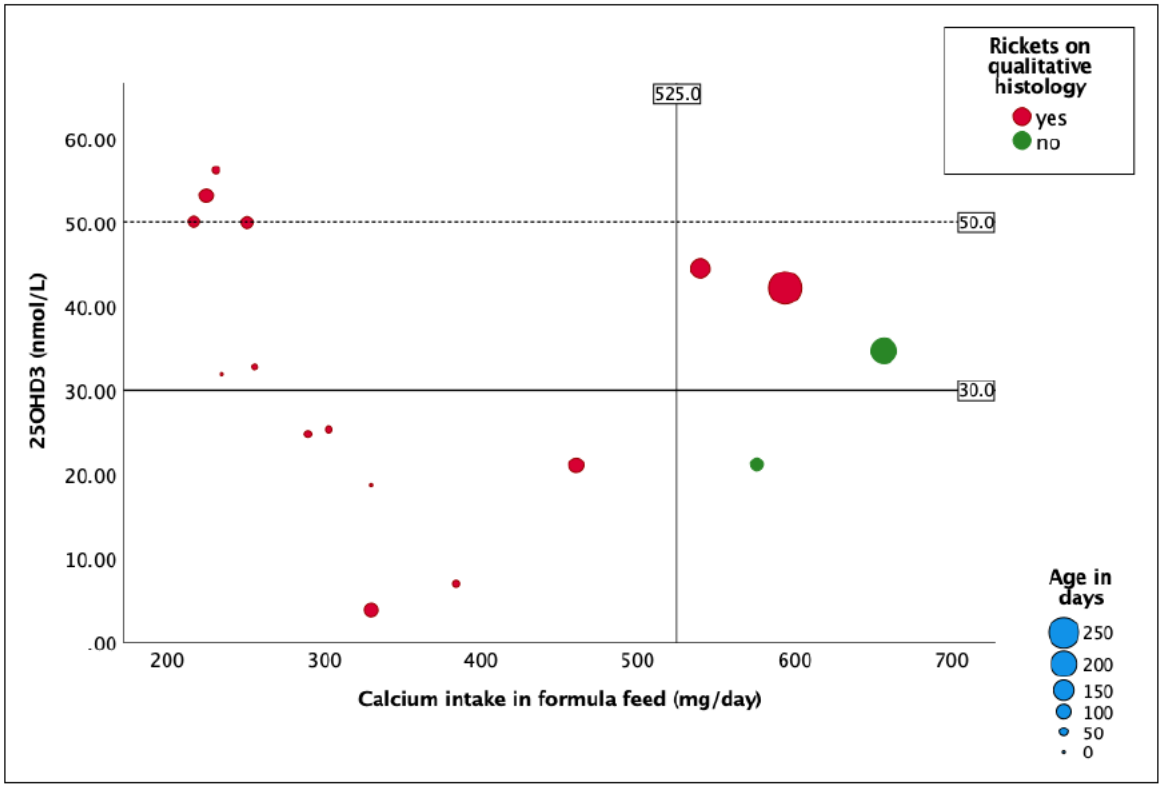


Figure 6.32: Scatter plot demonstrating relation between calcium intake and 25OHD$_3$ levels in subjects with and without histological rickets.

6.6.5 Radiology

6.6.5.1 Subject demographics

The subject demographics are as presented in **Section 6.6.2**. Radiographs were available on all subjects. All except one subject had a full skeletal survey. One subject who died of non-suspicious causes in hospital had pre-mortem chest X-rays and had a further babygram preceding post-mortem examination.

6.6.5.2 Radiological findings

The radiographs were reported to be normal by all three reviewers in 44.5% (n=15/33) of the subjects (number 1, 3, 4, 5, 8, 11, 13, 14, 17, 19, 20, 27, 29, 31 and 33 in **Table 6.8**). The radiological findings for the remaining subjects (54.5%, n=18/33) with any positive findings and/or discrepancy between different reviewers are detailed in **Table 6.12** below. **Figure 6.33** demonstrates the comparison or a normal wrist radiograph (from subject number 1) and a radiograph with cupping of the ulna with metaphyseal lucency (subject number 9). No subjects were reported to have any radiological signs of rickets on the formal Coroner's radiology reports. Only one subject (number 24) was considered to have significant changes of rickets (RSS of 2/10) by two of the three reviewers (Reviewer 2 and 3) **(Figure 6.34).** An additional eight subjects **(Table 6.12)** were found to have varying changes of the ulna by reviewer 2 and 3.

Table 6.12: Details of radiology findings in subjects with positive findings and/or discrepancy between reviewers

	Formal report by designated radiologist (Reviewer 1)	**Lead researcher review (S Uday) (Reviewer 2)**	**Independent reviewer (Reviewer 3)**			**Any abnormalities of the ulna**
		Review for changes of rickets only	**RSS if rickets present**	**Rib fractures* R= right L= left**	**Comments**	
2	Normal	Mild cupping, splaying, fraying of ulna	0.5	no	Ulna metaphyseal lucency	yes
6	Multiple growth arrest and recovery lines on distal tibia and fibula	Mild fraying of ulna		no	Subjective low bone density	no
7	Normal	Mild cupping of ulna		no	Apparent cupping of ulna due to position- within normal appearance	yes
9	Rib fractures (R 3-5, L5-6)	Mild cupping of ulna		On CT/X-ray normal	Ulna metaphyseal lucency - within normal limits	yes
10	Rib fractures 4-6 bilateral	Fraying of ulna	0.5	R- 4, 5, 6 L- 4, 5	Ulna metaphyseal lucency	yes
12	Rib fractures L2-7, R 2-5	Normal		L- 2, 6 R- 4, 5		no
15	Rib fracture, pneumothorax	Mild fraying of ulna		no	Relative osteopaenia	no
16	Normal	Ulna cupping		no	Ulna cupping but in keeping with age	yes

18	Rib fracture,	Normal		R- 2 to 6 L- 2 to 6		no
21	Irregular rib-histology advised, otherwise normal	Normal		R- 2 to 4 L- 2 to 4		no
22	Normal	Normal		L- 3 to 6		no
23	Fragment of bone adjacent to 8th rib equivocal for fracture vs normal	Mild fraying of ulna		no	Coarse trabecular pattern but difficult to assess due to poor quality images	yes
24	Normal	Fraying and splaying of ulna and fibula.	2	no	Cupping and lucency of distal metaphysis of ulna and fibula	yes
25	Normal	Mild fraying and cupping of ulna		R- 2,3 L- 2 to 4	Ulna distal metaphysis changes – in keeping with normal limits	yes
26	Normal	Normal		R- 2 to 5 L- 2 to 5		no
28	Rib fracture, no rickets	Cupping of ulna		? L 3, 4 minimal displacement	Cupping of ulna – within normal limits	yes
30	Acute right 4th rib fracture, metaphyseal fracture at 2-4 weeks of age, physiological periosteal reaction	Normal		R- 4		no
32	Normal	Normal		R- 4, 5		no

*a positive history of cardio-pulmonary resuscitation involving chest compressions was noted in all cases of rib fractures.

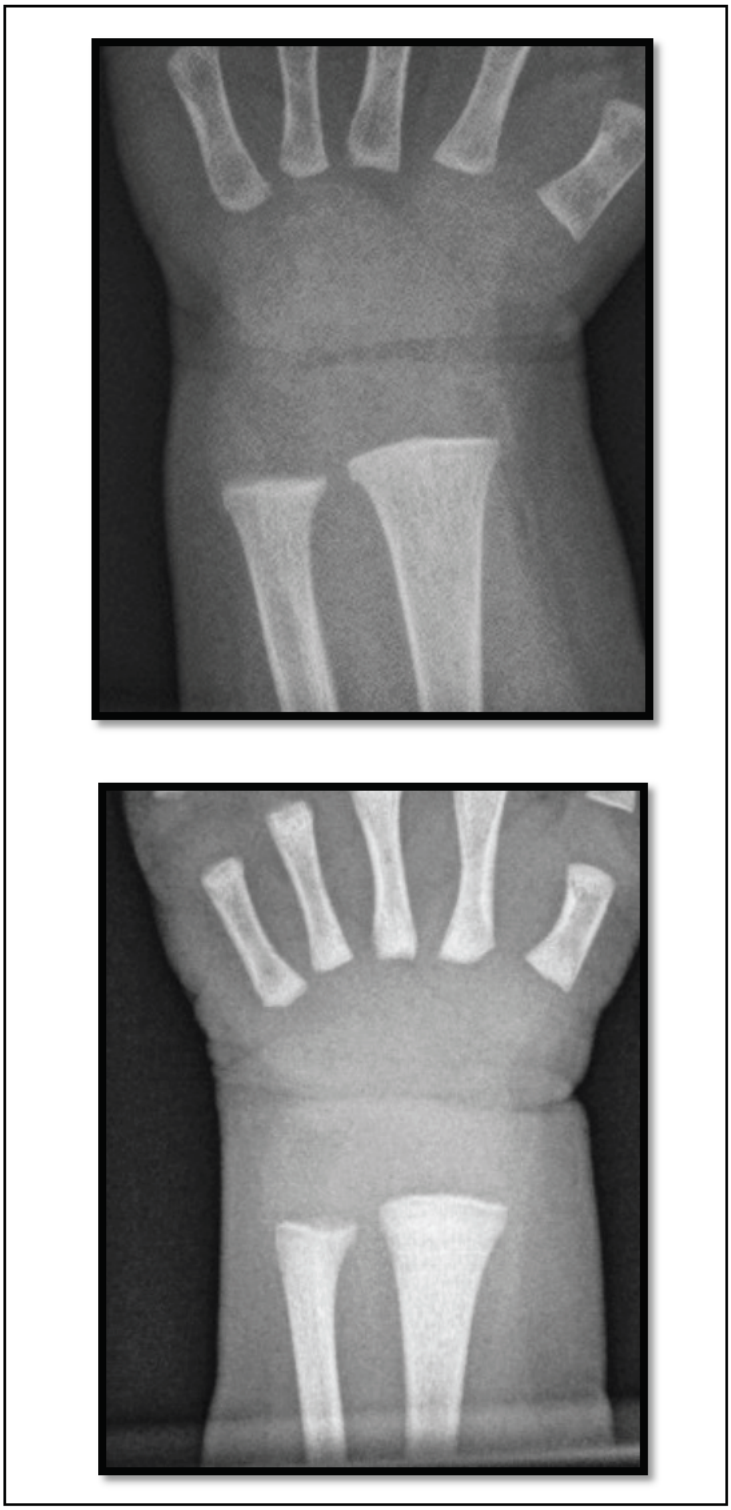


Figure 6.33: Radiographs demonstrating normal ulna (above) and cupping with some metaphyseal lucency (below)

The radiograph from subject number 1 (above) shows normal ulna and radius. The radiograph from subject number 9 (below) shows normal radius but the ulna was reported to demonstrate mild cupping by reviewer two and metaphyseal lucency by reviewer three.

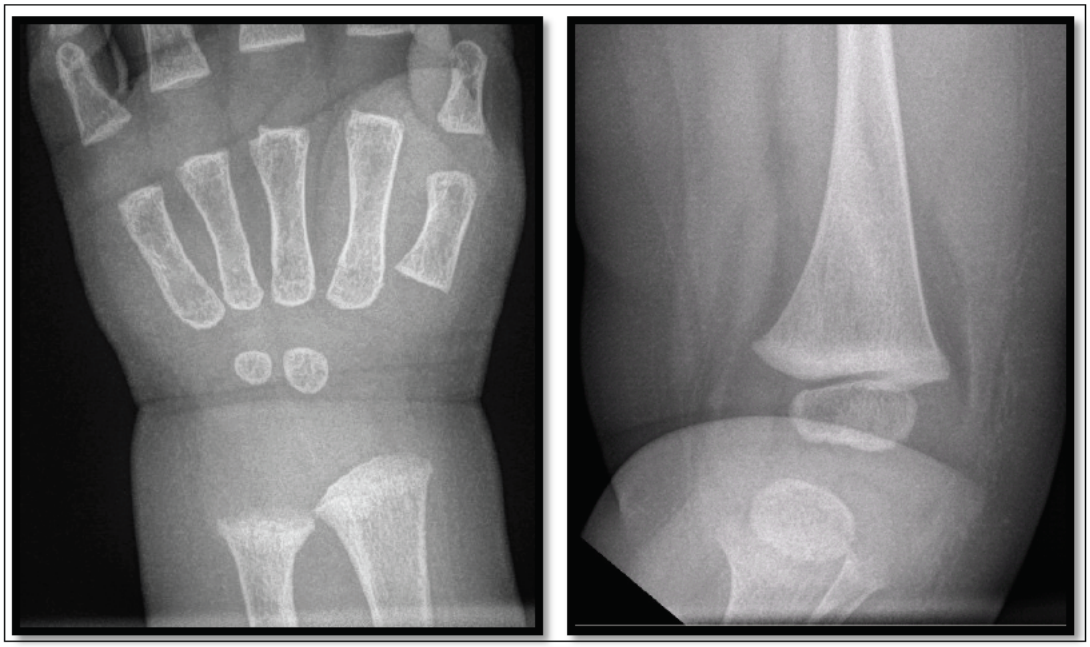


Figure 6.34: Wrist (left) and knee (right) radiographs demonstrating changes of rickets (cupping, fraying and splaying) at the ulna and fibula respectively.

Radiographs of subject number 24 demonstrating changes of rickets at the wrist (left) and knee (right). Only the ulna at the wrist and fibula at the knee demonstrate changes consistent with rickets. The Rickets severity score was 2 (on a scale of 0-10) for the changes noted at the ulna. The fibula is not scored as part of the RSS, knee scores are allocated for changes at the femur and tibia only.

6.6.5.3 Association between radiological and histological findings

A Fisher's exact test identified a significant (p=0.03) association between the presence of any radiological abnormalities in the ulna (cupping, metaphyseal lucency, fraying, splaying) and rickets on qualitative histology of the growth plate (**Table 6.13**). There was no association between the presence of rib fractures and signs of rickets on the growth plate (p>0.05).

Table 6.13: Association between the presence of radiological abnormalities of the ulna and qualitative changes of rickets of the growth plate

		Ulna abnormalities		
		Yes	No	Total
Rickets on qualitative histology	Yes	9	12	21
	No	0	9	9
	Total	9	21	

Examples of radiographic changes identified at the ulna with corresponding histological changes of rickets at the growth plate are illustrated below in **Figure 6.35** (subject 16) and **Figure 6.36** (subject 10).



Figure 6.35: Radiological sign of cupping and corresponding histological finding at the growth plate

Radiograph (above left) of subject 16 demonstrating cupping. The corresponding histological H&E section of the subject's rib growth plate (x1 magnification above left and x4 magnification below) demonstrates concave cupping of the growth plate, disarray of hypertrophic chondrocytes and expansion of hypertrophic zone with loss of distinction between zones.

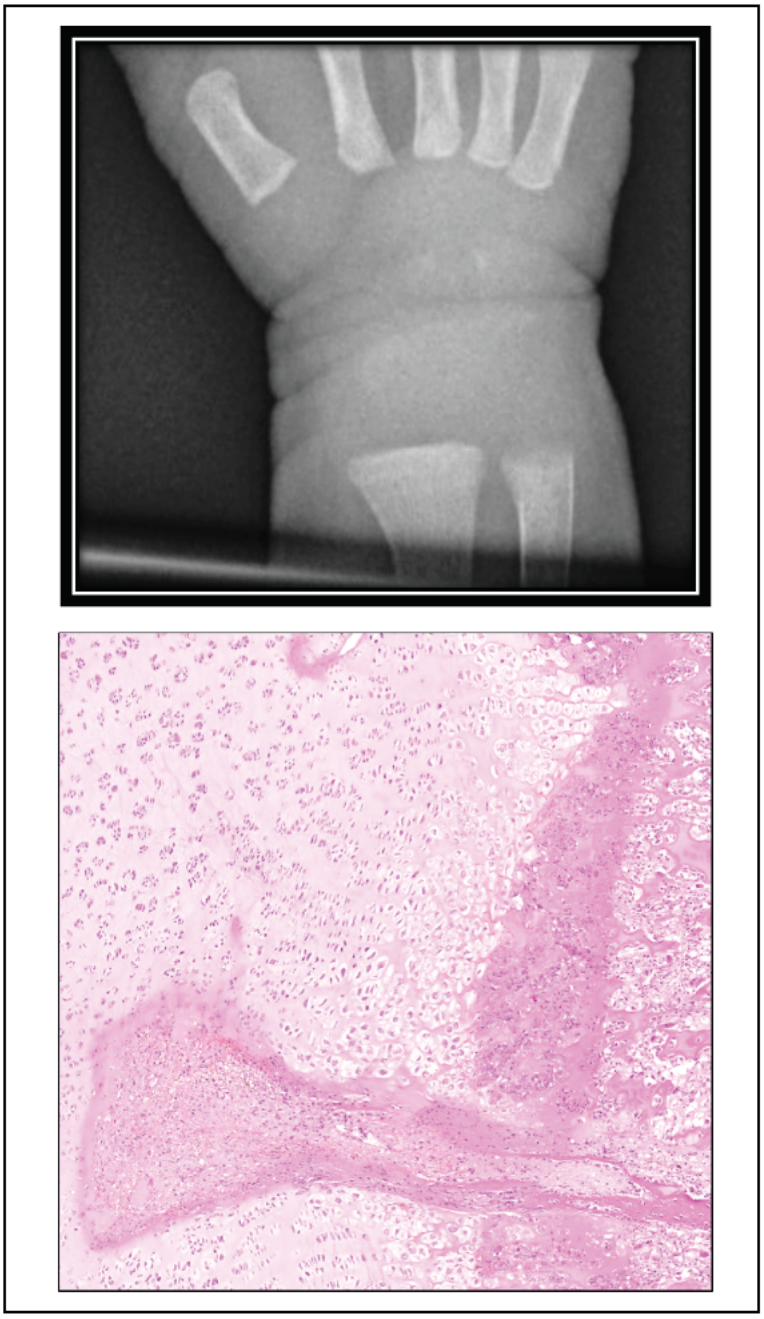


Figure 6.36: Subtle radiological signs (above) of rickets with corresponding histological findings at the rib growth plate (below).

Illustration of radiograph (above) from subject 10 showing mild fraying of ulna with metaphyseal lucency and cupping; histology (below) on H&E stained section of the growth plate (x 4 magnification) demonstrating classic features of rickets including disarray of hypertrophic chondrocytes with loss of columnar arrangement and distinction between hypertrophic and proliferative zones, penetrating blood vessel in the chondrocyte zone and tongues of hypertrophic chondrocytes extending into the primary spongiosa.

6.6.6 Histomorphometry

6.6.6.1 Subject demographics

Histomorphometry data from transiliac bone biopsies were available on 21 subjects (male=11). The mean (SD) age of the cohort was 131 (158) days and median (range) age was 72 (0-545) days. The majority were infants under one year of age (90.5%, n= 19/21), of whom 89.5% were under 6 months of age (n=17/19). Subjects of white ethnic origin were the majority at 71.4% (n=15/21), 24% (n=5/21) were of south Asian ethnicity and 5% (n=1/21) of mixed ethnic origin. The season distribution of sample collection was: 38% (n=8/21) in winter, 33% (n=7/21) in spring, 24% (n=5/21) in autumn and 5% (1/21) in summer. The majority were exclusively formula fed (66.7 %, n=14/21), one subject was on exclusive breast feeds and one on mixed breast and formula feeds. A total of five subjects (23.8%) were weaned, of whom four were fully weaned onto solids and one subject was reported to be on additional formula feeds.

6.6.6.2 Static histomorphometry

The mean (SD) Osteoid Surface/Bone Surface (OS/BS) was 28.5 (16.6) % and median (range) was 30.3 (1.5 - 57.0) %. The mean (SD) Osteoid Volume/Bone Volume (OV/BV) was 4.3 (2.9) % and the median (range) was 4.3 (0.09 - 10.6) %. The mean (SD) osteoid thickness (O.Th) was 4.5 (1.2) µm and the median (range) was 4.7 (2.0 – 6.5) µm.

Individual patient data for OS/BS **(Figure 6.37)**, OV/BV **(Figure 6.38)** and O.Th **(Figure 6.39)** in relation to age are presented in the scatter plots below. Age was positively correlated to OV/BV (r=0.47, p=0.03) and O.Th (r=0.46, p=0.03) but not OS/BS (r=0.32, p=0.16).

Subjects who had changes of rickets on qualitative histology are colour coded red and those without are coded green, the colour code black indicates patients where histology data was not available. Due to lack of histomorphometry reference data for children below 1.5 years of age the reference lines indicated in the graphs denote the mean values from normative data for apparently healthy children aged 1.5 - 6.9 years (n=10) [86].

In comparison to the above reference data [86] the OS/BS, OV/BV and O.Th were above the reference mean in 33.3% (n=7/21), 61.9% (n=13/21) and 14.2% (n=3/21) of the cohort respectively.

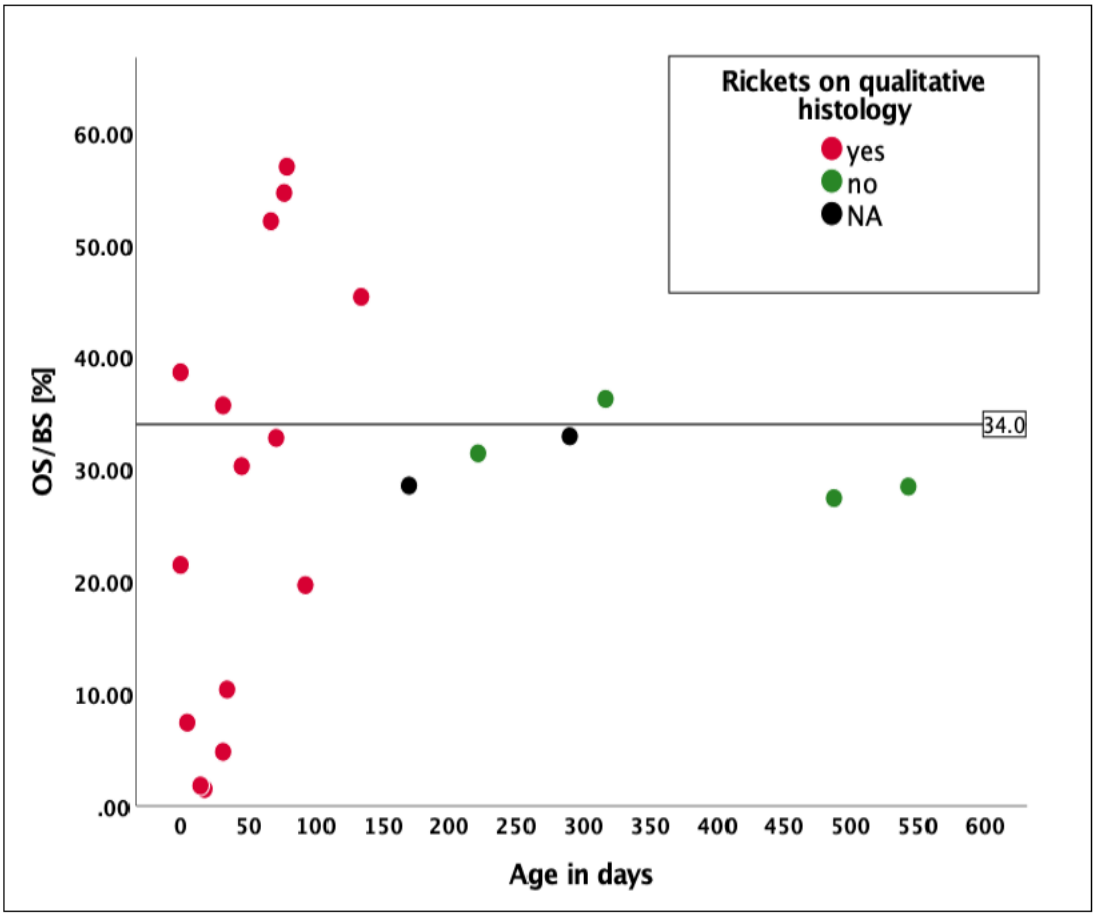


Figure 6.37: Scatterplot of age (in days) vs Osteoid Surface/ Bone Surface (in %).

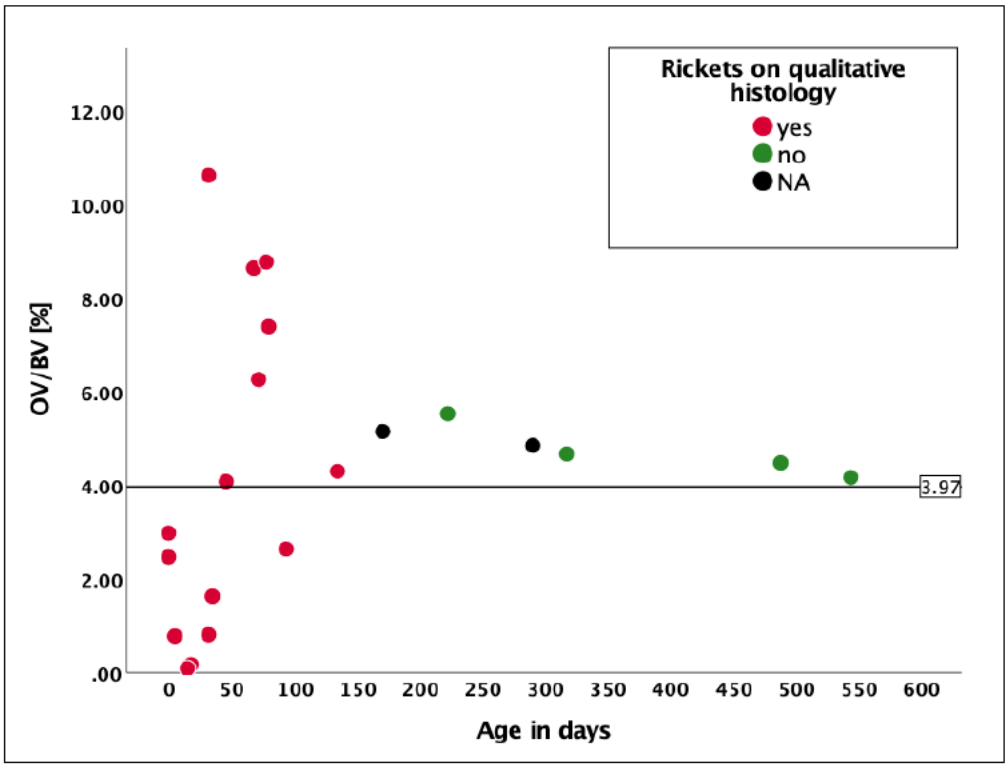


Figure 6.38: Scatter plot of age (in days) vs Osteoid Volume/ Bone Volume (in %)

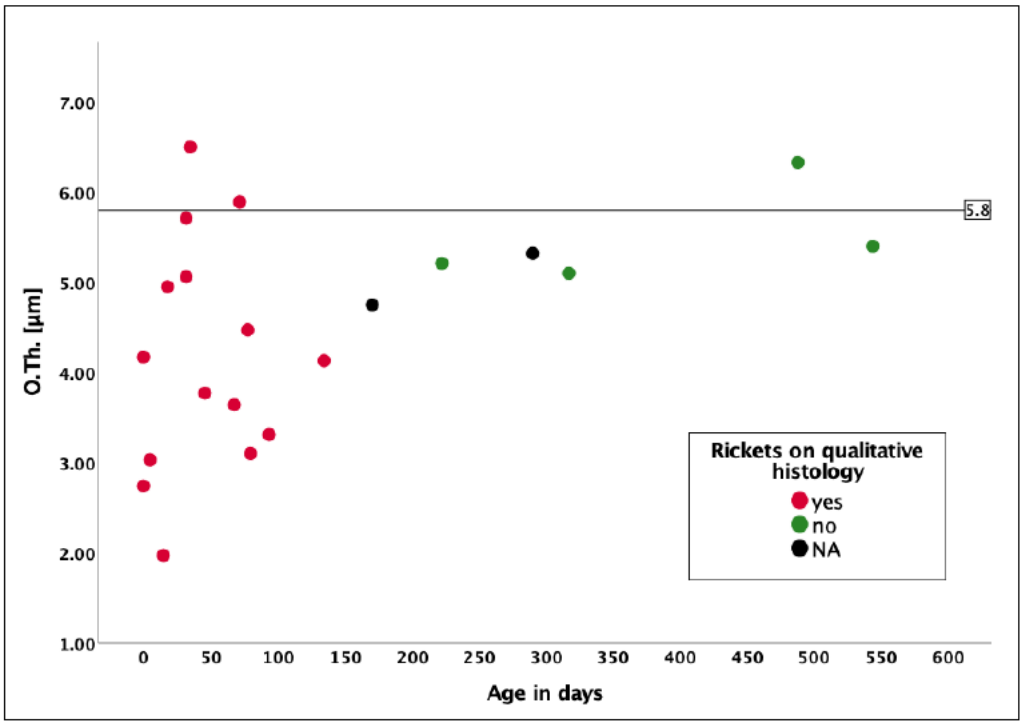


Figure 6.39: Scatter plot of age (in days) vs Osteoid Thickness (in µm)

The static histomorphometry parameters for subjects with and without rickets on qualitative histology are detailed in **Table 6.14** below. Due to the small number of patients without histological rickets, statistical analysis was not performed.

Table 6.14: Static histomorphometry parameters in subjects with and without evidence of rickets on qualitative histology

	With evidence of rickets on qualitative histology (n=15)	**Without evidence of rickets on qualitative histology (n=4)**
Osteoid surface/ Bone Surface (OS/BS) % Mean (SD) Median (range)	27.6 (19.6) 30.2 (1.5 - 57.0)	30.8 (4.0) 30.0 (27.4 – 36.3)
Osteoid Volume/ Bone Volume (OV/BV) % Mean (SD) Median (SD)	4.1 (3.4) 3.0 (0.09 - 10.6)	4.7 (0.6) 4.6 (4.2 – 5.5)
Osteoid thickness (O.Th) µm Mean (SD) Median (range)	4.2 (1.3) 4.1 (2.0- 6.5)	5.5 (0.6) 5.3 (5.1 – 6.3)

6.6.6.3 Quantitative backscattered electron imaging (qBEI) and BMDD

Trabecular Bone Mineral Density Distribution assessed on qBEI was available on seven subjects (5 male). BMDD was available for subjects 1,2,3,4,5,7 and 26. The mean (SD) age was 103 (72) days. One subject was of mixed ethnic origin and 3 each were or white and south Asian ethnic origin. Six were exclusively formula fed and one was weaned onto solids with addition formula feeds. One sample was collected in summer and 2 each in winter, spring, and autumn.

The mean (SD) and median (range) for CaPeak, CaMean, CaWidth, CaLow and CaHigh for the cohort are presented in **Table 6.15** below. As no reference data is available for children below 1.5 years of age, the mean of available reference data for healthy controls aged 1.5 to 20.9 years [161] is presented in **Table 6.15** and compared to the mean of the cohort.

Table 6.15: Trabecular Bone Mineral Density Distribution in the cohort compared to reference data from healthy children aged 2-20.9 years.

	CaPeak **Weight % calcium**	**CaMean** **Weight % calcium**	**CaWidth** **Δ weight % calcium**	**CaLow** **% bone area**	**CaHigh** **% bone area**
Median (range)	21.5 (19.2 – 22.7)	20.0 (18.3 – 21.8)	5.2 (4.5 – 7.3)	18.0 (11.4 – 30.6)	6.4 (0.6 – 9.5)
Mean (SD)	21.3 (1.0)	20.0 (1.0)	5.3 (0.9)	18.7 (6.7)	5.2 (3.5)
Reference mean [161] (2–20.9 years, n=50)	23.39	22.48	3.76	6.14	1.82
p value 95% CI	0.002 -3.0, -1.1	<0.001 -3.4, -1.5	0.005 0.6, 2.4	0.002 6.4, 18.8	0.04 0.2, 6.6

6.6.7 Three-point bending test

Fracture toughness data were available on thirteen subjects (8 males). The mean (SD) age of the cohort was 105.7 (149.2) days. Nine subjects were of white ethnic origin (69.2%), two of south Asian origin (15.4%) and one each of Black and mixed ethnic origin (7.7% each). Six samples were collected in spring (46.2%), one in summer (7.7%) and three each in autumn and winter (23.1% each).

The mean (SD) and median (range) Kc P_Q (fracture toughness at crack initiation) was 44.3 (34.8) and 31.9 (8.4 to 124.6) MPa√m respectively. The mean (SD) and median (range) Kc P_{max} (fracture toughness at maximum load) was 61.2 (39.4) and 43.9 (16.2 to 151.2) MPa√m respectively. P_Q and P_{max} ($r=0.92$, $p<0.001$) were strongly correlated. The Kc P_Q and P_{max} for subjects with and without histological rickets are presented in **Table 6.16**. The number of subjects without rickets was very small (n=3) for statistical comparison.

Table 6.16: Fracture toughness (Kc) at P_Q and P_{max} in subjects with and without rickets

		With rickets on qualitative histology (n=9)	**Without rickets on qualitative histology (n=3)**
Kc P_Q MPa√m	Mean (SD)	44.0 (33.0)	25.1 (24.1)
	Median (range)	31.9 (18.0- 124.6)	14.1 (8.4 – 52.8)
Kc P_{max} MPa√m	Mean (SD)	59.8 (37.1)	41.2 (25.1)
	Median (range)	43.9 (24.9 – 151.2)	41.0 (16.2 – 66.4)

Higher Kc values indicate higher resistance to fractures or increased ductility

There was no statistically significant correlation between Kc P_Q or P_{max} and $25OHD_3$ and its metabolites (n=10), static bone histomorphometry indices (n=12) and BMDD (n=6). The

mean Kc P_Q was significantly higher in males compared to females (59.0 ± 37.1 vs 20.6 ± 9.3 MPa√m respectively; p= 0.04). The mean Kc P_{max} was also higher in males compared to females but did not reach statistical significance (74.2 ± 44.9 vs 40.3 ± 16.1 MPa√m respectively, p=0.13).

A qualitative description of the load displacement curve for subjects without rickets, with histological rickets and radiological rickets is illustrated and detailed below in **Figure 6.40**.

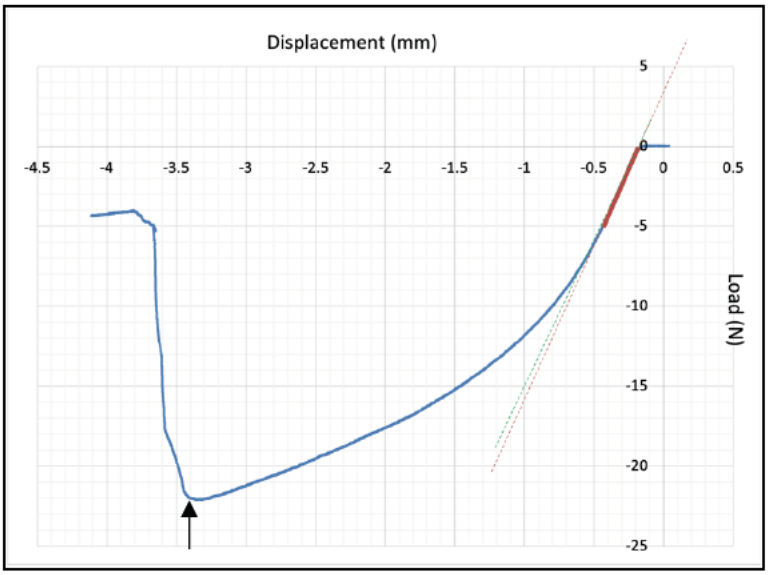


Fig A: Subject 3 with no evidence of histological or radiological rickets

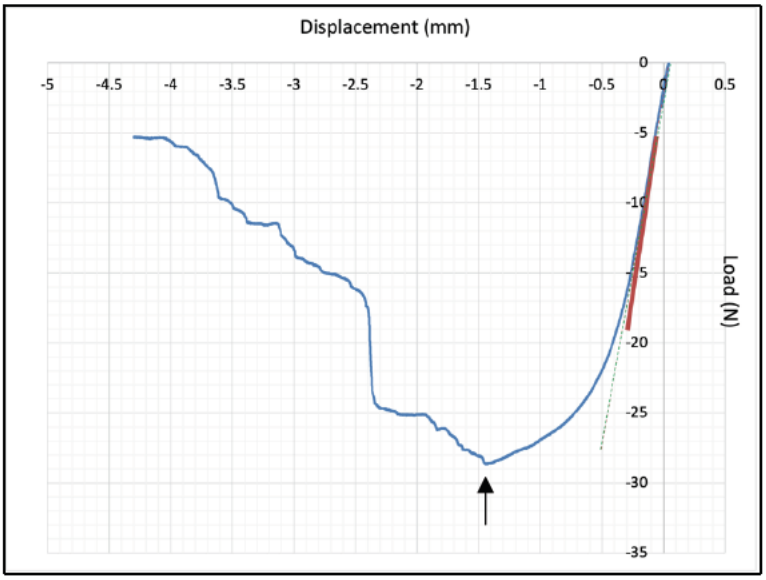


Fig B: Subject 9 with evidence of histological rickets and metaphyseal lucency of ulna

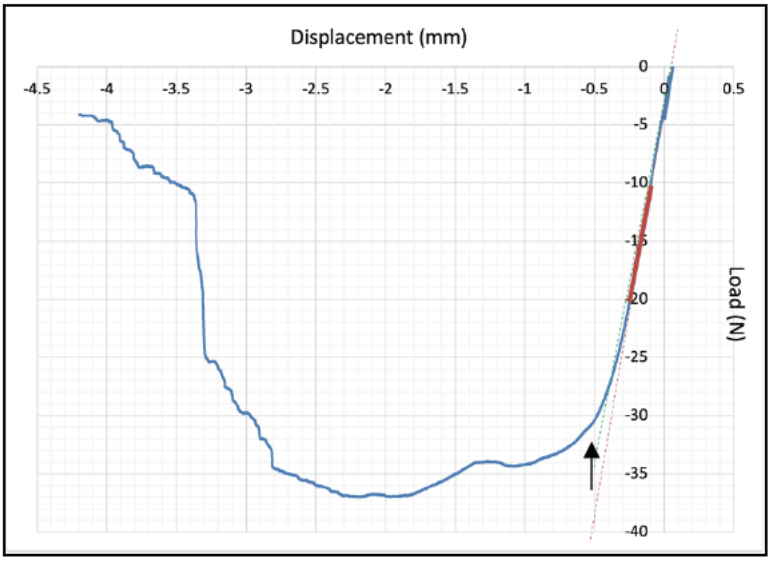


Fig C: Subject 10 with evidence of histological rickets and radiological rickets with a rickets severity score (RSS) of 0.5 on a scale of 0-10.

Figure 6.40: Qualitative description of load displacement curve on subjects without rickets when compared to subjects with histological rickets and radiological rickets.

The above figure illustrates the load displacement curve (interpreted from right to left) of the rib samples where load on Y axis is expressed in newtons (N) and displacement on X axis in millimetres (mm). The green dotted line represents the point at which the curve changes from being linear to non-linear. The linear part of the curve represents elastic deformation (not permanent deformation or returns to original form if load is withdrawn) and the non-linear part represents plastic deformation (permanent deformation). The black arrows represent the points where the curve line transforms from being smooth (representing proportionate displacement in response to load) to jagged (disproportionate displacement in response to load) indicating a change in the behaviour of the material to load.

Figure A is the load displacement curve from subject 3 with no evidence of rickets demonstrating a gradual progression from linear to non-linear curve at 1.4 mm. The material behaves appropriately in response to load for longer (indicated by location of black arrow) when compared to B and C.

Figure B is the load displacement curve from subject 9 with evidence of histological rickets demonstrating a rapid transition from linear to non-linear curve at 0.7 mm. The material behaviour in response to load transforms to abnormal (arrow) at a lower threshold when compared to A.

Figure C is the load displacement curve from subject 10 with histological and radiological evidence of rickets also demonstrating a rapid transition from linear to non-linear curve at 0.5 mm. The abnormal behaviour in response to load (arrow) is seen at a much lower threshold when compared to A and also B.

6.7 Discussion

6.7.1 Summary of the main findings

Vitamin D deficiency defined as 25OHD < 30 nmol/L was present in 40% of the infants and children with sudden unexpected death **(Figure 6.41)**. The proportion of subjects with histological signs of rickets at the growth plate (70%) far exceeded the proportion of subjects with radiological signs of rickets (9%) **(Figure 6.41),** with only one subject having a rickets severity score of 2/10 and the other two a score of 0.5/10.

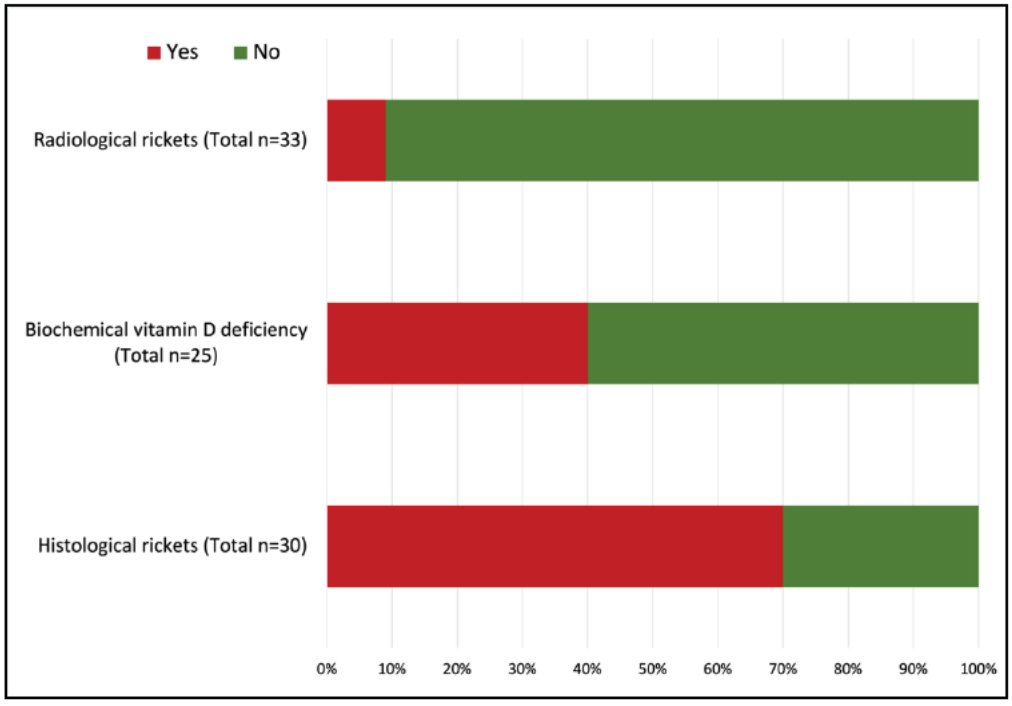


Figure 6.41: Proportions of the study cohort with radiological rickets, biochemical vitamin D deficiency (25OHD < 30nmol/L) and histological rickets in increasing order of prevalence.

The proportion of subjects with overlap in biochemical vitamin D deficiency, histological and/or radiological rickets are presented in a Venn diagram in **Figure 6.42.** In subjects with

biochemical vitamin D deficiency, histological rickets was noted in 34% whereas radiological rickets was only noted in 4%, indicating that histological changes precede radiological changes.

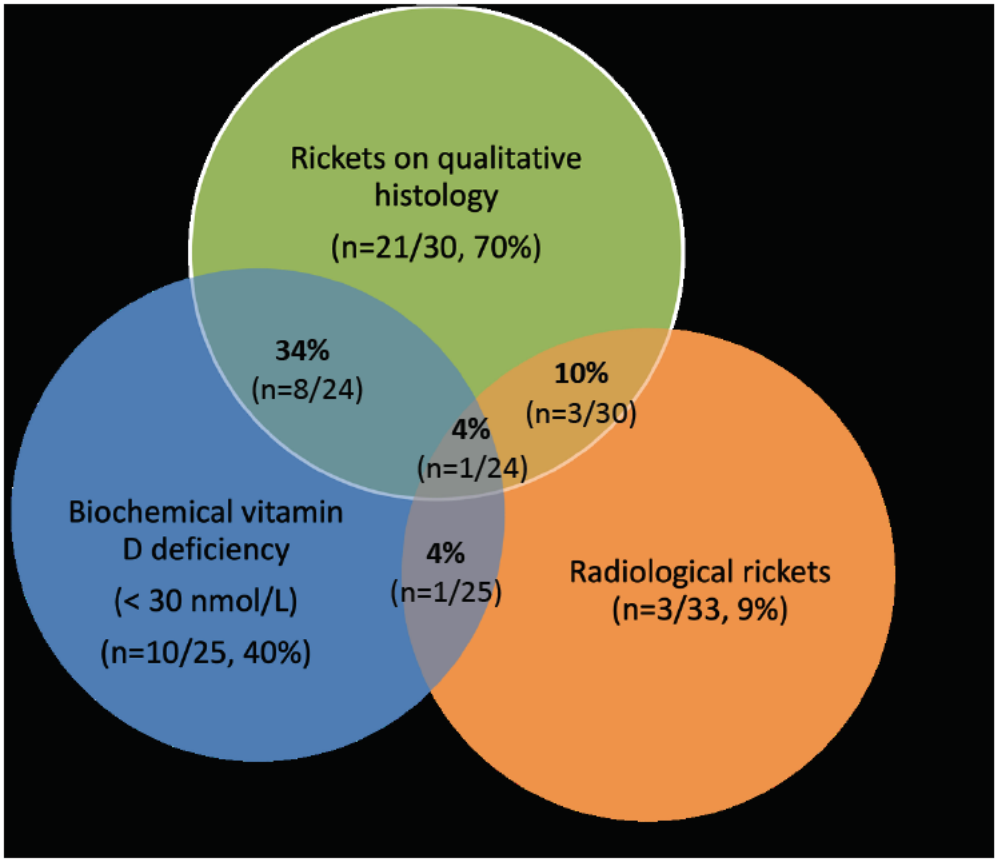


Figure 6.42: Venn diagram demonstrating the proportion of subjects with overlap in biochemical vitamin D deficiency, histological and radiological rickets.

Subtle radiological signs of rickets such as metaphyseal lucency of the ulna, which are often unrecognised/unreported or considered to be physiological, had a significant association with histological signs of rickets. The presence of histological rickets was greater in younger children, indicating the possible role of both antenatal and post-natal factors. Static histomorphometric indices were widely variable in younger infants and the OS/BS, OV/BV and O.Th were above the available reference mean (1.5-6.9 years) in 33.3%, 61.9% and

14.2% of the cohort respectively. The BMDD reference curve was shifted to the left indicating significantly lower mineralization density compared to older children. The presence of histological rickets compromises bone material properties making bone more plastic and ductile.

6.7.2 General discussion

The SUDIC cohort were predominantly of white ethnicity which is in keeping with known statistics in England and Wales [167]. The majority were exclusively formula fed and therefore not on any vitamin D supplements. Of the 11 individuals where vitamin D supplementation was indicated as per national recommendations [67], only one child (9%) was receiving it. The uptake of supplements was similar to that reported in the literature which has been discussed in detail in **Chapters 3 and 4**.

In the current cohort, one male white infant (subject 10) reported to have a history of poor feeding and sweating preceding death was found to have dilated cardiomyopathy on examination with no genetic cause found. The child had significant changes of rickets on growth plate histology **(Figure 6.36**) and deficient 25OHD levels **(Table 6.8)**, potentially implicating hypocalcaemic dilated cardiomyopathy due to nutritional rickets as the cause of death in the absence of other identifiable causes.

6.7.3 Dietary calcium intake

The mean calcium intake in the formula fed cohort was in keeping with the reports in the literature [151]. None of the formula fed subjects had a calcium intake lower than 200 mg daily. With the exception of vitamin D and K, most mineral and vitamin recommendations for

the first half of infant life are set based on breastmilk content [168]. Assuming a human milk intake of 0.8 L/day and a calcium content of 250 mg/L, an exclusively breast-fed infant would consume 200 mg of calcium per day during the first six months of life [151], hence the calcium requirement for this age group is set at this value [168]. Although we were unable to evaluate the calcium intake in the breastfed infants, as they were adequately demand feeding the requirement can be assumed to be met. There is a significant difference in calcium adequacy recommendations between the IOM and the UK Department of Health guidance. The IOM recommend an average intake (or adequate intake) of 200 and 260 mg daily calcium for infants aged 0-6 months and 6-12 months respectively [28], which is reiterated in the global consensus recommendations for prevention of rickets [29]. The UK Department of Health recommends an estimated average requirement (EAR) of 400 mg/day and recommended nutrient intake (RNI) of 525 mg/day of calcium [169]. It is however recognised that, based on the Royal College of Nursing feeding guidelines, the EAR cannot be achieved on average before 6-8 weeks of life and the RNI before 4-5 months of age [170] as noted in our cohort. In our cohort, the calcium intake increased with age in the first half of infancy, in parallel with the increased consumption of formula. Owing to the predominantly milk based diet in the first year, particularly the first six months of life, calcium deficiency as the cause of rickets is recognised to be more common beyond infancy in children aged 1-16 years and particularly in low and middle income countries [26,171]. However, the high prevalence of histological rickets noted in our cohort warrants the need to review the threshold for both 25OHD and calcium intake adequacy in this age group. The current thresholds for prevention of rickets are based on radiological diagnosis and not histological diagnosis.

Calcium deficiency as the cause of rickets was reported in eight UK children in the recent BPSU survey, however 50% of these children were also vitamin D deficient (25OHD < 30

nmol/L) [172]. Only two of the eight children were under the age of one year, aged nine and ten months, who also had 25OHD levels of 27.3 nmol/L and 31 nmol/L respectively [172]. The two subjects with calcium intake >525 mg/day and insufficient $25OHD_3$ levels who had histological rickets in our cohort were both of south Asian origin **Figure 6.32**.

6.7.4 25OHD and metabolites

Previous studies have demonstrated that 25OHD levels can be reliably measured on post-mortem samples [137], however metabolites of vitamin D have never been assessed. This is the first study to measure vitamin D metabolites on post-mortem samples. The findings of positive correlation between $25OHD_3$ and $24,25(OH)_2D_3$ [173,174]and a negative correlation between age and 3-epi-$25OHD_3$ [139] similar to reports from live subjects confirms the reliability of measuring vitamin D metabolites on post-mortem samples.

Although the total 25OHD levels were lower in subjects with histological rickets compared to those without, it did not reach statistical significance. However, we detected significantly lower $24,25(OH)_2D_3$ levels, higher $25OHD_3$:$24,25(OH)_2D_3$ ratios and a higher percentage of $25OHD_3$ as 3-epi-$25OHD_3$ in subjects with histological rickets **(Table 6.11)**. These findings confirm the added value of measuring other vitamin D metabolites in the assessment of nutritional rickets, especially in the early stages in the absence of radiological rickets. The amount of circulating $24,25(OH)_2D_3$ depends on its substrate $25OHD_3$ and activity of the enzyme 24 hydroxylase (CYP24A1). Expression of CYP24A1 is enhanced in the presence of elevated $1,25(OH)_2D_3$. It is therefore interesting to note that in a small number of initial samples analysed, we were unable to detect any serum $1,25(OH)_2D_3$ despite detectable levels of $25OHD_3$. The inability to detect $1,25(OH)_2D_3$ may also be due to its very short half-life. Nonetheless, in studies using mice with knockout of the genes for 24-hydroxylase (*CYP24A1*)

or 1α-hydroxylase (*CYP27B1*), Meyer and Pike showed that poor bone health was associated with elevated serum levels of $25(OH)D_3$ and suppressed levels of $24,25(OH)_2D_3$ in the setting of elevated PTH [175]. This was evident even in mice lacking just the *CYP27B1* gene as these mice lacked the ability to make sufficient $1,25(OH)_2D_3$ to drive expression of *CYP24A1*, and thus had low levels of $24,25(OH)_2D_3$ and elevated $25(OH)D_3$. In these animal model studies, poor bone health was associated with an elevated ratio of $25OHD_3/24,25(OH)_2D_3$, and in the current human study evidence of rickets was also associated with significantly higher ratios of $25OHD_3/24,25(OH)_2D_3$.

From the current data it is possible to speculate that an elevated ratio of $25OHD_3/24,25(OH)_2D_3$ reflects very low serum $1,25(OH)_2D_3$ concentrations and provides a marker for poor bone health. Additionally, $24,25(OH)_2D_3$ itself is known to exert biological activity through its action on the chondrocytes in the resting zone [176] and direct action on osteoblasts differentiation [138]. Lack of $24,25(OH)_2D_3$ is particularly thought to affect mineralization at sites of intramembranous ossification[176] and it has been implicated to play a crucial role in fracture healing [177]. Whether presence of higher levels of $24,25(OH)_2D_3$ confers protection against the development of rickets is to be evaluated.

Vitamin D metabolite ratio of $25OHD:24,24(OH)_2D$ is considered to be a superior indicator of sufficiency compared to 25OHD alone [136] and has been shown to bear a positive correlation with PTH [178] thereby serving as a surrogate marker. Whilst no reference ratios are established in the paediatric population, healthy adults are reported to have a ratio between 7 and 23 [174] with ratios greater than 25 indicating vitamin D sufficiency [173] and those above 80 of inactivating *CYP24A1* mutations [136]. A limiting factor is the inability to calculate a ratio when $24,25(OH)_2D_3$ levels are very low. In our cohort, two subjects, both of

south Asian origin who were breastfed, had undetectable $24,25(OH)_2D_3$ levels and their total 25OHD levels were 5.4 and 3.4 nmol/L **(Table 6.8)**. A $25OHD_3$:$24,24(OH)_2D_3$ ratio could not be computed in these cases. Despite excluding the most severe cases a significant difference in the ratio was noted between subjects with and without rickets confirming its utility.

Whilst the physiological role of 3-epi-$25OHD_3$ is yet to be elucidated, it is known that higher levels are seen in infants contributing to overestimation of 25OHD levels [139] which can have a significant impact on the recommended 25OHD thresholds for deficiency in this age group and subsequently on public health policies relating to supplementation. The higher percentage of epimers in infants may explain the higher levels of 25OHD noted in previous post-mortem studies [47,48]. In children below the age of one, it has been recommended that serum 25OHD should be measured with an assay that either does not cross-react with 3-epi-25OHD or allows unequivocal separation of 3-epi-25OHD from 25OHD [136]. One of the major limitations however is the lack of availability of metabolite assays for routine clinical use. 3-epi-25OHD can be hydroxylated to 3-epi-$1,25(OH)_2D_3$ but this form is less effective in binding the vitamin D receptor and results in lower bioactivity relative to conventional $1,25(OH)_2D_3$ [179]. It is therefore possible to speculate that a higher proportion of 3-epi-25OHD relative to 25OHD could be associated with lower capacity to induce key genes such as *CYP24A1*. In this respect it is interesting to note that rickets was more evident at earlier ages when the proportion of 3-epi-$1,25(OH)_2D_3$ was higher.

6.7.5 Histology

We identified a high prevalence of rickets on qualitative histology, predominantly in the younger age group, similar to previous retrospective post-mortem studies [47,48]. In a recent large retrospective study of SUDIC cases we have shown that nearly 80% (n=132/167) have

changes of rickets at the growth plate on qualitative histology, of whom 63% have established or severe changes and 37% have mild changes [180]. Additionally, we have detailed the previously unreported associated periphyseal changes [widening of the bark bone, exuberant ossification at the periphyseal corner ("step-off") and abnormal metaphyseal collar] [180]. Both the epiphyseal and perichondrial changes were more prevalent in infants less than three months of age confirming the importance of maternal health including adequate vitamin D status in pregnancy. We noted histological evidence of rickets in all three subjects aged D0-D5 **(Table 6.8)** implicating antenatal factors which emphasises the importance of adequate antenatal vitamin D supplementation.

No previous post-mortem studies have systematically evaluated the biochemical differences in subjects with and without histological rickets, which we have detailed in **Section 6.7.4** above. This is the first study to quantify the height of the hypertrophic zone in infants and children with rickets. Based on quantitative histology > 83% of the cohort would be classed as having rickets, even after using a generous cut off value from normative data. It must be noted that the normative data may have underestimated the height of the zones as it utilised photographed (x 4 magnification) prints which were enlarged (x 7.5 magnification) and zones marked visually by overlaying transparent films [135]. All our analyses were based on the presence or absence of qualitative rickets as these changes have been well documented in the literature. Irrespective of the classification used (qualitative, quantitative or both) the proportion of subjects with histological rickets is very high in comparison to subjects with biochemical vitamin D deficiency or radiological rickets.

The high proportion of histological rickets, especially in the younger age groups, raises the question of whether these changes are likely to be physiological in the growing infants. The

clinical relevance of these changes in the current cohort is unknown. However, the changes of rickets reported here have been well established in the literature [32]. Moreover, not all infants displayed these changes to solely attribute it to normal physiology. Additionally, 95% (n=20/21) of the subjects with histological changes had ≥ 3 changes of qualitative rickets. Clarification of physiological adaptation vs pathological changes which heal with time, as 25OHD and calcium intake increase, would require longitudinal assessment to study the natural progression of the histological changes in relation to variations in serum 25OHD levels and calcium intake. However, such a study would prove challenging due to the invasive nature of the samples required and the age group in consideration.

The high prevalence of histological rickets in 70% with only a 34% overlap with biochemical vitamin D deficiency warrants reconsideration of the threshold for defining vitamin D and dietary calcium adequacy, particularly in infants. Since adequate calcium intake is not established until at least 4-5 months of age it is important to maximise 25OHD levels to ensure optimum absorption of enteral calcium.

6.7.6 Radiology

Radiological rickets, quantifiable by a rickets severity score was only noted in 9% (n=3) of the subjects. Two of the three subjects had a low score of 0.5/10 at the ulna and only one subject had a score of 2/10 who had additional changes at the fibula. None of them had any changes at the radius, femur or tibia. In stark contrast, significant histological changes of rickets were noted in 70% of the subjects. All three subjects with radiological rickets had skeletal surveys double reported by two radiologists previously where these changes were missed. One possible explanation is that post-mortem skeletal surveys focus on the presence

of non-accidental injuries and fractures and not on the presence of rickets. Nonetheless, disagreement on the presence of rickets among radiologists is not unknown and was also reported by Cohen et al [48]. It is recognised that radiographic assessment of rickets remains subjective despite the use of objective scoring systems [157], especially when the changes are mild. Subtle changes at the ulna such as metaphyseal lucency and cupped appearance were noted in 30% of the subjects. Subtle changes of metaphyseal lucency of the ulna are attributed to physiological changes and are reportedly a frequent occurrence in post-mortem skeletal surveys (personal communication with R Santos). To the best of our knowledge there is no literature alluding to these changes and/or attributing them to physiological or pathological changes. Additionally, cupped appearance of the ulna is recognised as a positional artefact. Histological rickets at the growth plate was noted in subjects with the above-mentioned subtle changes. These changes were frequent in the ulna, therefore wrist radiographs are more likely to capture rickets than knee radiographs alone. Hence, restricting to knee radiographs for evaluation of rickets is likely to lead to missed diagnosis. A problem that may be more relevant to infants or non-ambulatory children who are not weight bearing. We have previously reported the pattern of higher rickets severity scores at the wrist compared to knee in non-ambulatory children in other forms of hereditary hypophosphataemic rickets [181]. A previous study comparing radiological and pathological signs of rickets at post-mortem in 124 foetuses and infants identified the highest prevalence of rickets at the rib, moderate in the ulna and lowest in the radius [182]. It has been shown that the rib bone, being the most rapidly growing bone in infants, reflects growth arrest at the onset of the insult which makes it the ideal bone to study in this age group [183] and therefore study of the costo-chondral junction forms part of routine post-mortem examination. In our cohort, the presence of abnormal vitamin D metabolites [lower $24,25(OH)_2D_3$ levels and higher $25OHD_3$:$24,25(OH)_2D_3$ ratios]

in the setting of histological rickets indicates the early insult at the growth plate and likely precedes radiological changes.

6.7.7 Histomorphometry, qBEI and BMDD

Currently, there are no histomorphometric reference data available in infants and children younger than 1.5 years of age. The existing normative histomorphometric data for children aged 1.5-23 years (n=58) was derived from tetracycline labelled bone biopsies obtained from apparently healthy children undergoing orthopaedic surgery with no evidence of metabolic bone disease, however bone metabolism markers or 25OHD were not measured [86]. To the best of our knowledge, this is the first study to evaluate bone histomorphometry in infants with SUDIC.

There was a huge variation in static histomorphometry parameters in the younger age group in our cohort which appeared to plateau with age **(Figure 6.37, Figure 6.38 and Figure 6.39)**. A significant proportion had increased osteoid surface (OS/BS, 33%) and osteoid volume (OV/BV, 62%) when compared to normative data for children aged 1.5- 6.9 years (n=10), whereas increased osteoid thickness was noted in fewer subjects (O.Th, 14%) [86]. In osteomalacia due to vitamin D deficiency in adults, a hyperbolic relationship between osteoid surface and osteoid thickness has been defined [33]. The osteoid surface increases first and only when it exceeds 70% of the bone surface, osteoid thickness and osteoid volume start to increase [33]. Not all subjects with histological rickets at the growth plate displayed high OS/BS and OV/BV. We hypothesise that the dissociation between histological findings at the growth plate and histomorphometric findings at the iliac crest may be caused by the different speeds in chondrogenesis and in bone remodeling, where histological changes of rickets at the growth plate hypertrophic zone appear faster than changes of osteomalacia in bone tissue.

During the bone remodeling process, the bone bone resorption phase precedes the formation phase; and the formation phase lasts 4-5 times longer than the resorption phase **(Figure 6.43)** [184]. Only during the bone formation phase would excessive osteoid become apparent. Additionally, bone remodeling is known to be heterogenous across different skeletal sites [184].

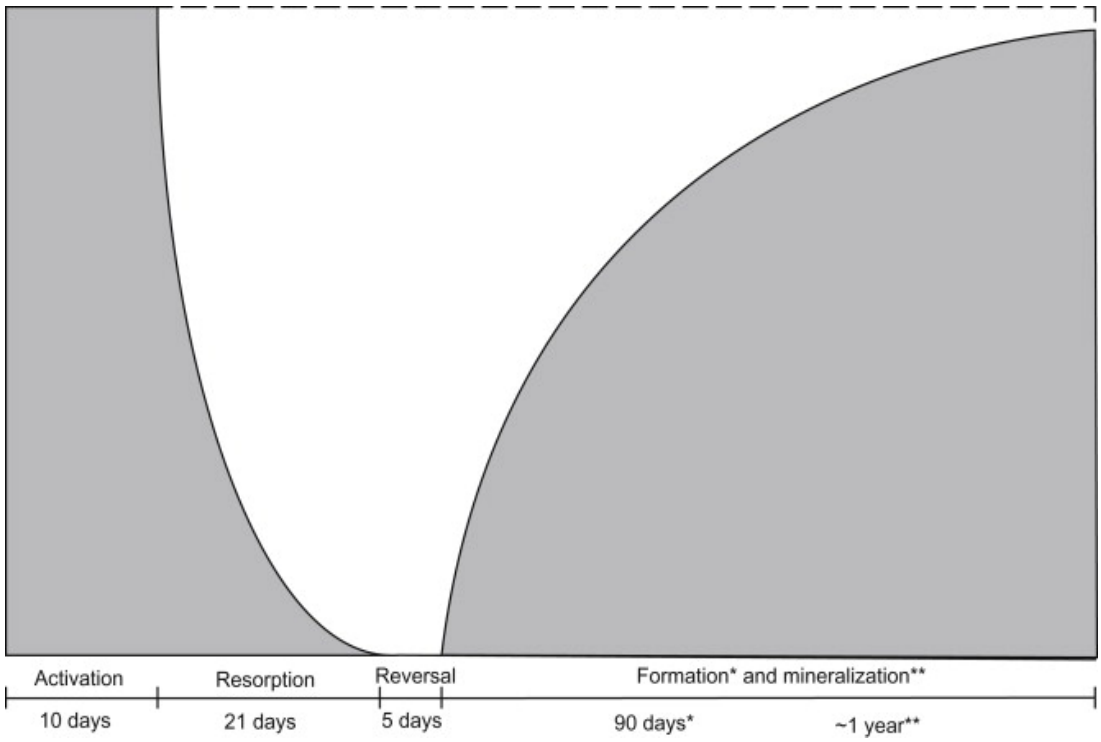


Figure 6.43: Stages of bone remodeling occurring over different time frames with formation phase taking 4-5 times longer than resorption phase.

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The phases of remodeling include activation (recruitment and differentiation of osteoclast precursors to form fully functional osteoclasts), resorption (osteoclasts actively dissolve minerals), reversal (cessation of osteoclast resorption and initiation of bone formation), formation and mineralization (osteoblasts lay down unmineralized organic matrix or osteoid which is followed by two stage mineralization) and finally a quiescent or resting phase (bone surface is covered by bone lining cells and the matrix within the remodeling unit continues to mineralize).

In our cohort, osteoid volume and thickness were noted to increase with age but not osteoid surface. The normative data reported static O.Th from 1.5 through to 23 years of age but OS/BS and consequently OV/BV decrease with age, with a peak in adolescence. Like adolescence, infancy is also a phase of rapid growth with infants increasing their length by about 50% from birth in the first year of life. Establishing normative data for this age group is crucial. Unfortunately, in our cohort there were very few infants without histological changes of rickets to assess the significance of difference in static histomorphometry parameters (OS/BS, OV/B and O.Th) between those with and without rickets.

In contrast to static histomorphometry parameters which vary with age, BMDD has been reported to be age independent [88]. The normative BMDD data from the same cohort of children included in histomorphometry analysis (aged 2-20.9 years) showed very little difference between the various BMDD curve parameters in different age groups [88] except for a moderate decrease of CaLow with increasing age [161]. Similarly, normative adult data (25–90 years, n=52) demonstrates striking similarity of the BMDDs irrespective of ethnicity and gender [160]. When compared to adults, the BMDD reference curve in children is shifted to the left [88]. The constant BMDD in healthy children of varying age groups is presumed to be because of high remodeling activity in the presence of continuing endochondral bone formation [88]. In our cohort, there was a significantly lower CaPeak and CaMean coupled with a higher CaLow; in simple terms the BMDD curve was shifted to the left compared to the reference curve for older children indicating hypomineralization. This is a feature which is seen in states of high bone turnover [185] or altered mineralization kinetics [87]. The lack of shift in the curve during adolescent growth phase in the healthy population [88] raises the question of whether the shift in the curve in our cohort is likely to be pathological. Further studies of healthy infants with sufficient 25OHD status are warranted to assess if the shift in

BMDD curve in this age group is due to altered mineralization kinetics or an evolving physiological state of high bone turnover.

6.7.8 Three-point bending and fracture toughness

Only a handful of studies to date have assessed mechanical properties of paediatric cadaveric bones [186-188]. This is the first study to compare material properties in relation to histological rickets. None of the above studies used the recommended notched approach [165] [166] precluding a direct comparison of findings. It is well recognised that the use of different testing conditions and methods to assess mechanical properties of bones precludes comparison between cohorts even for unnotched samples [166].

In our cohort, although statistical analysis was not feasible due to small number of subjects without histological rickets, higher Kc values indicating ductility of the material was documented in rickets. This notion is supported by the qualitative load displacement curve indicating increased plasticity in the presence of rickets.

Brittle fractures involve fracture without any appreciable plastic deformation (i.e. energy absorption) whereas ductile fractures are the converse and involves large plastic deformation before separation [189]. Subject 3 **(Figure 6.40A)** demonstrating elastic deformation was older but subject 9 **(Figure 6.40B)** and 10 **(Figure 6.40C)** demonstrating plastic deformation were younger. It is not possible to clarify whether the ductility is due to age or the presence of rickets. Ambrose et al reporting mechanical properties on the largest cohort of infant ribs (n=52) [186] found that a third of the rib samples tested were so flexible that the failure criterion of 30% strain after yield was used to end the test rather than the event of fracture [186], which is in keeping with our observation. If ductility was due to age it would be

expected to be consistent in all young children, however Ambrose et al found that most mechanical properties of the rib were correlated to age except ductility [186], a feature which is inherent to the presence of rickets and osteomalacia.

Fracture toughness was significantly higher in males when compared to females indicating increased ductility. Increased prevalence of rickets and bony deformities in males compared to females [190,191] has been reported in the literature. In the most recent UK BPSU survey of nutritional rickets, boys were significantly more affected than girls with an odds ratio of 2.17 [9]. In general, the increased predisposition of boys to nutritional deficiencies in the underdeveloped countries and in certain ethnic groups has been attributed to prolonged breastfeeding [192], however the changes noted in early life in our predominantly formula fed cohort suggests that other factors may play a role in gender differences, including growth rates in early life [193].

We did not find a correlation between fracture toughness and osteoid indices or BMDD parameters, however this will be explored further in the larger cohort. Studying a larger cohort will enable comparison of mechanical properties between subjects with and without rickets, different age groups and gender.

6.7.9 Limitations

There was a lack of complementary biochemical data including phosphate, calcium, alkaline phosphatase and PTH given the nature of the study. Whilst additional biochemical data would help translate the histopathological findings to clinically measurable parameters, it is known that biochemical abnormalities are a late feature of vitamin D deficiency, and the aim of this thesis was to explore the hidden histopathological findings.

The nature of the study did not allow for evaluation of dynamic histomorphometry parameters, nonetheless this is the first study to report static histomorphometry and BMDD data in children under 1.5 years of age.

Due to COVID restrictions we have not been able to obtain full datasets on all recruited subjects but analysis is under progress, including ongoing recruitment, and further data will enable evaluation of the differences in various parameters between groups with and without histological rickets.

6.7.10 Planned future work

Additional data gathered through further recruitment will be used to compare histomorphometric data in children with and without rickets, provided sufficient number of subjects without histological rickets are identified. The histomorphometry and BMDD data generated from subjects without a pathological diagnosis at post-mortem examination could serve as reference data for future studies. Histomorphometry and BMDD data will be correlated to fracture toughness for clinical translation of findings to bone strength and ductility. Most importantly, data generated through this study will be used to attenuate the burden of disease due to vitamin D deficiency by influencing UK public health policies relating to vitamin D through patient, public and political engagement.

6.8 Conclusions

In a predominantly white and formula fed cohort of UK infants, a high prevalence of occult histological rickets was identified in comparison to biochemical vitamin D deficiency and radiological rickets uncovering the true scale of the problem. A very small proportion of

children with histological rickets have radiological signs. Furthermore, subtle radiological signs of rickets are easily missed even by experienced radiologists leading to a significant underestimation of the scale of the problem which has significant implications on public health prevention policies. Altered bone material properties are evident in the presence of histological rickets in the absence of radiological signs.

The first report on the significant differences in vitamin D metabolites as opposed to the standard 25OHD levels in children with and without histological rickets is presented. Measuring 24,25$(OH)_2$D and 3-epi forms have been informative in this cohort and will be explored further in the ongoing post-mortem study.

The highly variable osteoid indices and left shift of the BMDD curve towards lower mineralization in comparison to older children require further evaluation.

Chapter summary

- Histological rickets is widespread, affecting 70% of infants, and far outweighs the prevalence of biochemical vitamin D deficiency (40%) and radiological rickets (9%).
- Histological rickets in infants was not influenced by season, ethnicity or mode of feeding, which has significant impact on public health policies relating to vitamin D supplementation.
- The subtle signs of rickets at the ulna which are significantly associated with histological rickets are frequently missed.
- Vitamin D metabolites and ratios were useful markers demonstrating significant variation between subjects with and without histological rickets.
- The osteoid indices were highly variable in infancy and the bone mineralization density distribution curve was shifted to the left indicating lower mineralization compared to older children.
- Presence of histological rickets demonstrated a trend towards higher fracture toughness and plastic deformation on qualitative assessment which requires further evaluation.

Chapter 7: **Final discussion**

7.1 Introduction

Vitamin D deficiency is well recognised as a growing concern in the UK population, specifically in children, as detailed in previous chapters. The presentation of vitamin D deficiency ranges from asymptomatic osteomalacia to symptoms of hypocalcaemia (seizures, tetany, dilated cardiomyopathy) and hypophosphataemia (hypotonia, delayed development, bowing of legs, swollen wrists). Rickets, principally diagnosed on radiographs, manifests as a late feature of the disease and is therefore unsurprisingly thought to have a lower incidence [9]. The ongoing reports of hospitalisation due to vitamin D deficiency in UK children indicate that the current supplementation policies **(Table 3.2)** are not effective in eradicating this preventable disease. The true extent of the hidden disease burden remains unexplored. Studying the true burden of rickets requires a study of the histopathological changes involving invasive bone biopsy and growth plate samples.

The overall aims of the studies described in this thesis were to examine the hidden burden of vitamin D deficiency, nutritional rickets and osteomalacia in UK infants and children. In this chapter the main results of each study are summarised. The implications of the findings from this thesis on the UK public health policies relating to vitamin D are discussed.

7.2 Summary of the thesis

7.2.1 Hidden pathology in infants presenting with symptomatic vitamin D deficiency and their family members

Chapter 4 set out to systematically explore the hidden burden of disease in infants presenting with symptomatic vitamin D deficiency and their family members. This was a prospective

case series of infants presenting to a single tertiary children's centre in the West Midlands. This is the first report highlighting the extent of growth plate abnormality, osteomalacia and mineralization defect in an infant dying of cardiac failure secondary to hypocalcaemic dilated cardiomyopathy in the setting of nutritional rickets due to severe vitamin D deficiency.

Four infants, of BAME mothers, presented with acute life-threatening and severe features of vitamin D deficiency including hypocalcaemic seizures, cardiac failure and cardiac arrest. All infants were relatively well and subtle signs were missed by parents and also healthcare professionals until presentation. The true disease burden of rickets, osteomalacia and cardiac dysfunction **(Table 4.1)** were unveiled on further investigations. All infants had 25OHD levels <12.5 nmol/L. Severe vitamin D deficiency, especially in the setting of cardiac involvement, is associated with significant morbidity. Patient 1 had repeated visits to the general practitioner and emergency department with pale floppy episodes (seizures due to hypocalcaemia) before ultimately presenting in cardiac failure. Despite a 100% compliance with all recommended heath care checks and immunisation visits, the need for supplementation was not highlighted in any of the four infants at any of the contacts with healthcare professionals.

The extent of hidden disease burden was expanded when asymptomatic family members were investigated **(Table 4.2)** and all of them found to have 25OHD levels below 50 nmol/L with nearly 77% having biochemical osteomalacia (elevated ALP and/or PTH).

7.2.2 Newborn blood spot screening study

Chapter 5 details a prospective cross-sectional study designed to investigate the prevalence of vitamin D deficiency in a multi-ethnic newborn cohort capturing the peak and nadir of

25OHD levels in summer and winter respectively. This is the first study in the UK to measure 25OHD levels on dried blood spots from the national newborn screening to assess the effectiveness of the current UK antenatal vitamin D supplementation programme **(Table 3.2)**.

25OHD levels were measured on 3000 (1500 summer-born and 1500 winter-born) Dried Blood Spots (DBS) obtained at routine newborn screening, received in the West Midlands regional laboratory. Nearly 70% of newborn babies had suboptimal 25OHD concentrations below 50 nmol/L with a third each having deficiency, insufficiency and sufficiency. Winter-born babies and those of non-white ethnicity had significantly lower 25OHD concentrations compared to summer-born and babies of white ethnic origin respectively. Irrespective of ethnicity only 15% of winter-born babies had sufficient 25OHD levels > 50 nmol/L. Approximately 50% of babies of non-white ethnic background and 35% of babies of white ethnic background had 25OHD levels below the UK/SACN [66] recommended levels of 25 nmol/L in the winter.

Across both seasons, the proportion of deficiency was highest at nearly 50% in babies of Asian and black ethnic background when compared to babies of white British ethnicity at 30%. Season of birth, ethnicity, gestation at birth and maternal age contributed to 24% of the variation in newborn 25OHD concentrations. Mother's socio-economic status as determined by index of multiple deprivation did not have a bearing on the 25OHD status of the newborn.

The limitation of this study was the lack of information on maternal vitamin D supplementation but compliance with national recommendations was expected. Whilst there was no follow up to assess the implications of these low 25OHD levels, the potential consequences of increased presentation of symptomatic vitamin D deficiency in spring and in dark-skinned individuals is already well documented in the literature [5,6,9]. The widespread

prevalence of histopathological changes of rickets, especially in infants in the first 100 days of life, explored in the post-mortem study supplement the findings from this study.

7.2.3 Rickets and osteomalacia in SUDIC

Chapter 6 details a prospective post-mortem study designed to uncover the true disease burden by investigating the hidden histopathological changes of rickets in infants and children under the age of two years with SUDIC. This is the first and unique study in infants to investigate static bone histomorphometry, BMDD and correlate histopathological changes to fracture toughness and vitamin D metabolites. The study is ongoing and open to recruitment until August 2022.

The prevalence of histological rickets far exceeded (70%) the prevalence of biochemical vitamin D deficiency (40%) and radiological rickets (9%) confirming the main hypothesis of the thesis. Subtle radiological signs of rickets, which were significantly associated with histological changes of rickets at the growth plate, were frequent at the ulna and often subjective or missed or attributed to physiological changes leading to a significant underestimation of the scale of the problem.

A 34% overlap between histological rickets and biochemical vitamin D deficiency (< 30 nmol/L) suggests the need to review the 25OHD and dietary calcium thresholds in infant. Highest prevalence of histological rickets in the youngest subjects implicates maternal factors.

Vitamin D metabolites can be reliably measured on post-mortem samples and were better indicators of underlying pathology when compared to measuring 25OHD levels in isolation. Subjects without histological rickets had a significantly higher level of $24,24(OH)_2D_3$ indicating vitamin D sufficiency or potentially a direct protective action of the metabolite.

Subjects with histological rickets also had a high percentage of $25OHD_3$ in its epimer form, supporting the notion that the epimer forms are less biologically active. Inability of routine assays to differentiate between 25OHD and its epimer forms can lead to misclassification of vitamin D status and dismissal of deficiency as the cause of pathology especially in infants. The data presented are also further evidence that simple measurement of serum 25OHD, as commonly employed for most paediatric studies across the globe, provides only a limited perspective on the biological function of vitamin D in any given individual. In recent years there has been a subtle shift to a more 'metabolomic' approach to vitamin D, and the current project strongly supports this development.

Highly variable osteoid indices were noted in the first year of life. Given the dearth of histomorphometry data in infants, osteoid indices were compared to mean from older children identifying raised OS/BS in 33.3%, OV/BV in 61.9% and O.Th in 14.2%. The BMDD curve was shifted to the left towards lower mineralization when compared to reference data from older children.

Higher fracture toughness in subjects with histological rickets, and in males compared to females, indicates increased ductility. Subjects with histological changes of rickets in the absence of radiological rickets also displayed altered material properties with abnormal displacement in response to load and increased plastic deformation.

7.3 Implications of the thesis for public health policies

The ongoing reports on the burden of nutritional rickets in the UK have indicated the need to intensify the current public health policies relating to vitamin D. This notion is further strengthened by the findings of this thesis which has uncovered the hidden disease burden.

Infants, irrespective of the mode of feeding and ethnicity, are the most vulnerable to the consequences of deficiency. The key study findings and their specific implications for policies are discussed below.

7.3.1 Antenatal supplementation

To give infants the best start in life, there is no doubt that good nutrition begins in utero. It is recognised that the effects of maternal 25OHD status may have long lasting effects on the offspring bone health [94]. **Chapter 4** highlighted the lack of adequate antenatal and post-natal vitamin D supplementation where infants presented with symptomatic vitamin D deficiency. All the mothers of infants with symptomatic vitamin D deficiency had suboptimal 25OHD levels. Whilst **Chapter 4** systematically explored compliance with supplementation in specific cases, **Chapter 5** highlights the overall lack of effectiveness of the current antenatal supplementation programme which leaves many infants at high risk especially those born in winter and to mothers of BAME background. **Chapter 6** demonstrates the impact of poor antenatal supplementation on the infant. All infants less than a week old had rickets despite the well-known fact that the foetus is basically a parasite and draws on all the maternal reserves. All but one subject in the first 100 days of life had rickets on histology, once again emphasising the importance of robust antenatal supplementation and ongoing early post-natal supplementation.

Adult vitamin D requirements have been extended to pregnant women due to the lack of pregnancy-specific data. Since these requirements cannot be met through diet, the IOM [28] and global consensus for prevention of rickets [29] recommend 600 IU/day supplements in pregnancy. The UK recommends 400 IU/day and pregnant women are not regarded as high risk groups but as the general population [66,67].

A study of pregnant women in Denmark (n=107) reported cord blood 25OHD concentrations < 50 nmol/L in 61% of newborns (of whom 15% had concentrations <25 nmol/L) despite the use of 400 IU/day supplements in 79% of the women [194]. Antenatal supplementation should aim at preventing deficiency in the newborn, bearing in mind that 25OHD concentrations in the neonate are roughly 50-70% of maternal concentrations [194]. A supplementation trial of pregnant white women (n=144) in Ireland (51.9^{o} N) identified that 1200 IU/day supplement safely achieve sufficiency (25OHD ≥50 nmol/L) in almost all women and prevented deficiency (cord 25OHD < 30 nmol/L) in 95% of neonates [113]. Similar dose-finding studies in ethnic minority population are warranted.

7.3.2 Infant supplementation

Whilst symptomatic vitamin D deficiency has predominantly been reported in children from ethnic minority background as noted in **Chapter 4**, in **Chapter 5** we report significantly low 25OHD levels in the majority of winter born babies irrespective of ethnicity and in **Chapter 6** we highlight that histopathological changes of rickets are not restricted to dark skin or breastfed babies. The cohort in the post-mortem study was predominantly of white ethnic background and the typical seasonal and ethnic variation noted in the newborn blood spot study **(Chapter 5)** was not identified. The newborn blood spot study findings confirm that the newborn 25OHD status is determined predominantly by maternal 25OHD status and possibly maternal sunlight exposure. The post-mortem study however highlights that all infants, irrespective of ethnicity or season of birth or mode of feeding, are at risk of developing histological rickets. The lack of seasonal and ethnic variation in infants, especially in the first half year of life, is plausible because of the lack of sun exposure and/or sun avoidance [195].

The high proportion of histological rickets in formula fed infants warrants a review of the current UK policy. Subjects in the post-mortem cohort were predominantly formula fed and were expected to be receiving vitamin D via formula feeds in the range of 9.9 -12 μg (396 – 480 IU) daily based on the mean volume consumed (712 ml) and the vitamin D content of the formula feed **(Appendix 14)**. 25OHD levels and calcium intake were noted to increase with age, which is possibly due to increased formula consumption with age. The high prevalence of histological rickets in infants despite being on formula feeds suggests that the amount of vitamin D and possibly calcium in formula feeds is not sufficient to prevent histological rickets. Optimising vitamin D is likely to enhance calcium absorption and a daily dose higher than 400 IU may be required.

The presence of histological rickets in babies in the first week of life indicates that the mothers were likely vitamin D deficient, and it is not known whether 400 IU in the infant would suffice if born to a mother with sufficient vitamin D status. A Danish study (n=108) demonstrated that universal supplementation of both breast and formula fed infants and greater adherence (>90%) to infant supplementation can reduce the proportion of newborns with 25OHD concentrations < 50 nmol/L from 61% at birth to 4% at 4 months; with none having concentrations below 25 nmol/L [194].

Most European countries recommend a dose of 200 - 400 IU daily in infants [196] irrespective of whether they are formula or breast fed [42]. France is the only country which recommends 1000 – 1200 IU daily in breastfed infants and additional 600 - 800 IU daily in infants consuming fortified formula [197]. France has retained these recommendations for over two decades and eradicated rickets with no safety concerns [197]. Further studies are required to assess the amount of vitamin D required to prevent histological rickets in infants.

Future supplementation trials should consider measuring vitamin D metabolites as opposed to 25OHD levels alone to assess sufficiency.

7.3.3 Implementation of policy

7.3.3.1 Antenatal

Supplement use in the UK population is low [77], as seen in the case of folic acid supplementation in pregnancy [198]. Only 27% of women aged 19-64 years reported taking vitamin D supplements in the National Diet and Nutrition Survey [77]. Only 15% (n=30/198) reported using vitamin D supplements in pregnancy in a longitudinal study of pregnant women in Southampton, UK [94]. The higher prevalence of deficiency in the UK population is in stark contrast to reports from Nordic countries where supplement use is generally high [58,199] and/or widespread fortification is adopted [58]. In Finland where food fortification is adopted, reportedly <1% of pregnant women and their newborns have 25OHD <50 nmol/L (n=584) with maternal consumption of fortified food and supplement use predicting umbilical cord 25OHD concentrations [112].

Universal supplementation of pregnant women (irrespective of risk factors) has been reported to enhance adherence [45]. All pregnant women, irrespective of their ethnic background, must be considered high risk and vitamin D supplements recommended at antenatal booking visits. A review of the UK antenatal supplementation policy with regards to monitoring of the uptake is warranted to address the preventable health consequences of vitamin D deficiency in the mother and the infant.

7.3.3.2 Infant

Akin to antenatal supplementation, uptake of infant supplementation in the UK is also poor [42,44]. Other high latitude countries across the globe with poor adherence to supplements [42,72] and/or high proportion of immigrant population [38,3,75] have also seen a resurgence in nutritional rickets [7,40]. Whilst sunlight exposure is a modifiable factor, the lack of UVB in high latitude countries is hardly modifiable [200] necessitating robust public health policies. A high proportion of deficiency in the UK newborn cohort and undiagnosed/hidden histological rickets in neonates suggests shortfalls in the current UK antenatal supplementation policy [201].

A 90-166 fold higher incidence of rickets seen in UK children of ethnic minority background, compared to children of white ethnic background [9,90] is explained by the lower 25OHD concentrations seen at birth and lack of rigid infant supplementation system. All infants presented in **Chapter 4** and **Chapter 6** had received all immunisation and attended necessary routine childcare visits but compliance to vitamin D was not checked at any of these visits. Furthermore, unlike other European countries, in the UK, primary paediatric health care is provided by health visitors and or general practitioners **(Figure 3.5)** who have not received any paediatric training [90]. Therefore, the subtle signs of rickets and its clinical presentations such as floppy episodes due to hypocalcaemic seizures can easily go undiagnosed. Introducing mandatory monitoring of vitamin D supplementation at healthcare visits is likely to reduce the dependence on healthcare professionals. In the long term, delivery of paediatric health care by appropriately trained clinicians must be a healthcare priority.

Early neonatal presentation as seen in patient 4 in **Chapter 4** could have been prevented by provision of supplements and information on vitamin D to the family at discharge from the

neonatal unit in addition to monitored antenatal supplementation. National monitoring of supplement uptake [42] and clear operational policies are warranted in the UK [69]. None of the parents of children presented in **Chapter 4** were aware of the devastating consequences of vitamin D deficiency, especially dilated cardiomyopathy and seizures. Empowering parents with this information at birth not only asserts the importance of supplements but ensures compliance.

7.3.3.3 General population

The high proportion of affected family members of symptomatic cases emphasises the importance of recommending supplements to family members who share the common risk factors of dark skin pigmentation, high latitude residence and lack of supplement use. One way of addressing widespread undiagnosed vitamin D deficiency is through strategies such as food fortification with vitamin D.

Countries such as Finland who have adopted widespread vitamin D fortification and increased supplement use (11% to 41%) have seen a steady improvement in 25OHD status (48 nmol/L in 2000 to 65 nmol/L in 2011) [79]. Even among non-supplement users, 91% are reported to achieve sufficiency through fortification [79]. A combined approach with fortification and supplementation of high risk groups has been shown to be the cost-effective way forward for the UK [78] not only to improve population health but also to eliminate social inequalities [202].

7.4 Future research recommendations

There is a significant gap in the knowledge of the nutritional intake (of vitamin D and calcium) and 25OHD status in infants in the first half of life in the UK. The diet and nutrition survey of infants only provides data in infants over 4 months of age [195]. It is crucial to address this gap given the higher-than-expected neonatal mortality in the UK compared to similar European countries. In European Union, the UK has dropped from being ranked 7th in 1990 to 19th in 2015 for neonatal mortality [203] which is concerning. Further large-scale prospective studies are required to assess the role of nutritional vitamin D deficiency, rickets and cardiomyopathy in neonatal and infant mortality. Post-mortem studies can provide invaluable insights into the cause of death where currently 60% remain unascertained.

The lack of adequate data in infants on vitamin D intake and 25OHD levels has led to the declaration of safe vitamin D intakes [66] rather than reference nutrient intake. Despite the knowledge of rapid growth and the notion of higher nutritional requirements in infancy the vitamin D recommendations are lower than that of older children. Further studies are essential to ascertain vitamin D requirements in this age group and studying vitamin D metabolites as opposed to 25OHD levels alone may be one way to ascertain sufficiency thresholds. Evaluation of epimers in healthy children will allow better assessments of vitamin D status and review of the threshold for deficiency, particularly in infants.

We are currently reviewing the association between subtle radiological features of rickets at the ulna and growth plate abnormalities in a larger cohort where we have previously reported the histological features [180]. Data on histomorphometry, BMDD and fracture toughness in infants without histological rickets are required to assess the impact of age independent of rickets.

7.5 Conclusions

The studies described in this thesis have uncovered the hidden biochemical and histopathological burden of vitamin D deficiency in UK children; additionally, undiagnosed biochemical osteomalacia in asymptomatic family members are revealed. In addition to advancing the scientific knowledge of histomorphometry and mechanical properties of bone in infants, these studies confirm that the cases of rickets or symptomatic vitamin D deficiency in the UK only represent the tip of the iceberg of this public health crisis. The high prevalence of histopathological rickets in infants impacting on bone health warrants special attention to antenatal and postnatal vitamin D supplementation irrespective of ethnicity, season and mode of feeding. Existing policies need to be reviewed to incorporate mandatory supplementation and monitoring in all pregnant women and infants and in particular linking these to antenatal care and immunisation visits. Prevention of disease is not restricted to vaccines only. Serious consideration must be given to food fortification.

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Appendices

Appendix 1: Summary of national infant vitamin D supplementation policies ranked by adherence rates in the first year of life in different European countries.

Country	Adherence rate	National Vit D recommendation	Vit D recommended for breast and formula fed	Vit D started on D1-D5 of life	Information on Vit D at NNU discharge	Free Govt Vit D supply	Family financial support	Recomm-ended number of childcare visits	Adherence to Vit D checked at childcare visits	Mandatory childcare visits to claim benefit	National Vit D adherence monitoring	Vit D food fortificat-ion
Austria	99%	√	√	√	√		√	√	√	√		
Hungary	98%	√	√		√		√	√	√	√		√
Israel	80-97%	√	√		√		√	√	√		√	-
Czech Republic	95%	√	√	√	√	√	√	√	√			
Netherlands	90-95%	√	√		√		√	√	√			√
Portugal	90%	√	√		-		√	√	√	-		
Sweden	90%	√	√		√	√	√	√	√			√
France	90%	√	√	√	√	√	√	√		√		√
Estonia	90%	√	√		√	√	√	√	√			√
Lithuania	90%	√	√		√		√	√	√			
Macedonia	90%	√	√	√	√		√	√	√	√		

Germany	70-90%	√	√	√	√	√	√	√	√			
Finland	85%	√	√		√		√	√	√			√
Russia	80%	√			√		√	√	√			√
Turkey	80%	√	√	√	√	√	√	√	√		√	
Albania	80%	√	√			√	√	√	√	√		√
Bulgaria	70-80%	√	√		√		√	√		√		
Norway	75%	√	√		√	√*	√	√	√			√
Romania	70%	√	√	√	√	√	√					
Switzerland	70%	√	√	√	√		√	√				
Poland	65-70%	√	√	√	√		√	√				√
Denmark	60-70%	√	√		√		√	√	√		√	
Italy	50-60%			√	√	√*		√		√		
Ireland	59%	√	√	√	√		√	√	√			√
Spain	50%	√	√	√	√			√				
Serbia	45%	√		√	√			√				√
Greece	30%	√										√
UK	20%	√				√*	√	√				√

- **Not answered** **√* Free only for certain groups**

Appendix 2: Ethnic group codes indicated on the newborn blood spot screening cards

Ethnic category	**Code**	**Description**
White	A	British
	B	Irish
	C	Any other white background
Mixed	D	White and black Caribbean
	E	White and black African
	F	White and Asian
	G	Any other mixed background
Asian	H	Indian
	J	Pakistani
	K	Bangladeshi
	L	Any other Asian background
Black	M	Caribbean
	N	African
	P	Any other black background
Other	R	Chinese
	S	Any other ethnic category
	Z	Not stated
	99	Not known

Appendix 3: Health Research Authority approval

Ymchwil Iechyd a Gofal Cymru
Health and Care Research Wales

NHS
Health Research Authority

Dr Wo fgang Hoeg er
Honorary Reader, Un vers ty of B rm ngham, IMSR
Un vers ty of B rm ngham
Inst tute of Metabo sm and Systems Research
Edgbaston
B rm ngham
B15 2TT

Email hra approval@nhs net
Research-permissions@wales nhs uk

09 January 2019

Dear Dr Hoeg er

HRA and Health and Care Research Wales (HCRW) Approval Letter

Study title:	**What is the prevalence and ethnic and seasonal variation of vitamin D deficiency on blood spot screening in newborns in the UK?**
IRAS project ID:	**245670**
Protocol number:	**2**
REC reference:	**19/EM/0019**
Sponsor	**Birmingham Women's and Children's Hospital**

I am p eased to conf rm that HRA and Hea th and Care Research Wa es (HCRW) Approva has been g ven for the above referenced study, on the bas s descr bed n the app cat on form, protoco , support ng documentat on and any c ar f cat ons rece ved. You shou d not expect to rece ve anyth ng further re at ng to th s app cat on.

How should I continue to work with participating NHS organisations in England and Wales?
You shou d now prov de a copy of th s etter to a part c pat ng NHS organ sat ons n Eng and and Wa es, as we as any documentat on that has been updated as a resu t of the assessment.

Th s s a s ng e s te study sponsored by the s te. The sponsor R&D off ce w conf rm to you when the study can start fo ow ng ssue of HRA and HCRW Approva .

It s mportant that you nvo ve both the research management funct on (e.g. R&D off ce) support ng each organ sat on and the oca research team (where there s one) n sett ng up your study. Contact deta s of the research management funct on for each organ sat on can be accessed here.

Appendix 4: Research ethics committee approval

NHS
Health Research Authority

East Midlands - Leicester South Research Ethics Committee
he Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

09 January 2019

Dr Wo fgang Hoeg er
Inst tute of Metabo sm and Systems Research, Un vers ty of B rm ngham
Professor n paed atr cs, Kep er Un vers tätsk n kum
Edgbaston, B rm ngham
B15 2TT

Dear Dr Wo fgang Hoeg er

Study title:	**What is the prevalence and ethnic and seasonal variation of vitamin D deficiency on blood spot screening in newborns in the UK?**
REC reference:	**19/EM/0019**
Protocol number:	**2**
IRAS project ID:	**245670**

The Proport onate Rev ew Sub comm ttee of the East M d ands Le cester South Research Eth cs Comm ttee rev ewed the above app cat on on 08 January 2019.

We p an to pub sh your research summary word ng for the above study on the HRA webs te, together w th your contact deta s. Pub cat on w be no ear er than three months from the date of th s favourab e op n on etter. The expectat on s that th s nformat on w be pub shed for a stud es that rece ve an eth ca op n on but shou d you w sh to prov de a subst tute contact po nt, w sh to make a request to defer, or requ re further nformat on, p ease contact hra.studyreg strat on@nhs.net out n ng the reasons for your request. Under very m ted c rcumstances (e.g. for student research wh ch has rece ved an unfavourab e op n on), t may be poss b e to grant an exempt on to the pub cat on of the study.

Ethical opinion

On beha f of the Comm ttee, the sub comm ttee gave a favourab e eth ca op n on of the above research on the bas s descr bed n the app cat on form, protoco and support ng documentat on, subject to the cond t ons spec f ed be ow.

Appendix 5: Newborn screening research committee approval

Public Health England

NHS

PHE Screening
Floor 5N
Wellington House
133-155 Waterloo Road
London
SE1 8UB

Screening helpdesk
+44 (0)20 3682 0890

Website
www.gov.uk/topic/population-screening-programmes
witter @PHE_Screening
Blog phescreening.blog.gov.uk

Dr Suma Uday
Dr Wo fgang Hög er
Birmingham Women's and Children's Hospital,
Stee house Lane, B rm ngham B4 6NH

15th January 2019.

Dear Drs Uday and Hög er

Re: Ethnic and seasonal variations in blood spot vitamin D at birth from neonatal screening cards

Thank you for your response to comments and adv ce g ven by the antenata and newborn screen ng programmes research adv sory comm ttee (RAC).
The RAC are sat sf ed that the rev sed protoco e m nates d rect pat ent dent f ers, ensures pat ent anonym ty and therefore e m nates the need for sect on 251 approva . You have conf rmed that the Laboratory Manager w anonym se the data before re eas ng t to the research team and bab es o der than 21days when the r b ood spot samp e was taken w be exc uded from the study.

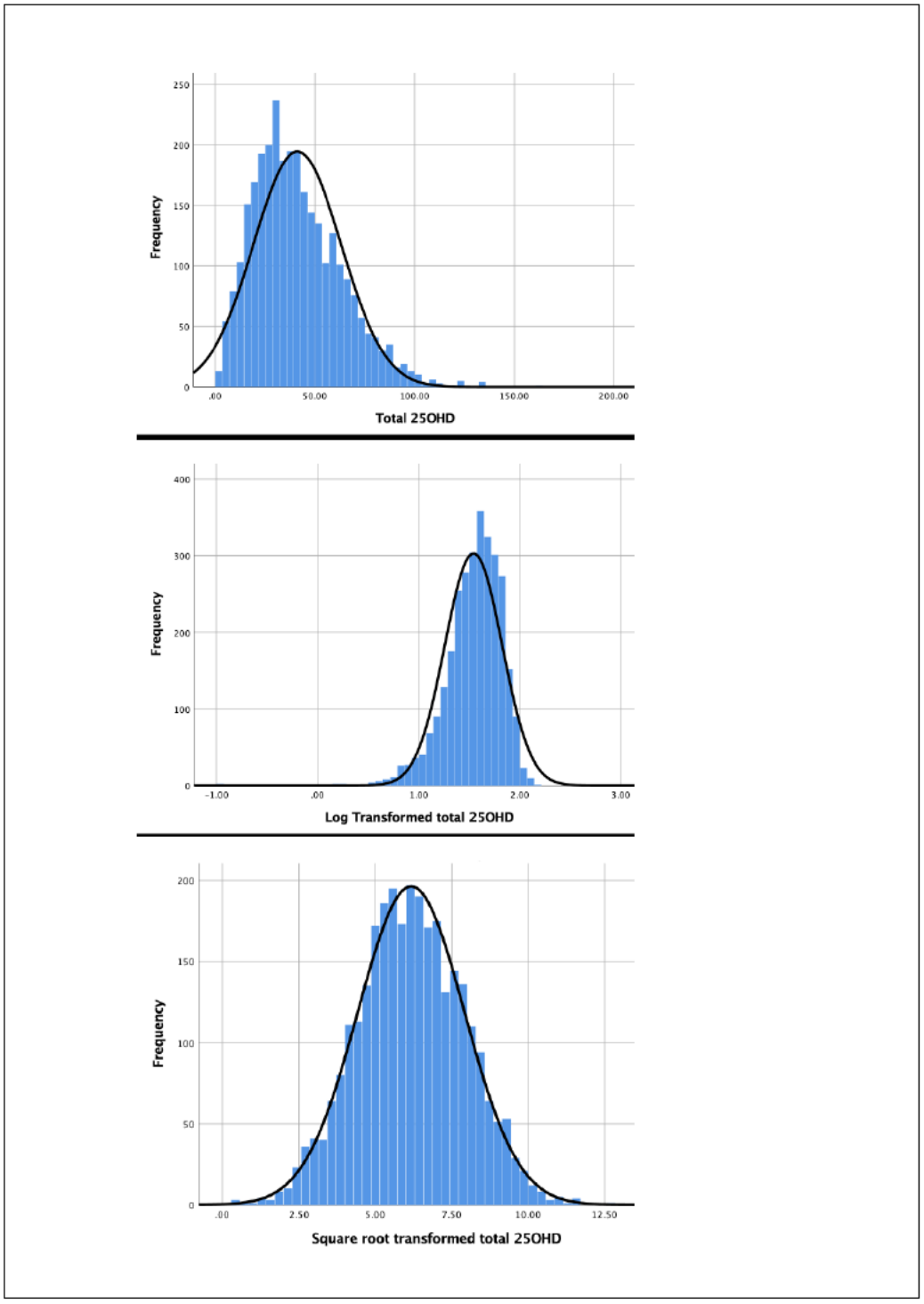
The RAC are p eased to approve your app cat on and ook forward to a progress report n t me for the August 2019 meet ng.

Yours s ncere y

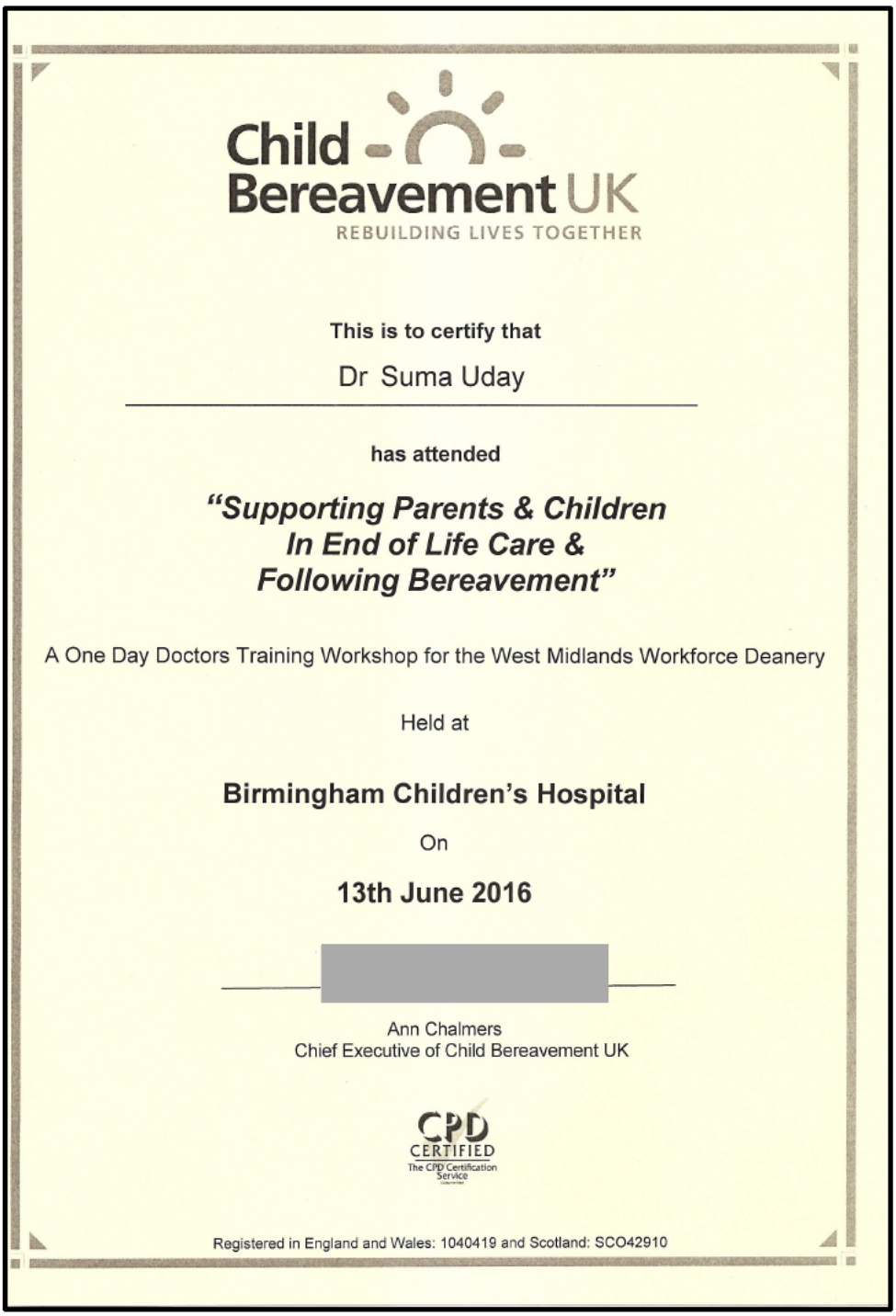
[redacted]

Professor Cather ne Peckham
Cha r of the Antenata and Newborn Research Adv sory Comm ttee.
p ease rep y to screen ng.research@phe.gov.uk

Appendix 6: Normality distribution following log and square root transformation



Appendix 7: Training on supporting parents and children in end of life care and following bereavement



Child Bereavement UK
REBUILDING LIVES TOGETHER

This is to certify that

Dr Suma Uday

has attended

"Supporting Parents & Children In End of Life Care & Following Bereavement"

A One Day Doctors Training Workshop for the West Midlands Workforce Deanery

Held at

Birmingham Children's Hospital

On

13th June 2016

Ann Chalmers
Chief Executive of Child Bereavement UK

CPD CERTIFIED
The CPD Certification Service

Registered in England and Wales: 1040419 and Scotland: SCO42910

Appendix 8: Invitation to bereaved parents requesting participation in a focus group

Birmingham Children's Hospital NHS
NHS Foundation Trust
DEPARTMENT OF ENDOCRINOLOGY
AND DIABETES
Steelhouse Lane
Birmingham
B4 6NH
0121 333 9999
www.bch.nhs.uk

Dear Parents

would like to invite you as a bereaved parent to a focus group for your valuable opinion on our study First of all would like to say that am deeply saddened by your loss and cannot even begin to understand what you must have been through

am a paediatric doctor working at Birmingham Children's Hospital am keen to improve the care children receive through research

My current study looks at the role of vitamin D deficiency in fractures and also death in children Currently we do not know at what level of vitamin D the bone strength is compromised causing fractures Most importantly the cause of death in the majority of children dying suddenly and unexpectedly remains unidentified Vitamin D deficiency has been implicated in some cases

Our study will be looking at vitamin D levels in blood and tissue changes suggesting bone disease The study will be conducted alongside routine coroner's post mortem examination

would be most grateful for your help in how we should best approach parents for the research and would very much appreciate your valuable views on our participant information sheets and consent process We have therefore invited you to this focus group so we can discuss what parents might find most appropriate and helpful

Thank you for your time look forward to meeting you

Suma Uday



CHAIRMAN
Dame Christine Braddock DBE DL
CHIEF EXECUTIVE OFFICER
Sarah-Jane Marsh



Appendix 9: Research Ethics Committee approval

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

The REC favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements. Each NHS organisation must confirm through the signing of agreements and/or other documents that it has given permission for the research to proceed (except where explicitly specified otherwise).

Guidance on applying for NHS permission for research is available in the Integrated Research Application System, www.hra.nhs.uk or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of management permissions from host organisations

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant (for medical device studies, within the timeline determined by the current registration and publication trees).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to request a deferral for study registration within the required timeframe, they should contact hra.studyregistration@nhs.net. The expectation is that all clinical trials will be registered, however, in exceptional circumstances non registration may be permissible with prior agreement from the HRA. Guidance on where to register is provided on the HRA website.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
IRAS Application Form [IRAS_Form_24012017]		24 January 2017
IRAS Checklist XML [Checklist_20022017]		20 February 2017
Letter from sponsor		23 January 2017
Other [Materials Transfer Agreement V1 13.01.17 - BCHFT tracked changes]	1	13 January 2017
Other [Revised protocol]	2.2	20 February 2017
Other [Revised participant information sheet v2.3]	2.3	20 February 2017
Other [Revised Participant Brief Information Sheet v1.1 20_2_17]	1.1	20 February 2017
Other [Revised consent form v2.2 9_2_17]	2.2	09 February 2017
Other [Coroner's letter]	NA	13 February 2017
Other [Supporting letter]	NA	20 February 2017
Summary CV for Chief Investigator (CI) [CI CV]	NA	03 November 2016
Summary CV for student [CV student]	NA	
Summary CV for supervisor (student research) [CI or Supervisor CV]	NA	03 November 2016

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol

- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website: http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

17/EM/0061	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project.

Yours sincerely

[redacted]

Mr John Aldridge
Chair

Email:NRESCommittee.EastMidlands-Nottingham1@nhs.net

Enclosures: "After ethical review – guidance for researchers"

Copy to: *Mrs Theresa Morton*
Ms Rachel Rikunenko, Birmingham Children's Hospital

Appendix 10: Health Research Authority approval

NHS
Health Research Authority

Dr Wolfgang Hoegler
Birmingham Children's Hospital
Department of paediatric endocrinology and diabetes
Level 3, Ladywood house,
Steelhouse lane,
Birmingham
B4 6NH

Email: hra.approval@nhs.net

10 March 2017

Dear Dr Hoegler

Letter of HRA Approval

Study title:	**Prevalence of occult bone disease due to vitamin D deficiency in sudden unexpected death in childhood: A post-mortem study.**
IRAS project ID:	**207267**
Protocol number:	**2.1**
REC reference:	**17/EM/0061**
Sponsor	**Birmingham Children's & Women's Hospital**

I am pleased to confirm that HRA Approval has been given for the above referenced study, on the basis described in the application form, protocol, supporting documentation and any clarifications noted in this letter.

Participation of NHS Organisations in England

The sponsor should now provide a copy of this letter to all participating NHS organisations in England.

Appendix B provides important information for sponsors and participating NHS organisations in England for arranging and confirming capacity and capability. **Please read *Appendix B* carefully**, in particular the following sections:

- *Participating NHS organisations in England* – this clarifies the types of participating organisations in the study and whether or not all organisations will be undertaking the same activities
- *Confirmation of capacity and capability* - this confirms whether or not each type of participating NHS organisation in England is expected to give formal confirmation of capacity and capability. Where formal confirmation is not expected, the section also provides details on the time limit given to participating organisations to opt out of the study, or request additional time, before their participation is assumed.
- *Allocation of responsibilities and rights are agreed and documented (4.1 of HRA assessment criteria)* - this provides detail on the form of agreement to be used in the study to confirm capacity and capability, where applicable.

IRAS project ID	207267

Further information on funding, HR processes, and compliance with HRA criteria and standards is also provided.

It is critical that you involve both the research management function (e.g. R&D office) supporting each organisation and the local research team (where there is one) in setting up your study. Contact details and further information about working with the research management function for each organisation can be accessed from www.hra.nhs.uk/hra-approval.

Appendices

The HRA Approval letter contains the following appendices:

- A – List of documents reviewed during HRA assessment
- B – Summary of HRA assessment

After HRA Approval

The document *"After Ethical Review – guidance for sponsors and investigators"*, issued with your REC favourable opinion, gives detailed guidance on reporting expectations for studies, including:

- Registration of research
- Notifying amendments
- Notifying the end of the study

The HRA website also provides guidance on these topics, and is updated in the light of changes in reporting expectations or procedures.

In addition to the guidance in the above, please note the following:

- HRA Approval applies for the duration of your REC favourable opinion, unless otherwise notified in writing by the HRA.
- Substantial amendments should be submitted directly to the Research Ethics Committee, as detailed in the *After Ethical Review* document. Non-substantial amendments should be submitted for review by the HRA using the form provided on the HRA website, and emailed to hra.amendments@nhs.net.
- The HRA will categorise amendments (substantial and non-substantial) and issue confirmation of continued HRA Approval. Further details can be found on the HRA website.

Scope

HRA Approval provides an approval for research involving patients or staff in NHS organisations in England.

If your study involves NHS organisations in other countries in the UK, please contact the relevant national coordinating functions for support and advice. Further information can be found at http://www.hra.nhs.uk/resources/applying-for-reviews/nhs-hsc-rd-review/.

If there are participating non-NHS organisations, local agreement should be obtained in accordance with the procedures of the local participating non-NHS organisation.

IRAS project ID	207267

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please email the HRA at hra.approval@nhs.net. Additionally, one of our staff would be happy to call and discuss your experience of HRA Approval.

HRA Training

We are pleased to welcome researchers and research management staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

Your IRAS project ID is **207267**. Please quote this on all correspondence.

Yours sincerely

Aliki Sifostratoudaki
Assessor

Email: hra.approval@nhs.net

Copy to: *Mrs Theresa Morton, Birmingham Children's Hospital, Sponsor Contact*
Ms Rachel Rikunenko, Birmingham Children's Hospital, R&D Contact

Appendix 11: Participant information leaflet

Birmingham Women's and Children's NHS Foundation Trust

Birmingham Community Healthcare NHS Foundation Trust

Hidden bone disease due to vitamin D deficiency in sudden childhood death

Detailed study information sheet for parents and carers
Version 3.0 7th December 2018

We would like to offer sincere condolences to you and your family. We are sad to hear about the loss of your child. We really appreciate you taking the time to read this information and consider this study during this difficult time.

I am Dr Suma Uday. I am a children's doctor working at Birmingham Children's Hospital (BCH).
Dr Wolfgang Högler (Children's doctor specialising in bone disease at University of Birmingham), Dr Tamas Marton (Doctor examining the cause of death on Coroner's post-mortem examination at Birmingham Women's Hospital), Dr Joanna Garstang (lead doctor for unexpected child death) and I are conducting an important study about the bone health of children with vitamin D deficiency. This might help understand more about why babies die unexpectedly.

We would like to invite you to give your consent for tissue and blood samples from your child to help with this study. In order to help you make a decision we would like to tell you why the study is being done and what it involves.
Part 1: tells you about the purpose of the study and what will happen if you consent for these samples to be used
Part 2: gives you more details about the conduct of the study

Part 1 - Why we are doing this study

What is the purpose of the study?

Background
Long standing vitamin D deficiency can cause a bone disease called rickets (soft bones) in children.

- Rickets can present with soft skull and delayed closure of soft spot, bowed legs, knock knees, low muscle tone, delayed development milestones. Severe rickets in babies can cause seizures due to low calcium and affect heart muscles causing it to fail; both of which can potentially lead to infant death.
- Rickets is diagnosed on x-ray changes in bones. However, x-ray changes are only seen very late in the disease when bone strength is very low and the child is prone to fractures.
- We have little understanding about the level of vitamin D that softens bone at the tissue level (osteomalacia) before X-rays become abnormal.

This issue can only be studied by:

- Looking at tissue samples from the rib and hip bones under the microscope
- Directly testing the strength of a small piece of bone using a specific device

Why?

- To determine the true occurrence of hidden osteomalacia and rickets.
- To identify the level of vitamin D at which bones soften
- To inform child health care programs for prevention of disease
- To help understand how bone softness contributes to bone strength

1

Birmingham Women's and Children's NHS Foundation Trust

Birmingham Community Healthcare NHS Foundation Trust

Why have I been approached?

You have been invited to take part in the study because your child will be undergoing a coroner's post mortem examination to look into the cause of death. Tissue samples will be taken as part of post mortem examination. This study will involve taking one additional bone sample.

Do I have to consent?

Consenting for the study is ***entirely your decision***. Once you have read through the information given below you can contact Dr Uday if you have any questions.

What will happen if I give my consent?

If you are willing to consent we will be studying blood and tissue samples already collected during your child's post mortem examination as well as one additional bone sample. This will involve two things:

1. Using part of the blood and tissue already collected for further examination
   a. A part of the blood sample will be used to measure vitamin D levels
   b. The growing part of the rib bone will be examined for changes specific to rickets
   c. Radiographs (x-rays) will be studied in detail for the presence of rickets.
   d. The size and weight of the heart measured will be documented
   e. A small piece (3-4cm) of rib is already extracted. Bone strength will be tested on a part of this piece by a method called 3-point bending test, which is the gold standard for measuring bone strength. The test will be performed by biomechanical scientists at Aston or Sheffield University in batches. At the end of the study this sample will be dealt with as per your wishes expressed in the Coroner's consent form.
2. Taking an additional bone sample which does not form part of the post mortem examination
   a. A small 5 mm sample from the hip bone will be collected using a needle when the hip bone is routinely exposed for examination. This sample will be sent to a specialised laboratory in Vienna for further examination.

The additional bone sample collected at post-mortem examination will be dealt with as per your ***wishes expressed during telephone consent.*** At the time of consent we would like to request you to provide information on whether you (during pregnancy) and your child were on vitamin D supplements. We would like to access your child's case records to obtain details of age, sex, ethnicity, residence post code and details of any medical conditions.

Are there any benefits from taking part in the study?

There may be no direct benefit to your family from taking part in this study. However, undiagnosed vitamin D deficiency has previously been associated with child deaths, and as part of this study we will be looking into whether vitamin D deficiency may have a role in child death. Currently the cause of death remains unidentified in 60% of unexpected infant deaths. The findings of this study may help us prevent vitamin D deficiency related disease in the future by informing preventive strategies.

The results of blood vitamin D levels and rib growth plate changes will be included in the post mortem report; these will be explained to you by your paediatrician.

Are there any risks from taking part in the study?

As the study is conducted alongside the usual post mortem examination, we do not anticipate any risks from taking part in the study. There will be no delay in post mortem examination due to participation in the study.

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What will happen if I change my mind?

You can withdraw your consent from the study within 3 months of providing consent without giving a reason as participation is ***completely voluntary***. In case of withdrawal of consent the information collected will not be included in the study.

Is participation in the study anonymous?

Under the General Data Protection Regulation (GDPR), data will be stored on a password protected drive at Birmingham Women's and Children's Hospital. The data will be accessible to all study team members, however participants will only be identifiable by Dr Uday. All information published will be anonymised and no patient details will be identifiable.

How will the information collected be used?

Birmingham Women's and Children's Hospital is the sponsor for this study. We will be using information from your child's medical records in order to undertake this study and will act as the data controller for this study. This means that we are responsible for looking after the information and using it properly. Birmingham Women's and Children's Hospital will keep identifiable information about your child for 25 years after the study has finished.

Your rights to access, change or move your child's information are limited, as we need to manage your information in specific ways in order for the research to be reliable and accurate. If you withdraw your consent from the study, we will keep the information about your child that we have already obtained. To safeguard your child's rights, we will use the minimum personally-identifiable information possible.

You can find out more about how we use your information by contacting the research and development team at 0121 333 8707.

Birmingham Women's and Children's Hospital will collect information about your child for this research study from your child's medical records. This information will include their name, age, gender, contact details and health information, which is regarded as a special category of information. We will use this information only for this study.

What if there is a problem?

If you have any concerns about any aspect of the study you can contact me (**e-mail** [redacted]. I will do my best to answer your questions. If you do not wish to raise your concerns with me directly you can contact my supervisor (e-mail [redacted] or the Research and Development department (e-mail: bwc.research@nhs.net, Tel: 0121 4721377). Alternatively you may wish to contact Patient Advice Liaison Services (PALS) at Birmingham Children's Hospital (01213338403) who offer confidential advice, support and information on health and NHS related matters and also help you with complaints procedure.

What will happen to the results of the study?

The results of the study will be shared in the following manner:

1. The findings will form part of the post-mortem examination report , this will be discussed with you by your paediatrician or pathologist
2. A summary of the study for all professionals involved
3. Presentation at professional conferences to inform future health care for babies
4. Publication in peer reviewed scientific journals

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Who has reviewed this study?

All research in the NHS is reviewed by an independent group of people called the research ethics committee. This study has been reviewed by East Midlands - Nottingham 1 Research Ethics Committee (Ref: 17/EM/0061)

Who has funded the study?

The study has been funded by Nutricia early life nutrition in collaboration with Royal National Orthopaedic Hospitals NHS trust to support clinical research to improve nutrition and health in early years of life. The research fellow has been funded by a Global Challenges PhD scholarship by the University of Birmingham. The scholarship program aims to improve maternal and child health globally.

What do I do now?

If you have agreed to be contacted by the research team and are eligible you will be telephoned about the study. If you wish to give consent you can do this verbally over the telephone, which will be recorded. If you would like more time to think or need any clarifications I (Suma Uday) will telephone you the next day. After I have answered all your questions if you express your wish to participate, I will ask you to give recorded verbal consent to me at the time of this call.
Once you have consented over the telephone you will be sent a letter confirming your willingness to participate in the study. We will also send you the details of who to contact in case you change your mind as you are under no pressure and can withdraw from the study for up to 3 months from the end of the study.

Study team contact information

Lead Researcher:
Dr Suma Uday
Registrar in Paediatric endocrinology and diabetes, Birmingham Children's Hospital, Steelhouse lane, Birmingham, B4 6NH.
E-mail: , Tel:

Chief investigator/Principal investigator (Birmingham)
Dr Tamas Marton
Consultant Perinatal Pathologist, Birmingham Women's Hospital, Mindelsohn way, Birmingham, B15 2TG. E-mail:
, Tel:

Principal investigator (Sheffield)
Dr Marta Cohen
Consultant Paediatric Pathologist, Sheffield Children's Hospital NHS Trust, Western Bank, Sheffield, S10 2TH. E-mail:
Tel:

Research and development department contact:
Julie Kane
R&D Facilitator, Birmingham Children's Hospital, Steelhouse lane, Birmingham, B4 6NH. E-mail: bwc.research@nhs.net ,
Tel:

Appendix 12: Brief participant information leaflet

Birmingham Women's and Children's NHS Foundation Trust

Birmingham Community Healthcare NHS Foundation Trust

Hidden bone disease due to vitamin D deficiency in sudden childhood death

Brief study information sheet for parents and carers Version 1.62 7th Dec 2018

We would like to offer sincere condolences to you and your family. We are sad to hear about the loss of your child. We really appreciate you taking the time to read this information and consider this study during this difficult time, which might help understand why babies die.

I, Dr Suma Uday, along with Dr Wolfgang Högler, Dr Tamas Marton and Dr Joanna Garstang are interested in finding out whether vitamin D deficiency has a role in child death.

Background:

Long standing vitamin D deficiency in children can cause rickets. Rickets can present with soft skull and delayed closure of soft spot, bowed legs, knock knees, low muscle tone, delayed development milestones. Severe rickets in babies can cause seizures due to low calcium and affect heart muscles causing it to fail; both of which can potentially lead to infant death. As the cause of death remains unknown in 60% of unexpected infant deaths we would like to examine if vitamin D deficiency has a role in infant death.

What does the study involve?

- We would like to obtain your consent to examine in detail the tissues collected from your child during post-mortem examination.
- In addition to the already collected samples we would like to obtain one very small (5 mm) sample from your child's hip bone when it is exposed for examination to look for signs of bone disease related to vitamin D deficiency.
- We would also like to access your child's health records to look for any other relevant medical information which we feel may affect bone health.
- We would like to ask you about whether you took vitamin D supplements in pregnancy, during breastfeeding or gave these to your child.

What do I do now?

If you have agreed to be contacted by the research team and are eligible you will be telephoned about the study. If you wish to give consent you can do this verbally over the telephone, which will be recorded. If you would like more time to think or need any clarifications I (Suma Uday) will telephone you the next day. After I have answered all your questions if you express your wish to participate, I will ask you to give recorded verbal consent to me at the time of this call.

Once you have consented over the telephone you will be sent a letter confirming your willingness to participate in the study. We will also send you the details of who to contact in case you change your mind as you are under no pressure and can withdraw from the study for up to 3 months from the end of the study.

Participation in this project is completely voluntary and you can withdraw from the study within 3 months of the study end date.

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Birmingham Community Healthcare NHS NHS Foundation Trust

Are there any risks?

We do not anticipate any risks from participation in the study. There will be no delay in post-mortem examination as the research samples mostly form part of routine examination.

Benefits

The study is unlikely to be of benefit to your family but it may help us understand more about the relationship between Vitamin D and child death. The results will help us inform preventive health strategies to improve child health in the future.

Further information and study team contact:

Please refer to the attached "Detailed study information sheet for parents and carers Version 3.0 dated 7th Dec 2018". If you need any further clarifications please contact me on [redacted] or [redacted]

Principal investigator (Birmingham)
Dr Tamas Marton
Consultant Perinatal Pathologist
Birmingham Women's Hospital, Mindelsohn way, Birmingham, B15 2TG
E-mail: [redacted] Tel: [redacted]

Principal investigator (Sheffield)
Dr Marta Cohen
Consultant Paediatric Pathologist
Sheffield Children's Hospital NHS Trust, Western Bank, Sheffield. S10 2TH
E-mail: [redacted] Tel: [redacted]

Appendix 13: Informed consent form

Birmingham Women's and Children's NHS
NHS Foundation Trust

Birmingham Community Healthcare NHS
NHS Foundation Trust

Consent form V 2.4 29/5/2018

Study title: Hidden bone disease due to vitamin D deficiency in sudden childhood death

Researchers: Dr Suma Uday / Dr Tamas Marton / Dr Joanna Garstang / Dr Wolfgang Högler

Name of child: _____

Name of person giving telephone consent: _____

Relationship to the child: _____

Name of person obtaining telephone consent: _____

Signature: _____ Date: _____

You have understood and consented to the following over the telephone (which has been recorded with your permission and will be used as proof of consent):

For your child's samples to be sent to University of Birmingham, Aston/Sheffield University and the laboratory in Vienna for further examination as part of the research study. ☐

That data collected will be treated as confidential, will be fully anonymised and stored securely. ☐

That participation is voluntary and you are free to withdraw consent within 3 months from end of study without giving any reasons *(date will be inserted based on start date)* ☐

We discussed the options available in relation to the method of disposal of the additional, tiny hip bone tissue sample and you have opted for the remaining sample to be:

Retained for the purpose of future diagnosis ☐

Retained for the purpose of future diagnosis, audit, teaching health professionals and ethically approved research ☐

Disposed of in a respectful and legal manner ☐

Returned via their Funeral Director to deal with lawfully in accordance with their wishes
Name and address of Funeral Director: ☐

If any of the above information is incorrect or you wish to withdraw from the study please contact the lead researcher on the details provided below:
Dr Suma Uday, Registrar in Paediatric Endocrinology and Diabetes,
Birmingham Women's and Children's Hospital, Steelhouse lane, Birmingham, B4 6NH
Contact: [redacted], Tel: [redacted]

Consent form Version 2.4

Appendix 14: Calcium and vitamin D content of frequently used formula feeds

Formula feed **Recommended (minimum- maximum)**	**Calcium /100 ml of prepared feed** 34-94 mg/100 ml	**Vitamin D / 100 ml prepared feed** 1.3-2 μg/ 100 ml
Aptamil first (from birth)	60 mg	1.65 μg
Aptamil follow on (6-12 months)	73 mg	1.70 μg
Cow and gate first infant milk	53 mg	1.45 μg
Cow and gate hungry infant	61 mg	1.40 μg
SMA Pro first infant milk	43 mg	1.5 μg
SMA anti-reflux (birth – 12 months)	46 mg	1.70 μg

Vitamin D 1 μg = 40 IU