The differential response to vitamin D supplementation in neonates born with low vitamin D stores according to skin pigmentation

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Abstract

Vitamin D is a fat-soluble vitamin that can be obtained endogenously by exposing the skin to ultraviolet beta radiation from sunlight or exogenously from food and supplements. Due to the recommendation that infants should not be exposed to sunlight, and low vitamin D content in breast milk, this age group is recommended to be given a vitamin D supplement until a sufficient amount can be obtained from their diet. The Canadian Paediatric Surveillance Program (2002-2004) estimated a total of 104 confirmed cases of vitamin D deficiency rickets, most of which were diagnosed in the north or immigrant families. From the confirmed cases, 92 (89%) were children with intermediate to dark skin color, and 98 (94%) were children who were breastfed without suitable vitamin D supplementation. Given that vitamin D supplementation is highly recommended for infants with dark skin, how these infants respond to vitamin D supplementation if they are born with low vitamin D stores, and if a higher dosage is needed to overcome the deficiency is important to explore. Previous studies are difficult to interpret because either they studied infants with sufficient vitamin D status or lacked an objective method to measure skin color if any. The primary research objective of this thesis is to investigate if there is a difference in the response of serum 25-hydroxyvitamin D (25(OH)D) to vitamin D supplementation in neonates born with low vitamin D stores (25(OH)D <50 nmol/L) according to skin pigmentation, and if a higher dose of vitamin D supplementation will result in a faster rate of improvement of vitamin D status in infants with dark skin. Healthy term born breastfed infants were recruited from the Lakeshore General Hospital in the Greater Montreal area, latitude 45.5^{o}N (NCT02563015). Infants were screened for low serum 25(OH)D (Liaison, DiaSorin, Stillwater, MN 55082, USA) at birth and then randomized to a treatment group: 400 IU/d (n=36) or 1000 IU/d (n=36). Infants were stratified by skin pigmentation in male and female blocks. At baseline, 3, and 6 months,

anthropometry, body composition, skin pigmentation, and biochemistry measurements were examined. The study sample was 50% male, 24% infants with dark skin color, and 65% breastfed until 6 months; compliance rate was 70% overall. After 6 months of supplementation, all groups had mean serum 25(OH)D concentration above the 50 nmol/L threshold as suggested by the Institute of Medicine for achievement of bone health. Overall, infant skin color did not affect the infant serum 25(OH)D response to vitamin D supplement at any time point ($p=0.40$). The 1000 IU/d group had a significantly higher mean concentration than the 400 IU/d group at 3 and 6 months. This study shows that skin pigmentation was not a significant determinant of infant serum 25(OH)D concentration at 3 or 6 months of age. Given the high rate of compliance observed in this study, public health efforts to improve vitamin D status in infants of all skin colors might be best focused towards improving compliance through educational means.

Resume

La vitamine D est une vitamine liposoluble qui peut être obtenue de façon endogène par l'exposition de la peau aux rayons ultraviolets B du soleil ou de façon exogène sous forme de supplément ou apport alimentaire. Puisqu'il faut éviter d'exposer les nourrissons aux rayons du soleil et que le lait maternel contient peu de vitamine D, il est recommandé de donner à ce groupe d'âge un supplément de vitamine D jusqu'à ce que l'alimentation arrive à en fournir une quantité suffisante. Le Programme Canadien de Surveillance Pédiatrique a confirmé un total de 104 cas de carence en vitamine D (2002-2004), dont la majorité a été diagnostiquée dans le Nord du Canada ou chez des familles d'immigrants. Des cas confirmés, 92 (89 %) enfants avaient une peau de couleur intermédiaire à foncée, et 98 (94 %) enfants avaient été allaités sans supplémentation de vitamine D. Étant donné que la supplémentation de vitamine D est largement recommandée chez les nourrissons avec la peau foncée, il importe d'examiner comment ces nourrissons réagissent aux suppléments de vitamine D s'ils sont nés avec une carence en vitamine D, et si une dose plus élevée est nécessaire pour surmonter la carence. Les études antérieures sont difficiles à interpréter, soit parce qu'elles ont porté sur des nourrissons qui ne présentaient pas de carence en vitamine D, soit parce qu'elles n'ont pas eu recours à une méthode objective pour mesurer la couleur de la peau, le cas échéant. Le premier objectif de recherche de la présente thèse est de déterminer s'il y a une différence dans la réponse de la 25-hydroxyvitamine D (25[OH]D) sérique à la supplémentation de vitamine D chez les nouveau-nés ayant une faible réserve de vitamine D (25(OH)D <50 nmol/L) en fonction de la pigmentation de la peau, et si une dose plus élevée de vitamine D provoquera une hausse plus rapide du taux de 25(OH)D chez les nourrisons avec la peau foncée. On a recruté des bébés en bonne santé, nés à terme et allaités, à l'Hôpital Général du Lakeshore. L'hôpital se trouve à une latitude de 45,5

°N (NCT02563015) et dessert la grande région de Montréal. On a sélectionné les nourrissons avec un faible taux de 25(OH)D sérique à la naissance (Liaison, DiaSorin, Stillwater, MN 55082, États-Unis), puis ils ont été répartis aléatoirement dans deux groupes expérimentaux: 400 UI de vitamine D/jour (n = 36) ou 1 000 UI/jour (n = 36). Les nourrissons ont été stratifiés selon la pigmentation de leur peau et selon leur sexe. Les critères suivants ont été examinés au début de l'étude, à 3 mois et à 6 mois: données anthropométriques, composition corporelle, pigmentation de la peau et mesures biochimiques. Dans le groupe etudié, 50 % des bébés étaient de sexe masculin, 24 % avaient la peau foncée et 65 % étaient allaités jusqu'à 6 mois; le taux de conformité de supplémentation de vitamine D a été de 70 % dans l'ensemble. Après 6 mois de supplémentation, les nourrissons de tous les groupes avaient une concentration sérique moyenne de 25(OH)D supérieure au seuil de 50 nmol/L suggéré par l'Institute de Medicine pour assurer la bonne santé des os. La couleur de la peau des nourrissons n'a eu d'incidence sur la concentration sérique de 25(OH)D à aucun des points temporels évalués après la supplémentation de vitamine D ($p = 0,40$). Les nourrissons du groupe de 1 000 UI/jour présentait une concentration moyenne de 25(OH)D nettement plus élevée que le groupe des 400 UI/jour à 3 et à 6 mois. Cette étude montre que la pigmentation de la peau ne constitue pas un déterminant important du taux de 25(OH)D dans le sang à l'âge de 3 et 6 mois. Étant donné le taux de conformité élevé constaté dans le cadre de cette étude, les efforts de santé publique visant à améliorer le statut en vitamine D chez les nourrissons, sans égard à la couleur de leur peau, auraient avantage à passer par la sensibilisation des personnes concernées à l'importance du conformément aux recommandations de supplémentation.

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Contribution of authors

Norah was the primary author of the manuscript in this thesis and was a large contributor to the work included. She attended study visits and assisted with anthropometric and body compositions as part of the research team. She also had a role with study visit sampling; she analyzed infants blood ionized calcium and collected and stored infant blood and urine. She wrote the literature review, manuscript and discussion parts.

Dr. Hope Weiler was the principal investigator of the trial and coordinated all the study aspects. She was also Norah's direct supervisor.

ABBREVIATIONS

$1,25(OH)_2D$	1,25-dihydroxyvitamin D
25(OH)D	25-hydroxyvitamin D
AAP	The American Academy of Pediatrics
AI	Adequate Intake
ANOVA	Analysis of variance
BMI	Body mass index
BSA	Body surface area
CDA	The Canadian Dermatology Association
CHMS	Canadian Health Measures Survey
CIE	International Commission on Illumination
CLIA	Chemiluminescent immunoassay
CNF	Canadian Nutrient File
CONSORT	Consolidated Standards of Reporting Trials
CPS	Canadian Paediatric Society
CV	Coefficient of variation
CYP	Cytochrome P450
d	Day
DBP	Vitamin D binding protein
DEQAS	Vitamin D External Quality Assessment Scheme
DIN	Drug Identification Number
DRI	Dietary Reference Intakes
DXA	Dual-energy x-ray absorptiometry
FFQ	Food frequency questionnaire
IOM	Institute of Medicine
ITA°	Individual typology angle
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
NIST	National Institute of Standards and Technology
RCT	Randomized controlled trial
RDA	Recommended Dietary Allowance
REDCap	Research Electronic Data Capture
SD	Standard deviation
SPF	Sun protection factor
UL	Tolerable upper intake level
UVB	Ultraviolet beta
UVR	Ultraviolet radiation
Vitamin D_2	Ergocalciferol
Vitamin D_3	Cholecalciferol
WHO	World Health Organization

UNITS

cm	Centimeter
g	Gram
IU	International unit
kg	Kilogram
L	Liter
m	Meter
ml	Milliliter
mmol	Millimole
nmol	Nanomole
nm	Nanometer
ng	Nanogram
µg	Micrograms

CONVERSION FACTORS

1 ng/mL of 25(OH)D_3=2.50 nmol/L
1 ng/mL of 25(OH)D_2=2.42 nmol/L
1 µg of vitamin D=40 IU

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1.0 Literature review

1.1 Introduction

Vitamin D is considered as an essential nutrient needed to maintain adequate serum calcium and phosphate concentrations and ensured normal mineralization of bone and to prevent rickets [2]. Vitamin D is a fat-soluble vitamin that can be obtained endogenously by exposing the skin to ultraviolet beta (UVB) radiation from sunlight or exogenously from food and supplement. Due to the recommendation that infants should not be exposed to sunlight [3], and low vitamin D content in breast milk [4], this age group is recommended to be given a vitamin D supplement until a sufficient amount can be obtained from their diet [2]. Very early in the history of the prevention of rickets, Canadian mothers were encouraged to give their infants cod liver oil which is a rich source of vitamin D [4, 5]. In 1967, 400 IU/d was recommended for all infants from two weeks of age, which aligns with the current recommendation [6]. The Canadian Paediatric Surveillance Program (2002-2004) estimated a total of 104 confirmed cases of vitamin D deficiency rickets, most of which were diagnosed in the north or immigrant families [7]. From the confirmed cases, 92 (89%) were children with intermediate to dark skin color, and 98 (94%) were children who were breastfed without suitable vitamin D supplementation [7]. Although there are studies that link vitamin D deficiency with dark skin color in infants, there is a gap in knowledge about how they respond to vitamin D supplementation and if a higher dosage is needed to overcome the deficiency.

1.2 Vitamin D: background

1.2.1 Metabolism

Vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol) are the two forms of vitamin D that can be found in food or supplements [2, 8]. Vitamin D_3 is produced after exposure of

mammalian skin to sunlight [9]. Details of the metabolism of vitamin D are beyond the scope of this thesis, and these are outlined in recent reviews [10, 11] as the following: in the skin, 7-dehydrocholesterol absorbs UVB radiation from the sun, which causes changes in the double bonds and forms previtamin D_3 after opening the B ring. This process occurs only under the influence of UVB, and there is no enzymatic factor involved, whereas the rest of the hydroxylation steps are implemented by cytochrome P450 (CYP) mixed-function oxidases. Then previtamin D_3 goes through further rearrangement of the bonds that makes it more stable. When D_3 is formed, it is transported by the vitamin D bonding protein (DBP) to the circulation [9]. Dietary vitamin D is integrated into chylomicrons and then carried by the lymphatic system into the venous circulation. Vitamin D from both sources can be stored and then released from adipocytes. Vitamin D, which binds to DBP in the circulation, transports into the liver where the 25-hydroxylase (apparently CYP2R1) can convert vitamin D to 25-hydroxyvitamin D (25(OH)D). The active form of vitamin D is 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) known as calcitriol, which can be formed after metabolism of 25(OH)D mediated by 1α-hydroxylase (CYP27B1), mainly in the kidneys. Other sites where this enzyme can be found are the placenta, macrophages and monocyte.

All major vitamin D intermediate metabolites can be epimerized through a C-3 epimerization pathway [12]. Epimers have identical chemical structures except for the C-3 position where the A-ring is transformed from the beta to alpha orientation [12, 13]. Infants under one year of age have a significant concentration of C-3 epimers that contributed between 8.7-61.1% of total 25(OH)D concentration with unclear biological importance [13].

1.2.2 Vitamin D isoforms

There is no difference in the hydroxylation steps between vitamin D_2 and vitamin D_3 [14] although there are two points of view about their role as supplements in the adult literature. The first one suggests that vitamin D_3 is more effective than vitamin D_2 [15] and the other argues that both of them are equally efficient in maintaining 25(OH)D if given on a daily basis [16, 17]. There is insufficient evidence in the pediatric area to measure the difference in effectiveness between vitamin D_2 and D_3 supplementation. One study found that after 3 months of infant life, both vitamin D_2 and D_3 supplementation equally elevated the circulating total 25(OH)D concentration [18]. Another study aimed to compare the efficacy of vitamin D isoforms in infants from 1 to 4 months of age, who were healthy and breastfed, concluded that both isoforms elevated plasma 25(OH)D concentration to the same level [19].

1.2.3 Vitamin D status

Three categories are mainly used to describe vitamin D status: sufficient, insufficient and deficient. By considering that 25(OH)D is the primary circulating metabolite of vitamin D with a long half-life in circulation between 2 to 3 weeks, it is the most widely accepted measurement of vitamin D status [17]. The Institute of Medicine (IOM) considers levels more than or equal to 50 nmol/L of 25(OH)D as sufficient and preferable for all people, between 30 nmol/L and 49.9 nmol/L as inadequate in some people. Moreover, it considers levels less than 30 nmol/L as high risk for deficiency [2].

The Canadian Paediatric Society (CPS) considers levels less than 25 nmol/L of 25(OH)D as deficient; between 25 nmol/L and 75 nmol/L as insufficient; and between 75 nmol/L and 225 nmol/L as sufficient [20] (**Table 1.1**). The American Academy of Pediatrics (AAP) recommends that infants should have serum 25(OH)D concentration ≥ 50 nmol/L [21]. However, to the best

of the author's knowledge, few publications can be found on the optimal 25(OH)D concentration for infants.

Table 1.1: Vitamin D status according to the IOM and CPS

Category	IOM[1] Serum 25(OH)D level (nmol/L)	CPS[2] Serum 25(OH)D level (nmol/L)
Deficient	< 30	< 25
Inadequate[1]/insufficient[2]	30-49.9	25-75
Sufficient	50-125	75-225

[1] Institute of Medicine [2].
[2] Canadian Paediatric Society [20].

1.2.4 Recommendations

Increased understanding of the metabolism of vitamin D and its impact on health has led to the development of revised recommendations for vitamin D. The Recommended Dietary Allowance (RDA) per day according to IOM, in 2011, is the following: 600 IU/d for age group 1 to 70 years including pregnant and lactating women; and 800 IU/d for adults more than 70 years [2]. Regarding the infant population, the IOM recommended an Adequate Intake (AI) value of 400 IU/d for infants from 0 to 12 months [2]. The CPS, Dietitians of Canada, Health Canada [22] (**Table 1.2**), and The AAP recommend the same dose [21]. Interestingly, Health Canada has a longstanding history of recommending that infants receive 400 IU of vitamin D on a daily basis. Very early in the history of the prevention of rickets, in 1930, Canadian mothers were encouraged to give their infants cod liver oil, which is a high source of vitamin D and 5 ml provides around 426 IU [4]. They were recommended to start from one week to two years of age starting from two or three drops to four teaspoons a day [5]. In 1967, 400 IU/d was recommended for all infants (breastfed or not) from two weeks of age, which aligns with the current recommendation [6]

The tolerable upper intake level (UL) was also set at 1000 IU/day for infants from birth to 6 months and 1500 IU/day for infants from 6 months to one year [2, 17]. Additionally, the IOM reported that there is no need for further recommendations for special groups such as high northern latitudes, or dark skinned people since the Dietary Reference Intakes (DRI) values have been estimated at the levels ensuring sun exposure is unnecessary to obtain adequate vitamin D [2]; on the other hand, the CPS recommends twice (800 IU/d) the IOM recommendation of vitamin D supplementation for infants in communities where deficiency is common such as northern communities in the winter months [20].

Furthermore, the IOM recommendations did not account for body weight which plays a fundamental role in the amount of dose [20, 23, 24] since an increase in body weight causes a

lower response to a given dose [25]. In that case, the effect of age on vitamin D status might be reduced when taking body weight into consideration [25]. Given these points, the IOM committee members explain that there is a lack of evidence to support if obese persons need more vitamin D intake than the general population [26]. Even if evidence is available, it might not be practical to apply it in the infant population where weight gain is fast, since that may cause confusion because the dose would change quickly.

Although the IOM announced that taking more than the new RDA is not linked with any more health benefits [2], currently many studies that focused on adult population suggest doses much higher for reaching the sufficiency level of 75 nmol/L [27]. In infant population, Gallo, Comeau et al.[28] found that doses more than 400 IU/d did not provide any more health benefits for bone mineral or growth.

Table 1.2: Canadian vitamin D recommendations for infants

Agency/Society	Vitamin D
Health Canada [22]	400 IU/d
Dietitians of Canada [29]	400 IU/d
Canadian Paediatric Society [20]	400 IU/d 800 IU/d[1]

[1]For Northern First Nations and Inuit communities in winter.

1.2.5 Toxicity

The primary descriptions of vitamin D toxicity are hypercalciuria, hypercalcemia, suppressed parathyroid hormone, and serum 25(OH)D more than 250 nmol/L [30]. The CPS considers serum 25(OH)D concentration more than 500 nmol/L as toxic and 225 nmol/L as linked with hypercalcemia and calcium deposition in tissue [20]. Symptoms of hypercalcemia are polydipsia, poor appetite, weight loss, abdominal pain, vomiting, polyuria and constipation [14]. The Canadian Health Measures Survey (CHMS) showed that less than 0.5% of the population age 6 to 79 had serum concentration more than 220 nmol/L [31]. In infants, high doses of vitamin D around 900000 to 4000000 IU over a period of 2-8 weeks can cause vitamin D toxicity [32]. Existing studies address vitamin D toxicity in two contexts: from dietary sources or from supplements. One example was in 1950 when pediatrician noted an outbreak of hypercalciuria thought to be due to high doses of vitamin D in infants' diets in the United Kingdom [33, 34]. Moreover, misunderstanding of physician instruction by parents can cause excess intake of vitamin D supplementation or over the counter supplementation errors due to dosages and concentrations [35, 36].

Regarding manufacturing errors, this could occur with any supplement. Kara, Gunindi et al. reported cases of 7 children suffering from vitamin D intoxication as a result of using a local supplement (fish oil) which contianed a massive amount of vitamin D [37]. In general, vitamin D toxicity is uncommon in children [14], yet it is advisable to take precautions to prevent it from happening.

1.2.6 Testing of vitamin D deficiency

The rate of vitamin D testing has grown in developed countries as a result of raised awareness regarding the possible association between vitamin D deficiency and unfavorable

health outcomes [38]. Screening people who are not at risk is not recommended by the Endocrine Society [17]. In like manner, periodic testing for normal children is not recommended [30, 39]. More research is needed about the cost-effectiveness of the test. In Canada, vitamin D is measured in a representative sample in the ongoing cross-sectional CHMS which is repeated every two years [40, 39]. The CHMS in 2009-2011 suggested that 32% of Canadians aged 3 through 79 had vitamin D insufficiency, where 25(OH)D was below 50 nmol/L [40] and the percentage was slightly decreased for the next cycle in 2012-2013 to 25% [41]. Unfortunately, infants were not included in this national Canadian data.

Unlike adult, infant and child serum 25(OH)D is usually measured in a capillary blood sample via heel prick or finger not only in research, but also in clinical settings [42]. Although this method needs a small volume of blood and is more cost-effective, it has been demonstrated in adults to overestimate 25(OH)D by around 19 nmol/L in comparison to venous blood with no clear explanation for that [42]. A study in preschool children has shown that for a wide range of values these two methods are closely correlated [43].

For measuring vitamin D deficiency, several assays are used such as automated immunoassays, radioimmunoassay, and liquid chromatography-tandem mass spectrometry (LC-MS/MS) [44]. Total 25(OH)D reflects both of the isoforms, D_3 and D_2, and can be measured with almost all immunoassays, excluding the Roche assay which measures D_3 only [44]. All automated immunoassays cannot accurately measure 25(OH)D concentration less than 20 nmol/L [44]. For infants from 4 to 6 weeks of age, it is recommended to use LC-MS/MS to measure vitamin D metabolites [45]. LC-MS/MS can capture the important metabolites: 25(OH)D_2, 25(OH)D_3, and the 3-epimer 25(OH)D [13]. It must be borne in mind that the world of vitamin D assays is in constant growth, not only because assay manufacturers have

acknowledged some of their assay limitations, but also because of the increase in vitamin D knowledge in general [46]. The question remains whether there is a possibility to develop a standardized test which eliminates confusion when researchers want to collect results from different studies [46]. This increased the need for monitoring the performance of laboratories in the measurement of vitamin D led to the foundation of the Vitamin D External Quality Assurance Scheme (DEQAS), in 1989 [46, 47].

1.2.7 Public awareness

It is importance to measure vitamin D deficiency awareness of the community in general as well as for parents and health care providers to help the policy makers in different countries to set a clear framework for education programs. Although there are vitamin D recommendations for infants in many countries, there is a need for further education and awareness in this area. In the United Kingdom, a number of authors have reported that health care staff and parents need more education about the risk of vitamin D deficiency and positive outcomes of vitamin D supplement since 85.71% of parents were unfamiliar with vitamin D recommendations [48]. In the US, vitamin D supplements are not usually recommended by health workers for infants. While there are several possible explanations for that, one is that they consider breastfeeding enough to provide the vitamin D requirement [49, 50]. In Canada, very few exclusively breastfeeding mothers (6%) said that they had not been advised to use vitamin D supplements for their infants [51]. In British Columbia, Canada, nurses are asked not only to educate mothers about breastfeeding, but also to encourage the use of vitamin D supplementation [52].

1.3 Sources of vitamin D

1.3.1 Endogenous synthesis

1.3.1.1 Sun exposure

Vitamin D can be endogenously produced in the skin upon exposure to solar UVB radiation, where energy is used to convert 7-dehydrocholesterol to pre-vitamin D_3 at 290-315 nm wavelengths [53]. In addition, prolonged sun exposure will not cause toxicity [2]. Different factors can affect the amount of vitamin D production such as environmental [54], individual [55, 56] and social factors [57]. In some cultures, traditional covered dress can limit solar UVB radiation exposure [57-59]. Also, the use of sunscreen can limit vitamin D production since vitamin D_3 synthesizing capacity declines by 95% if sunscreen with a sun protection factor (SPF) of 8 is applied and by 98% if SPF of 15 is applied [60, 61]. While it is suggested that sunscreen might reduce vitamin D synthesis in the laboratory settings in practice there is no clear evidence if it does the same in free living settings [62].

With all this mentioned, infants have sensitive skin that can burn easily. The Canadian Dermatology Association (CDA) recommends that infants should be kept out of direct sun exposure especially between 11 am and 4 pm [3]. Sunscreen can be used for infants over 6 months in areas where the skin is not covered such as the face [3]. The AAP, as well, advises avoiding exposure to direct sunlight, and if this cannot be avoided by clothes or shade, it is possible to use sunscreen on limited areas such as the face [63].

Although there have been studies on infant sun exposure and serum 25(OH)D, past [64] and present [65], there is still a need for more studies about a safe sun level of exposure that might contribute to changing the current recommendations. Presently, there are movements to find a safe ultraviolet product that is also effective in treating vitamin D deficiency [66],

knowing that according to the author's knowledge there have been no experiments completed on infants as of yet.

1.3.1.2 Skin color

1.3.1.2.1 The role of melanin

Normal human skin consists of two main layers: the outer epidermis and the inner dermis [67]. In the epidermis layer of the skin, there are two types of cells that are present in abundance: the keratinocytes and melanocytes [67]. The keratinocytes produce and excrete keratin which has a role in strengthening the skin and give it a waterproof feature [67]. Melanocytes synthesize melanosomes where melanin is formed [67, 68]. Melanin is the final product of a series of chemical reactions [69]; in short, tyrosinase enzyme converts tyrosine to dopa to then dopa-quinone which is a precursor of melanin [67, 68].

Around 36 keratinocytes are linked with each melanocyte [68]. Through melanocytes' dendritic structures, melanosomes transfer to keratinocytes and form the melanin caps that protect DNA from ultraviolet radiation (UVR) [70]. In addition, melanin is found in two main forms: eumelanin (black-brown), and pheomelanin (yellow-red) [68, 71]. All types of human skin contain a fixed number of melanocytes, but what distinguishes them is the number, size, and distribution of melanosomes within keratinocytes [70]. This is the main reason for the differences in the human skin color in various racial groups (**Figure 1.1**) [1]. Moreover, the production of melanin -amount and type- differs with skin site, age, and sex [72]. As an illustration, a study that used spectrophotometer instrument to measure melanin index (a parameter for melanin content of the skin) for 148 Asian volunteers, their ages ranging from 5 months to 80 years, found that the forehead had the highest amount of melanin and the anterior chest had the lowest [73]. Moreover, melanin was more evenly distributed between the body

parts in younger volunteers than the older ones [73]. Another study by Post, Krauss et al. found that the forehead was darker than the upper arm in American black and white infants; taking into consideration that the measurements were made within hours after birth and there was no UVR exposure [74]. It is suggested that in all age groups the forehead contains more melanin as compared with the forearm [75]. For how melanin differs with age, infants reach young adult skin color within six months of age [75]; for studies interested in the relationship between skin color and other factors, this information should be considered.

1.3.1.2.2 The role of chromophores

Normal skin contains different types of chromophores that can be defined as molecules and particles that have light absorbing properties. The most prevalent chromophores in the skin are melanin and hemoglobin [76]. In the epidermis layer, there are the dark-brown eumelanin and the yellow-reddish phaeomelanin which can absorb different light ranges: visible and ultraviolet [71]. In the dermal layer, there are bright red oxyhemoglobin, bluish red reduced hemoglobin, and yellow bilirubin pigments [71]. It is not easy to notice the redness of oxyhemoglobin and the blueness of reduced hemoglobin in darker skin tones [71].

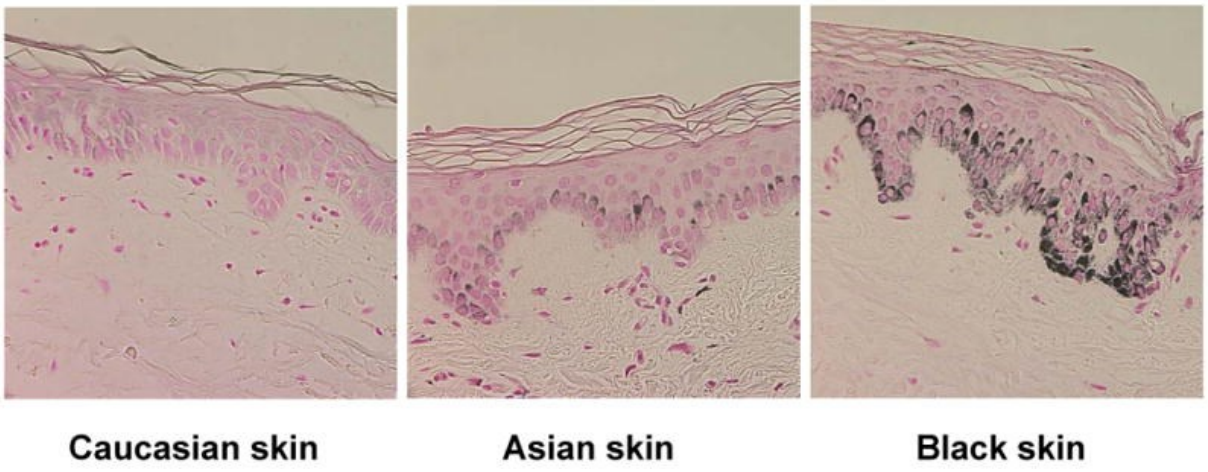

Figure 1.1: Melanin content in skin of different racial/ethnic groups

The number of melanocytes is almost identical in various racial/ethnic groups, yet the content of melanin in the basal layer of the epidermis is lower in Caucasian and Asian skin in compared to black skin [1].
Figure used with the permission from John Wiley and Sons [1].

1.3.1.2.3 Facultative versus constitutive skin color

The skin is considered as the first line of defense for the body; therefore, it has two methods to overcome the harmful effect of UVR: stimulate melanin production and increase epidermal thickness [1]. It is possible to describe the skin color by facultative skin color when it is the result of an external cause such as exposure to sunlight or by constitutive skin color which appears without any external influences, or in other words the effect of genes [68, 77, 78]. Facultative skin color can be considered as a useful indicator for plasma 25(OH)D as changes in facultative skin color from light to brown might correlate with 15 nmol/L increases in 25(OH)D [79]. To measure constitutive skin color, the buttocks area is considered a reliable site for Caucasian children and adults [80]. The outer forearm might be a significant predictor of 25(OH)D as facultative site [79], not the forehead which might be covered with hair, hat, or other accessories; and also, as mentioned earlier this site is more pigmented by nature. In addition, seasonal changes affect more facultative skin color than constitutive skin color [81]. For the infant population, to calculate the body surface area that is exposed to the sun, the Lund and Browder chart can be used; this method gives the percentage of any part of the body compatible with age [82, 83].

1.3.1.2.4 Skin color assessment

The way the human eye and brain perceive skin color depends on the amount of white light (wavelength range of 400-700 nm) absorbed or scattered from the skin [71, 76]. Although the human eye can distinguish hundreds of colors, people express these colors in different words [71]. Skin pigmentation can be classified either subjectively or objectively. One of the subjective methods that is used today to assess skin color is the Fitzpatrick scale that mainly depends on the individual's ability to tan and react with the sun (**Table 1.3**) [78]. At first this scale was

developed just as a four-point scales from 1 to 4, and in 1988 the darker skin colors 5 and 6 was added [78]. Currently, the L* a* b* colorimetric system proposed by the International Commission on Illumination (CIE) in 1976 [84] is widely used to objectively measure skin color following the guild line from the European Society of Contact Dermatitis [76]. In the CIE system, the L* value displays brightness, with the range of color from black to white; the a* value displays the range of color from red to green; and the b* value displays the range of color from yellow to blue [76]. These values are translated into skin color by calculating individual typology angle (ITA°) using the following formula: $ITA° = [\arctan((L^*-50)/b^*)] \times 180/\pi$; where $\pi = 3.14159$ [84]. Depending on ITA° value, skin color can be classified into categories that are differently described by researchers (**Table 1.4**). In general, the lower the ITA° value, the darker the skin. Not to mention, that the ITA° value has been validated as a reliable and reproducible method for measuring skin color [85]. The spectrophotometer instrument can objectively measure skin pigmentation by displaying the L* a* b* values as continuous or categorical variables. Athough many studies have used this tool to measure infant skin color, inferences drawn from this instrument must be taken cautiously because of the nature of infant skin. During the first year of life, infant skin continues to develop [86], especially, during the first three months of life where significant changes occur, such as increased skin peeling on the forehead and forearm [87]. One common condition that also affects newborn skin color is jandice causing yellowish skin color, cited in [88]. Exclusively breastfed newborns have a different jundice pattern, peaking by 5-15 days and ending around the third week of life, cited in [88].

Table 1.3: Fitzpatrick skin types

Skin type[1]	Skin color	Burn	Tan
1	White	Yes	No
2	White	Yes	Minimal
3	White	Yes	Yes
4	White	No	Yes
5	Brown	No	Yes
6	Black	No	Yes

[1] Based on [78].

Table 1.4: The range of individual typology angle and skin color classifications

ITA°[1]	Skin categorization			
	Fitzpatrick 1975 [78]	**Chardon 1991** [89]	**Del Bino 2006** [85]	**Reeder 2010** [90]
≥55	Type 1	Very light	Very light	Very fair
≥41 to <55	Type 2	Light	Light	Fair
≥28 to <41	Type 3	Intermediate	Intermediate	Medium
≥10 to <28	Type 4	Tan	Tanned	Olive
≥-30 to<10	Type 5	Brown	Brown	Dark
<-30	Type 6	Brown	Dark	Very dark/black

[1] Individual typology angle.

1.3.1.2 Season and latitude

Previous studies clearly indicate the significant effect of season and latitude on the concentrations of 25(OH)D [54, 93, 91]. Seasons are mainly caused by the earth's rotation around the sun and day and night around its own axis; all these phenomena cause periodic changes in the solar zenith angle and then attenuation of the solar radiation [54, 92]. There is an inverse relationship between solar zenith angle and the number of UVB photons reaching the earth's surface [92]. In higher latitudes UVB becomes less intense because of the increased sunlight angle of penetration and greater distances to travel through the earth's atmosphere [91]. Moreover, air pollution and reflectivity of the surface can be other interfering aspects of solar zenith angle [92]. Because UVB exposure is critically important to the production and maintenance of serum 25 (OH)D concentration, countries located at higher latitudes may face limited UVB which may contribute to risk of vitamin D deficiency [54, 93], yet several other questions remain to be addressed to understand the common incidence of vitamin D deficiency in sunny areas such as Brazil and the Middle East [57, 59, 94].

In latitudes above 35° N and below 35° S, there is no cutaneous synthesis of vitamin D in the winter months, cited in [92]. For example, in Edmonton (52° N) there is no cutaneous synthesis of vitamin D between October and March [54]. Whereas people residing in tropical regions can produce enough vitamin D year around [92]. In fact, living at higher latitudes is not only linked with vitamin D deficiency, but also with many other diseases such as multiple sclerosis, Crohn's disease, hypertension, cardiovascular disease and cancer, cited in [53]. The CPS recommend using 800 IU/d of vitamin D supplementation during the winter months for breastfed infants who live in the north. In general, children of all ages are less likely to have enough vitamin D stores during winter compared to summer [95].

1.3.2 Exogenous intake

1.3.2.1 Dietary sources

Vitamin D is naturally present only in a few foods, all of which are not frequently consumed by infants except for egg yolk. The two forms of vitamin D in food are vitamin D_2 which is derived from fungi such as yeast [53, 96-98] and vitamin D_3 which naturally found in fatty fish (salmon, mackerel, and sardines) [95, 96], fish liver oils and eggs [99, 100]. Salmon is the most common fish type that is consumed in North America [100]. Although liver and other meat organs are source of vitamin D, they are infrequently used, and people avoid them as being rich in cholesterol [100]. Moreover, vitamin D is mostly high in animal sources, which are unsuitable for vegetarians [95].

The amount of vitamin D content in food can be substantially affected by the techniques used in cooking. As an illustration, baking and microwaving fish can preserve the vitamin D content of fish while fifty percent of active vitamin D content reduces in frying fish [101]. In the same context, wild fish might have more vitamin D content than farmed ones [101]. Canadian Nutrient File (CNF) can be used to estimate the amount of vitamin D content in different food groups (**Table 1.5**) [4]. It is important to acknowledge that the amount of vitamin D in national food compositions databases considerably differs from country to country even for the same food [102]. Furthermore, a recent study indicated that pregnant Canadian women obtain most of their vitamin D from supplements, and the main food sources of vitamin D were milk, yoghurt, and fish [103]. Natural food alone might not be sufficient to meet the recommended vitamin D intake level for the general population and for infants who usually consume small amounts. Fortification of staple foods can be one way to solve the problem [102].

Table 1.5: Dietary sources of vitamin D from the Canadian Nutrient File 2016

Food items[1]	IU per 100 g serving
Fish	
Salmon, sockeye (red), canned drained solids, without skin and bones	859
Salmon, Atlantic, farmed, baked or broiled	274
Salmon, Atlantic, wild, baked or broiled	327
Salmon oil	177
Cod (scrod), Atlantic, baked or broiled	46
Tuna, light, canned in water, drained, unsalted and salted	48
Tuna, white, canned with water, drained, unsalted and salted	80
Mackerel, Atlantic, baked or broiled	104
Sardine, Atlantic, canned in oil, drained solids with bone	93
Sardine oil	332
Other food items	
Egg, chicken, whole, cooked, fried	89
Egg, chicken, yolk, cooked	188
Margarine, tub, hydrogenated, canola oil	659
Mushroom, Maitake, raw	1123

[1]Data obtained from the Canadian Nutrient File 2016 [4].

Table 1.6: Vitamin D content in different types of milk

Types of milk	Vitamin D (IU/100 ml)
Similac advance ® [104]	40.5
Enfamil A+ Infant Formula [105]	41
Whole goat milk, enriched [1]	40
Whole goat milk, unenriched [1]	4
Whole pasteurized – homogenized, 3.25% m. f [1]	41
Mature breast milk [1]	3

[1] Data obtained from the Canadian Nutrient File 2016 [4].

1.3.2.2 Fortified food

Considering that natural foods that are high in vitamin D are limited, in Canada, people widely rely on foods that are fortified and the use of the supplements [100]. The fortification of food in Canada is regulated by Food and Drug Regulations [106]. In Canada, vitamin D food fortification is under regulatory control because high doses of vitamin D for prolonged use can cause toxicity [100]. Correspondingly, in other countries such as the United Kingdom, Australia, Scotland and Ireland, fortifying food is not obligatory, but available as voluntary choice for some foods [107].

In Canada, infant food is not usually fortified [108]. In a study done on Canadian infants, it is suggested that from 0 to 6 months the mean daily intake of vitamin D from solid food was 16 IU and 32 IU from 7 to 12 months [108], taking into consideration that breast and formula milk dietary intakes were not analyzed. Formula milk is mandated by Health Canada to be fortified with vitamin D (40 IU/100 ml) [109]. In that case, for infants to reach the 400 IU/d recommendation, 1 L of formula per day should be ingested [110, 111]. For that reason, the AAP suggests at least 400 IU/d vitamin D for formula fed infants who consume less than 1 L/d [21]. In 1950, the fortification of evaporated and dried milks began in Canada and changed to include fluid milk in 1965, cited in [112]. Nowadays, fortification of milk and margarine is mandatory in Canada [109]; for each 250 ml of milk, 100 IU of vitamin D is added [4]. In fact, there is a direct relationship between milk consumption and 25(OH)D concentration in the Canadian population [31]. According to the CHMS, 39.1% of females 20 to 39 years consumed fortified milk less than once a day [31]. Similarly, in Toronto, Canada, a cross-sectional study, in women of child-bearing age (18-35y) showed that milk was not consumed by 46% [113].

For other products, in Canada, it is optional to add vitamin D_2-yeast to yeast-leavened bakery products at the level of 90 IU per 100 g of product; this yeast can be used with products such as bagels, croissants, pizza and bread mix [114]. For eggs, Hayes, Duffy et al. examined the outcome of using vitamin D_3 or 25(OH)D_3 enhanced eggs on serum 25(OH)D during winter (8wk) in healthy adults [115]. Interestingly, they found that eating 7 eggs/week compared with commercial eggs of ≤ 2 eggs/week can maintained serum 25(OH)D, but they did not evaluate the impact of this method on energy intake and fat [115]. The long-term impact of such intakes should be studied on a wide scale before applying it, considering the effect on pregnant women and small children.

1.4 Maternal-fetal transfer in utero

Worldwide maternal vitamin D deficiency continues to be a problem although it is possible to prevent it from occurring [116]. Acceptable vitamin D status is not only associated with decreases in the risk of various maternal health problems such as preterm birth, bacterial vaginosis, preeclampsia and gestational diabetes, but also helps to avoid infant complications later in life such as low bone density, asthma, and type 1 diabetes [117].

The level of maternal vitamin D status plays a fundamental role in infant vitamin D status because 25(OH)D crosses the placenta (the fetus does not produce vitamin D); this can explain the high correlation between the maternal vitamin D status and the infant at birth [20]. Canadian mothers, especially aboriginal women, were found to have low vitamin D status [118]. The results obtained by Waiters, Godel et al. [119] suggest that most northern Canadian mothers did not achieve the CPS 25(OH)D cut-off concentration of 75 nmol/L at delivery (33 Caucasian, 59.8 ±29.4 nmol/L; 37 Indian (known today as First Nations) 52.1±25.9 nmol/L; 51 Inuit 48.8±14.2 nmol/L). As reported by Weiler, Fitzpatrick-Wong et al. [120], 46% of mothers, white

and non-white (First Nation, Asian, Filipino, and black) were found to have 25(OH)D concentration less than 37.5 nmol/L after delivery, most of them in the non-white group (p=0.002).

The majority of pregnant and lactating women use a prenatal multivitamin supplement in Canada and the United States, such vitamins commonly have around 400 to 600 IU vitamin D [121]. In a randomized controlled trial (RCT), in Vancouver, British Columbia, Canada, pregnant women were randomized into one of the three treatments groups 400, 1000, or 2000 IU/d of vitamin D_3 from 13 to 24 weeks of gestation until 2 months postpartum. They found that even without directly supplementing the infant with vitamin D, 44% of infants born to mothers in the 2000 IU/d group achieved a 25(OH)D concentration more than 75 nmol/L, and around 98% achieved a 25(OH)D more than 30 nmol/L [121]. In comparison with directly supplementing the infant, maternal supplementation may be preferred considering the advantages of improving vitamin D for both of them at the same time [121]. For that reason, it is suggested that increasing vitamin D doses for mothers can help to increase the transfer of 25(OH)D to the fetus [121]. However, for the neonates born infant with low vitamin D status at birth, very little is known as to how much vitamin D is needed to build vitamin D stores rapidly and safely.

1.5 Infant vitamin D status

Nutrition during the first year of life is important as this is a period of rapid growth and development. According to the World Health Organization (WHO) infants must be exclusively breastfed for the first six months of life to obtain ideal health before introducing complementary food [122]. The health benefits of breastfeeding are well recognized and apply not only to the infant, but also to the mother, yet research confirms that breast milk vitamin D is low around 3 IU for each 100 ml [4] and does not provide enough exogenous vitamin D for infants. For this

reason, the WHO defines exclusive breastfeeding as only breast milk, but allows the infant to receive vitamins, minerals and medicines [122]. Moreover, stores that pass through the placenta during the last months of pregnancy remain only for several weeks.

There is also a difference in breast milk vitamin D and $25(OH)D_3$ concentration between black and white women due to various factors such as a higher compliance in taking prenatal vitamins in white women than in black women [123]. In Canada, vitamin D supplementation is recommended for exclusively or partially breastfed infants until one year of age [22, 124]. As mentioned earlier, formula milk is fortified with vitamin D (**Table 1.6**), but infant should consume 1L/d to reach the 400 IU/d recommendation. Some researchers believe that formula milk feeding is not enough to overcome the vitamin D deficiency [125]. In a study done to evaluate vitamin D deficiency rickets among Canadian children, three infants showed rickets during their early life although they received infant formula that supported normal growth [7].

One study suggested that 4000 IU vitamin D daily for lactating mothers can help to maintain enough vitamin D in breast milk for infant's needs [126]. On the other hand, the IOM committee members argue that pregnant and lactating women, like other community members, do not need high dosages of vitamin D supplementation [26].

Vitamin D plays a major role in physiological pathways since vitamin D receptors exist in nearly all tissues [53]. In children vitamin D deficiency linked with a large proportion of the health problem such as hypocalcemia seizures, growth disturbances, rickets, cardiovascular dysfunction, autoimmune disease and attention deficit hyperactivity disorders, cited in [48]. Numerous studies have argued that increasing vitamin D intake can reduce the risk of developing chronic disease [127]. In a study done in Finland, they found that infants who routinely received

vitamin D supplementation (2000 IU/d) had approximately 80% lower chance to develop type 1 diabetes in later life (relative risk, 0.22) [128].

1.5.1 Rickets

1.5.1.1 Overview

Rickets is a disease where there are skeletal system deformities and muscle weakness [50]. These skeletal deformities were linked with rickets by David Whistler in 1645 and have been under study since that time [129]. The disease was spread in children living in the northern Europe and the northeastern United States where pollution and lack of sun exposure were common [127]. Edward Mellan, in 1918, provided irrefutable evidence that rickets is a dietary deficiency disease caused by an inadequacy of fat-soluble vitamin D. In 1930, rickets was almost eradicated due to vitamin D fortification of milk in the United States. Recent reports, however, indicate that the disease is resurgence because of sun exposure avoidance and hypovitaminosis D among breastfed infants [130]. The Canadian Paediatric Surveillance Program (2002-2004) estimated a total of 104 confirmed cases of vitamin D deficiency rickets, most of which were diagnosed in the north or immigrant families [7]. From the confirmed cases, 92 (89%) were children with intermediate to dark skin color, and 98 (94%) were children who were breastfed without suitable vitamin D supplementation [7].

1.5.1.2 Types of rickets

This bone deformity in the skeletal system appears during the rapid growth of bone often before the age of one and half years in areas such as the costochondral junction and the long bone epiphyses [50]. Some examples of skeletal deformities are craniotabes, frontal bossing, delayed closure of fontanelles, genu varum, prominence of the costochondral junctions (rachitic rosary) and widening of the metaphyses of the wrists and ankles [95].There is a clear association between vitamin D status and muscle strength and function since vitamin D deficiency not only can cause rickets, but also can cause delays in growth and motor development which is associated with weak bones and muscles that effect crawling or walking, cited in [131]. There are different types of rickets: nutritional rickets which are associated with insufficient solar UVB exposure or insufficient intake of vitamin D, calcium, or phosphorus; vitamin D – dependent rickets type I caused by a deficiency of renal 25(OH)D_3-1-α- hydroxylase, whereas type II is caused by defective interaction between calcitriol and vitamin D receptor; and vitamin D resistant rickets due to impaired proximal renal tubular reabsorption of phosphorus and in the presence of an appropriately normal calcitriol level [132]. Among young infants, vitamin D deficiency remains the main cause of rickets [50]. For a medical diagnosis of rickets to be made, clinical, radiological, and laboratory evidence are required [95].

1.5.1.3 Treatment

Infants with rickets should be treated with higher than the normal recommended dose to build stores of vitamin D. The Global Consensus Recommendations on Prevention and Management of Nutritional Rickets, in May 2014, suggests that 2000 IU/d is the minimal recommended dose of vitamin D for at least 3 months to treat nutritional rickets [30]. The AAP guideline to treat rickets varies in doses and duration based on the age of the child, and vitamin

D supplements can be consumed between the period of two to three months to maintain normal 25(OH)D concentration and rebuild tissue stores. The recommendations are divided by age groups as the following: infants less than one month of age 1000 IU/d, between 1 to 12 months of age 1000 to 5000 IU/d, and more than 5000 IU/d for ages more than one year [95]. The Australian and New Zealand guidelines differ in doses and age groups as well; for infant less than 1 month of age 1000 IU/d is the dose within a 3 months, for 1 to 12 months old infants a dose of 3000 IU/d within three months or 300000 IU/d during 1 to 7 days; and for ages more than one year 5000 IU/d within three months or 500000 IU/d during 1 to 7 days [133]. For both guidelines, a dose of 400 IU/d is recommended to maintain vitamin D level [95, 133].

1.6 Vitamin D supplementation in infants

In Canada, before the start of the new generation of vitamin D supplements, cod liver oil was used for infant [5]. Currently in Canada, there are different brands of vitamin D supplements that commercially available with various dosages (**Table 1.7**). The highest dose that can be obtained without prescription is 1000 IU [2].

In Vancouver, Canada, one research group reported that the majority of medical staff (80%) prescribe D-Drops ®, which is oil based and only one tasteless drop is needed to provide 400 IU [134] for infants [52]. Another research group in Switzerland compered two forms of vitamin D supplements for infants and found that adherence was higher for oil based than alcoholic based products. That can be linked with not only the unpleasant taste of the alcoholic formulation but also the fact that four drops were needed to get the required dose, on the contrary, to the one drop from the oil-based product [135]. Adherence to vitamin D supplementation is one of the greatest challenges. In one Montreal hospital (the Royal Victoria Hospital), 98% of exclusively breastfeeding infants and 88% of mixed feeding infants, who were

born between 2007 and 2008, received vitamin D supplement at some point of their life before 6 months of age [51]. Among infants who were exclusively breastfeeding, 74% received 400 IU/d and 50% of mixed feeding infant did not meet the current recommendation. In addition, the primary reason for noncompliance with supplementation was that mothers thought that supplemental vitamin D was not needed during the transition to fortified formula [110].

Table 1.7: Vitamin D content of some commercially available supplements

Brand name[1]	DIN[2]	Dose	Manufacturer code
D3-DOL	80019649	400 IU	JAP
PediaVIT D	80003285	800 IU	EUR
Baby Ddrops	80001869	400 IU	DDP
D VI SOL	00762881	400 IU	MJO
Ddrops vitamin D	80001792	400 IU	DDP
JAMP-VITAMIN D	80003038	400 IU	JMP
Pedia VIT D	02231624	400 IU	EUR
Ddrops vitamin D	80001791	1000 IU	DDP

[1] Source: Government of Canada [136].

[2] DIN: Drug Identification Number.

1.6.1 Response to vitamin D supplementation

The relation between vitamin D supplementation and 25(OH)D concentration is influenced by many factors all of which are based on adult studies. One study [137] systematically reviewed the data for different ethnic and age groups from 10 to >65 years and found that the difference in circulating 25(OH)D as a response to vitamin D supplementation might be linked with body weight, type of supplement (D_2 or D_3), calcium intake, age and baseline 25(OH)D. Another systematic review [138] reported consistent factors with the previous study such as body weight, baseline 25(OH)D, age, and calcium intake. In addition, there were other factors some of which were ethnicity, genetics, fat content in the diet, environment and vitamin D regimen (type, dose and duration). To start with ethnicity, a study that investigated the difference in dose response between white and African Americans men and women (18-65 years) for 6 months. [139]. In this RCT, vitamin D dose at baseline was 2000 IU/d when 25(OH)D between 50-80 nmol/L and 4000 IU/d when 25(OH)D < 50 nmol/L. After that, during the study period the cases were followed every two months and the doses adjusted according to serum 25(OH)D to reach the following results: more than 80 nmol/L and less than 140 nmol/L. Although there was no racial difference in response to supplementation and both groups achieved 75 nmol/L by week 18, African Americans needed double the doses to reach this goal. This result can be explained by the lower baseline value in African Americans than white Americans for both males and females ($p< 0.01$). Also, researchers targeted healthy older African American and Caucasian women with insufficient vitamin D, they found that 97.5% of African American women respond to 800 IU vitamin D_3 by increases in serum 25(OH)D to more than 50 nmol/L, the same as Caucasian women [140, 141]. They interpreted that to the similarity, in both groups, in absorption and metabolism of the vitamin D supplement, and the knowledge

about low serum 25(OH)D in African American women can be linked with dark skin and its lower ability to produce vitamin D.

Regarding infants, one of the most significant factors that determines an infant's response to vitamin D supplementation is the 25(OH)D concentration at inception of the treatment. In a RCT where infants received 400 IU/d (D_2 or D_3), infants who had baseline 25(OH)D concentration less than 24.9 nmol/L had a significant increase in 25(OH)D concentration compared to those with 25(OH)D concentration more than 50 nmol/L [19]. A study done in Vancouver (49°N), BC, Canada, aimed to compare the difference in 25(OH)D concentration of healthy term born infants for Asian immigrant (Middle East, South Asia, China and all countries in South East Asia) and white ethnicity backgrounds (European background). Infants were 2–4 months of age and were receiving vitamin D supplements. They concluded that serum 25(OH)D concentration was not dependent on skin pigmentation or ethnicity [142]. However, there are limitations to this study such as the cross-sectional design and the small sample size. Also, most of the infants started with an average of 98.6 nmol/L of 25(OH)D which is considered sufficient according to the IOM cut-off [2]. In anther a study done between Hispanic and non-Hispanic Caucasian infants in Houston, Texas (29°N), they found that infants who received vitamin D supplementation for 3 months at 400 IU/d had 25(OH)D concentrations above the IOM cut-off [2] despite baseline value or ethnicity [143].

It should be noted that not all the reviewed studies measured the skin color and the terms used to describe the study population such as white and black were used exactly as mentioned in the paper. Because these terms are widely used to denote race or ethnicity (section 1.8), the terms light and dark will be used in this thesis to describe infant skin color. Despite current knowledge about how infants with dark skin are born with lower vitamin D status versus infants with light

skin [144, 145, 146] (**Table 1.8**), there is a relatively small amount of the literature about how they respond to vitamin D supplementation and if health care providers should individualize their treatment strategy for vitamin D deficiency.

Table 1.8: Vitamin D status of infants from different racial/ethnic groups

Reference	Country	Skin color measurement	Population		n	Sample time	Mean ± SD 25(OH)D (nmol/L)
Waiters, Godel et al. 1999 [119]	Canada	No	Caucasian		30	Cord blood	41.4±23.5
			Indian[7]		34	Cord blood	34.1±14.3
			Inuit		49	Cord blood	34.6±12.6
Abrams, Hawthorne et al. 2012 [143]	The US	No	Non-Hispanic Caucasian		19	Cord blood	57.5±23.5
						3 months [11]	94.3±19.0
			Hispanic		19	Cord blood	42.3±18.0
						3 months [11]	78.0±20.3
Green, Li et al. 2015 [142]	Canada	Spectrophotometer	Asian immigrant[1]		28	2–4 months[10]	80.0 (70.0-95.5)[6]
			White non-immigrant		37		75.0 (65.0-85.0)[6]
Cadario, Savastio et al. 2013 [144]	Italy	Subjectively	Italian mothers		32	Cord blood	67.0±32.5
						Neonatal serum[3]	42.8±10.0
			Non-Caucasian[2]	LOB[4]	10	Cord blood	26.5±17.3
						Neonatal serum[3]	20.8±10.0
				MBB[5]	20	Cord blood	36.8±15.5
						Neonatal serum[3]	38.3±20.0
Basile, Taylor et al. 2007 [145]	The US	No	African	Group1[8]	9	Cord blood	32.8±10.0
			American	Group2[9]	58		25.3±14.3
			Caucasian	Group1[8]	6		72.5±17.5
				Group2[9]	25		44.3±23.0
Bodnar,Simhan et al. 2007 [146]	The US	No	Black		200	Cord blood	39.0 (36.3-41.8)[6]
			White		200		67.4 (63.8-71.3)[6]

[1] Iranian (n=6), Pakistani (n=1), Chinese (n=16), Indian (n=2), Filipino (n=3).

[2] Asian, Latin American, North African, and African.

[3] It was venous blood sampling within 3 days after birth.

[4] Light olive/light brown.
[5] Medium brown/black skin.
[6] Mean (95% CI).
[7] known today as First Nations.
[8] April 1-October 31.
[9] November 1–March 31.
[10] Infant blood samples were collected by venipuncture.
[11] Not clear how infant blood samples were collected.

1.7 Demographics of Canada

Canada is a multicultural country; according to the 2016 census more than 250 ethnic origins or ancestries were reported [147]. This was an increase from 1871 when around 20 origins were counted [147]. There has been a change in immigrants' origins from Europe-based to Asia-based immigration [148]. Between 1980 and 2016, Canada received around 5.7 million immigrants and 860000 of them were refugees [149]. These numbers might indicate a demographic shift in the population of Canada. It is suggested that number of the visible minority population could increase between 11.4 and 14.4 million by 2031 [148]. A visible minority is defined by The Employment Equity Act as "person, other than Aboriginal peoples, who are non-Caucasian in race or non-white in colour" [150]. The following groups can be considered as visible minorities: "South Asian, Chinese, Black, Filipino, Latin American, Arab, Southeast Asian, West Asian, Korean, Japanese, Visible minority, n.i.e. ('n.i.e. ' means 'not included elsewhere'), multiple visible minorities" [150]. However, there are a few exceptions that excluded some groups from the visible minority category such as mixed race including 'Latin American' and 'white', 'Arab' and 'white', or 'West Asian' and 'white' [150]. Hence, not all visible minority groups have darker skin color.

1.8 Race and ethnicity terms

The terms race and ethnicity are so complex and open to debate despite their role in investigating some health problems in specific racial or ethnic groups [151, 152]. Ethnicity is described as "a multi-faceted quality that refers to the group to which people belong, and/or are perceived to belong, as a result of certain shared characteristics, including geographical and ancestral origins, but particularly cultural traditions and languages" [151]. It is suggested that

information such as language, country of birth, family origins and religion are necessary for ethnicity studies [151]. Currently, self-reported ethnicity is commonly used although it might alter with time or context [151]. Because there is no clear description about these term in the published studies, comparison between studies are not easy [151, 153]. In addition, the term "nationality" has been connected with race and ethnicity as well [154]. Race might not be suitable to approximate for other unmeasured social, cultural, and environmental confounders [153]. Many researchers combined these terms as race/ethnicity because of this overlapping [151].

1.9 Genetic factors

Research on the genetic influence on vitamin D has been ongoing for years. In one of the largest genome-wide association studies, it is believed that genetic variants in DHCR7, CYP2R1, and GC genes increase the risk of vitamin D insufficiency [155]. DHCR7 encodes the enzyme 7-hydrocholesterol reductase [155]. This enzyme converts 7-dehydrocholesterol to cholesterol which reduces the amount of 7-dehydrocholesterol available for photochemical synthesis of vitamin D [155]. GC encodes vitamin D binding protein [155] and also the group-specific component [156]. Despite the importance of the information provided by these studies, it was predominantly a reflection of white populations and only explains 1-4% of the variation in 25(OH)D concentration [155]. At the GC locus, two common alleles have been identified for GC: GC*1 and GC*2 [157]. In addition, there are two sub-alleles of GC*1: GC*1F and GC*1S, which unequally distribute between various ethnic groups [157]. Regarding skin color, white populations have frequencies of the GC*1F allele in comparison to populations with yellow and black skin pigmentation [157]. It is thought that GC*2 is linked with a lower level of 25(OH)D and $1,25(OH)_2D$ whereas GC*1S is linked with only 25(OH)D [158]. The current knowledge

about genetic factors is limited and factors such as sun exposure and lifestyle remain important factors to determine vitamin D status [159].

1.10 Rationale and objective

Canada and particularly Montreal has a multicultural population with different skin colors. Breastfed infants with darker skin pigmentation at risk for low vitamin D status. Given that vitamin D supplementation is highly recommended for them, how these infants respond to vitamin D supplements if they are born with low vitamin D stores, and if a higher dosage is needed to overcome the deficiency is important to explore. Previous studies are difficult to interpret because either they studied infants with sufficient vitamin D status or lacked an objective method to measure skin color if any.

Primary research objective: To investigate if there is a difference in the response of serum 25(OH)D to vitamin D supplementation in neonates born with low vitamin D stores according to skin pigmentation, and if a higher dose of vitamin D supplementation will result in a faster rate of improvement of vitamin D status in infants with dark skin.

The hypothesis is that infants with dark skin will have a lower response to vitamin D supplementation than infants with light skin and will need the higher dose to support faster improvements in serum 25(OH)D concentration. In addition, birth serum 25(OH)D concentration will be the main factor that determines infant's response to vitamin D supplementation.

2.0 Manuscript

The differential response to vitamin D supplementation in neonates born with low vitamin D stores according to skin pigmentation: a double-blind, randomized trial, in Montreal, Canada

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2.1 Abstract

Vitamin D deficiency is common in exclusively breastfed infants who have dark skin pigmentation. Some medical societies recommend a higher level of vitamin D supplementation for such infants; however, this is based on professional opinion more than evidence. Therefore, the objective of this study was to investigate if there is a difference in the response of serum 25-hydroxyvitamin D (25(OH)D) to vitamin D_3 supplementation in neonates born with low vitamin D stores (25(OH)D <50 nmol/L) according to skin pigmentation, and if a higher dose of vitamin D supplementation will result in a faster rate of improvement of vitamin D status in infants with dark skin. Healthy term born breastfed infants were recruited from a hospital in Greater Montreal (NCT02563015). Infants were screened for low serum 25(OH)D (Liaison, DiaSorin, Stillwater, MN 55082, USA) at birth and then randomized to 1 of the 2 treatment groups: 400 IU/d (n=36) or 1000 IU/d (n=36). Infants were stratified by skin pigmentation in male and female blocks. At baseline, 3, and 6 months, anthropometry, body composition, skin pigmentation, and biochemistry measurements were examined. Based on Fitzpatrick descriptions a spectrophotometer (CM-700d/600d, Konica Minolta, USA) was used to classify infant skin: light skin (1-3) and dark skin (4-6) by using constitutive pigmentation at the inner upper arm. The study sample was 50% male, 24% infants with dark skin color, and 65% were breastfed until 6 months; compliance rate was 70% overall. After 6 months of supplementation, all groups had mean serum 25(OH)D concentrations above the 50 nmol/L threshold. Overall, infant skin color did not affect the infant serum 25(OH)D response to vitamin D supplementation at any time point (p=0.40). The 1000 IU/d group had a significantly higher mean concentration of serum 25(OH)D than the 400 IU/d group at 3 and 6 months. This study shows that skin pigmentation

was not a significant determinant of infant serum 25(OH)D concentration'- at 3 or 6 months of age and that vitamin D recommendations for the general infant population are appropriate.

2.2 Introduction

Vitamin D is a fat-soluble vitamin that can be obtained endogenously by exposing the skin to ultraviolet beta (UVB) radiation from sunlight or exogenously from food and supplements. Due to the recommendation that infants should not be exposed to sunlight [3], and the low vitamin D content of breast milk [4], this age group is recommended to be given a vitamin D supplement until a sufficient amount can be obtained from their diet [2]. The Canadian Paediatric Surveillance Program (2002-2004) estimated a total of 104 confirmed cases of vitamin D deficiency rickets, most of which were diagnosed in the north or immigrant families [7]. From the confirmed cases, 92 (89%) were children with intermediate to dark skin color, and 98 (94%) were children who were breastfed without suitable vitamin D supplementation [7].

Although there are studies that link infant vitamin D deficiency with dark skin color, there is a gap in knowledge about how they respond to vitamin D supplementation and if a higher dosage is needed to overcome vitamin D deficiency. A study in Vancouver (49°N), BC, aimed to compare the difference in 25-hydroxyvitamin D (25(OH)D) concentration of healthy term born infants of Asian immigrant (Middle East, South Asia, China, and all countries in South East Asia) and white (European background) ethnicity backgrounds. Infants were 2–4 months of age and were receiving vitamin D supplements. The study found that infant 25(OH)D concentration was not dependent on skin pigmentation or ethnicity [142]. However, there are limitations to this study such as the cross-sectional design and the small sample size. Most importantly, the infants started with an average of 98.6 nmol/L of 25(OH)D which is considered sufficient according to the Institute of Medicine (IOM) cut-off [2]. In another study done between Hispanic and non-Hispanic Caucasian infants in Houston, Texas (29°N), they found that infants who received 400IU/d of vitamin D supplementation for 3 months had 25(OH)D

concentrations above the IOM cut-off [2] despite baseline status or ethnicity [143]. However, skin color was not measured, and it remains unclear whether skin color might be a better proxy for the response to supplementation than race or ethnicity. Therefore, the objective of this study is to investigate if there is a difference in the response of serum 25(OH)D concentration to vitamin D supplementations (400 IU/d or 1000 IU/d) in neonates born with low vitamin D stores (25(OH)D < 50 nmol/L) according to skin pigmentation (dark or light), and if a higher dose of vitamin D supplementation will result in a faster rate of improvement of vitamin D concentration in infants with dark skin.

2.3 Methods

2.3.1 Study design and subjects

The study objective was explored using preliminary data from a double-blind, randomized controlled trial (RCT) (NCT02563015) conducted at a single-center: the Mary Emily Clinical Nutrition Research Unit of McGill University, Montreal, Quebec, Canada. Participants were recruited from the Lakeshore General Hospital in the Greater Montreal area, latitude (45.5 ^{o}N). The recruitment period was between March 2016 and August 2017. Infant vitamin D status was screened within 24-36 h of life using serum 25(OH)D (Liaison, DiaSorin, Stillwater, MN 55082, USA). Infants with serum 25(OH)D < 50 nmol/L were stratified by skin pigmentation in male and female blocks; and then were randomized to treatment groups (400 IU/d or 1000 IU/d vitamin D_3). Infants were allocated to their groups at the end of the baseline visit (1-4 weeks).

The inclusion criteria were: healthy, term born infants between 37-42 weeks gestation, appropriate weight for gestational age (males weight between 2500 to 4300 g; and females between 2400 to 4200 g) based on a Canadian growth reference [160], and infants born to a healthy mother who intended to breastfeed to at least 3 months. Exclusion criteria were: maternal diagnosis with diseases such as hypertension, Crohn's disease, celiac disease, liver disease, diabetes of any type, pre-eclampsia and kidney disease, or medication(s) that might impact vitamin D metabolism other than vitamin/mineral supplement. Mothers who smoked during pregnancy were excluded.

Infants and their mothers were seen at baseline (1-4 weeks), 3, and 6 months. These time points allow enough time to evaluate changes in serum 25(OH)D concentration and infant skin color [75]. At all the visits, anthropometry, body composition, skin pigmentation, and biochemistry measurements were examined. Other information such as demographic data,

vitamin D supplement compliance, sun exposure, infant feeding, and general health information were collected via questionnaires.

2.3.2 Socio-demographic data

At screening and the baseline visit, mothers completed a demographic survey on income, education, self-identified ethnicity and race according to the methodology published by Statistics Canada [161, 162]. The hospital medical records were used to gather information about mothers such as weeks of gestation, pre-pregnancy weight, pregnancy weight gain; and infant birth data.

2.3.3 Vitamin D supplement

The trial vitamin D supplements were supplied pre-coded and *in kind* by Euro-Pharm International Canada Inc. (Montreal, Canada). All bottles contained a water-based vitamin D_3 supplement similar in taste, smell and texture. Double-blinding was accomplished using a unique code for each infant. This allowed researchers and families to remain blinded in case of an adverse event, these codes allow tracing back to the product dosage for that infant only. Mothers were provided 4 bottles of the supplement (50 ml for each) and given instructions to administer 1 ml of the supplement each day. Vitamin D content was regularly confirmed by Euro-Pharm International Canada Inc. (Montreal, Canada) to be in an acceptable range as (360-520 IU/ml) for the 400 IU dose and (900-1200 IU/ml) for the 1000 IU dose. The average supplement contents for this study were 424 IU/ml for 400 IU dose and 999 IU/ml for 1000 IU dose.

2.3.4 Compliance assessment

Parents were provided with a compliance record to record the number of daily doses their infants received between each visit. The percentage of compliance was calculated at each visit, by dividing the number of doses used by the number of days since the last visit. Supplement compliance was defined as 80-100% of doses taken between visits. Additionally, at each study

visit parents verbally reported frequency of compliance by answering a question with several options (everyday, almost every day, about 2 or 3 times a week, about once a week or never).

2.3.5 Skin pigmentation assessment

A portable spectrophotometer (CM-700d/600d, Konica Minolta, USA) was used for measuring skin reflectance; before each use the instrument was calibrated. This measurement was done in triplicate at four sites: the inner upper arm (constitutive), the forehead, mid-forearm and lower leg. The L* and b* values were used to calculate the individual typology angle (ITA°) [84]. Based on Fitzpatrick descriptions, infants were classified into two skin color groups: light skin (1-3) and dark skin (4-6) [78] using constitutive pigmentation before exposure to (UVB).

2.3.6 Sun exposure assessment

Infant sunlight exposure was parent-reported at each visit. Questions included: time of the day, duration, region of skin exposed to direct sunlight, sunscreen use and travel to sunny country (in latitudes between 35° N and 35° S). Based on the Lund and Browder chart [82], sun index was calculated by multiplying percentage body surface area (BSA) by the time spent outside per day (minutes/d) sunscreen use was not included [62, 163]. Degree of sun exposure was calculated by subtracting the average facultative skin pigmentation (forehead, mid forearm, and lower outer calf) from the constitutive skin pigmentation (inner upper arm). Birth during the vitamin D synthesizing period was defined as April through October and the non-vitamin D synthesizing period November through March [54].

2.3.7 Biochemical analysis

2.3.7.1 Blood samples

At screening, a 0.5 ml capillary blood sample was collected by a hospital nurse in a serum microtainer® tube at the same time as routine sampling for newborn screening tests (e.g.

phenylketonuria test). At baseline, 3, and 6 months, a non-fasting capillary blood sample (1.0 ml) was collected in serum microtainer® tubes after warming the heel (baseline and 3 months) or finger (6 months). Samples were centrifuged at 4000 x g for 15-20 min (6°C) to measure serum 25(OH)D, and then stored at (-80°C) for subsequent analysis. Blood-ionized calcium was measured immediately as a safety assessment (ABL80 FLEX Radiometer Medical A/S, Denmark) and the values were compared to reference data on breastfed, healthy, term-born, infants from the same research facility (**Table 2.4**) [164].

Serum total 25(OH)D concentration was measured using a chemiluminescent immunoassay (CLIA) (Liaison, DiaSorin, Stillwater, MN 55082, USA) which has a limit of detection of 10 nmol/L. The manufacture provided control samples were measured with a coefficient of variation (CV) of 8.8% (129.3±11.4) and 10.8% (38.7±4.2) for high and low kit controls, respectively. A laboratory pooled serum sample from healthy adults was tested throughout the study, revealing an inter-assay CV of <13.7%. In addition, the laboratory maintains certification with the Vitamin D External Quality Assessment Scheme (DEQAS) and participates in the National Institute of Standards and Technology (NIST) quality assurance programs. Accuracy for NIST 25(OH)D standard 972a level 1 was 98.6% (74.3±6.9 nmol/L; CV= 9.3%), and for level 4 was 89.7% (67.3±9.7 nmol/L; CV=14.3%).

2.3.7.2 Urine sample

A urine sample was obtained for safety assessments at each visit using infant urine bags and stored (-20°) for later measurement of urinary calcium corrected to creatinine at the Montreal Children's Hospital (certified by the provincial quality assurance program, the Laboratoire de sante publique du Quebec) (Beckman DxC600 California, USA) [165].

2.3.8 Adverse events

Although both vitamin D dosages (400 and 1000 IU) are considered at or within the tolerable upper intake level (UL) recommendations by the IOM [2], all infants were monitored for safety. For safety assessments, any abnormal results such as serum 25(OH)D concentration of 225 nmol/L or more were repeated within 24 hours; and plasma and urine mineral concentration were reviewed. Based on physician assessment, the infant would move to the standard of care 400 IU/d and be followed in an intent-to-treat protocol or remain in the respective treatment group.

2.3.9 Anthropometric measurements

Before each visit, a diaper, urine bag, and gown were weighted then used during the visit. By using an electronic pediatric scale with a movement program (Mettler-Toledo Inc., Switzerland), infant total weight (to the nearest 0.1 g) was obtained by subtracting the total weight of the diaper, bag, and gown. Infant crown-heel length was measured by using an infant length board (O'Learly Length Boards, Ellard Instrumentation Ltd., US) to the nearest 0.1 cm. Head circumference was measured by using a flexible, non- stretched tape (Perspective Enterprises, US). Z-scores for weight, length, and head circumference measurement were calculated using World Health Organization (WHO) software (WHO AnthroPlus, Switzerland).

2.3.10 Dietary data

Usual food and supplement intakes of the mothers were estimated by using a validated food frequency questionnaire (FFQ) (the modified Willett/Harvard) solely for the pregnancy period [166, 167]. Infant feeding practices (exclusively breastfed, mixed, or formula feeding) were collected each visit by questionnaire. Exclusive breastfeeding was defined as only breast milk, but allows the infant to receive vitamins, minerals, or medicines. Dietary information was analyzed by an excel sheet specially designed for this study using nutrient composition data from the Canadian Nutrient File 2010b.

2.3.11 Body composition assessment

Infant body composition was measured by using dual-energy x-ray absorptiometry (DXA) (APEX version 13.3:3, Hologic 4500A Discovery Series, Bedford, MA). Infants wore a gown and diaper with no metal or plastic. In addition, infants were scanned using the infant whole-body software while swaddled with a light blanket to limit movement. This scan provides estimates of lean mass and fat mass [168, 169]. Lean and fat mass accretion values (g/months) were calculated as change in lean and fat mass from baseline to 3 months and 3 to 6 months, adjusted for actual time between visits. To maintain quality assurance, a lumbar spine phantom (Hologic phantom No. 14774) was scanned daily. The coefficient of variation (CV%) of the phantom was 0.6% for bone mineral content and 0.4% for bone mineral density.

2.4 Ethical approval

The study was reviewed and approved by the Research Ethics Committee of St. Mary's Hospital Center that oversees research conducted at the Lakeshore General Hospital. Consent forms were completed by parents/ primary caregivers at both screening and baseline visits before

entering into the trial. All study visit data were entered during the visit in a secure, online databases system: Research Electronic Data Capture (REDCap).

2.5 Statistical analysis

The sample size was estimated using data from a previous study in infants [143]. Changes in 25(OH)D concentration from 1 to 3 months between non-Hispanic Caucasian and Hispanic infants had an effect size of 16.3 nmol/L and pooled standard deviation of 19.6 nmol/L. The minimum sample size was calculated to be 23 infants for each group at the 5% significance level with 80% power (two-sided test).

Normality and homogeneity of variances were tested using Shapiro-Wilk and Levene's tests. Non-normally distributed data were log transformed prior to further analysis. For all tests, the traditional significance level of 0.05 was used after correction for multiple comparisons where appropriate

Participant characteristics were tested for baseline differences among treatment (400 IU and 1000 IU) and skin colors (light and dark) using Student's t-tests for continuous variables; and Chi-square and Fisher exact tests for categorical variables. Characteristics with significant differences between groups were included as covariates in a mixed model analysis of variance (ANOVA) either as fixed effects or random effects. This model, in general, uses all available infant data. ANOVA was used to test for statistically significant differences between the groups accounting for fixed effects (skin color, treatment, time) and random effects (demographics, season of birth, and actual time between visits). A *post hoc test* (Tukey Kramer) was conducted to adjust for multiple comparisons. Data are presented as mean ± standard deviation (SD) for continuous data and number (%) for categorical variables unless otherwise stated. A p-value ≤

0.05 was accepted as significant, and all tests were two tailed. Intent-to-treat analyses were conducted using SAS version 9.4 statistical software (SAS Institute Inc, Cary, NC).

2.6 Results

2.6.1 Participants Characteristics

Of 765 infants screened at birth, 693 were excluded, and 72 were included in this study (light skin (n=55), dark skin (n=17)) (**Figure 2.3**). Maternal and infant characteristics were similar among groups except for income (p=0.038), maternal race/ethnicity (p=0.008), and pregnancy weight gain (p=0.007) between the two skin color groups. All infants were from two parent families, 60% of them had a household income above 70,000 Canadian dollars, and 75% of mothers and 58% of fathers completed university education. In addition, 45.8% (n=33) of mothers and 47.2% (n=34) of fathers were born outside of Canada. The mean age of mothers at the time of delivery was 31.4±4.6 years, 91.7% of mothers took a multivitamin during pregnancy, and 65% (n=43) reported doing so daily. Vitamin D intake from foods and supplement(s) during pregnancy showed that 52% consumed more than 600 IU/d with no differences between the treatment or infant skin color groups. A higher proportion of mothers with lower vitamin D intake (< 600IU/d) were white 59% (p=0.04). Pre-pregnancy body mass index (BMI) was <25 kg/m^2 in 61% (n=44), and the mean pregnancy weight gain was 13.2±6.0 kg. Before entering the trial, 93% of the mothers reported giving their infants a vitamin D supplement, and 87% (n=58) of infants met the Health Canada recommendation of 400 IU vitamin D in a supplement every day.

The study sample included equal numbers of male and female infants. All infants, by study design, were term born and appropriate for gestational age (**Table 2.1**). In general, anthropometric measurements increased over time, but did not differ by groups (**Table 5.1**). No

significant (time*treatment) or (time*infant skin color) interactions were observed for infant weight for length z-score, weight for age z-score, length for age z-score or BMI for age z-score. To assess lean and fat mass accretion, repeated measures ANOVA were used accounting for fixed effects including skin color, treatment, and time; and random effects including income and actual time between visits. The results indicated no significant (time*treatment) or (time*infant skin color) interactions for infant lean and fat mass or the respective accretion rates. At follow-up, 81% of infants were exclusively breastfed to 3 months of age and 66% to 6 months of age (**Table 2.2**). Almost two-thirds of the mothers 64% (n=39) reported giving their infants solid food, mainly cereal, before 6 months of age. At 6 months, no significant difference was found between the treatment groups in compliance (80-100%) of doses taken between visits (p=0.135), but compliance was higher in infants with dark skin (p=0.006) (**Table 2.5**). No significant (time*treatment) or (time*infant skin color) interactions were observed for infant sun exposure. The results of blood ionized calcium and urinary calcium creatinine ratio were within the normal range with no (time * treatment) or (time * infant skin color) interactions (**Table 2.4**).

Three and eight infants in the dark and light group, respectively, were lost to follow up at 6 months. Infants who dropped out had significantly higher serum 25(OH)D concentration (p=0.01) at birth, and 91% (n=10) were born during the vitamin D synthesizing period (p=0.041). There were no other differences by any screening or baseline characteristics between completed versus dropped out infants.

2.6.2 Vitamin D status

As shown in **Table 2.1**, at birth there was no difference between infants with light or dark skin in serum 25(OH)D concentration (p=0.46) screened at birth or between the two treatment groups (p=0.28). Serum 25(OH)D concentration was significantly higher (p=0.005) for infants

who were born from April through October (34.6±9.7 nmol/L) than November through March (27.2±12.0 nmol/L). There were also no differences in the proportions of infants who reached ≥ 50 nmol/L at 3 months (p=1.00) or 6 months (p=0.23) for both skin color groups. For treatment groups, 91% (n=29) of infants in the 400 IU/d group and all infants (n=32) in the 1000 IU/d group reached ≥ 50 nmol/L of serum 25(OH)D at 3 months (p=0.24). At 6 months, 97% (n=28) of infants in the 400 IU/d group and all infants (n=32) in the 1000 IU/d group reached ≥ 50 nmol/L (p=0.47). No infant had serum 25(OH)D ≥ 225 nmol/L at 3 months or 6 months. After 6 months of supplementation, all groups had a mean value of serum 25(OH)D above the 50 nmol/L threshold (**Figure 2.1)**. Overall, infant skin color did not affect the infant serum 25(OH)D response to vitamin D supplementation at any time point (time*infant skin color=0.40). The 1000 IU/d group had significantly higher mean concentration than the 400 IU/d group at 3 months (p<0.0001) and 6 months (p<0.0001). The relationship between serum 25(OH)D and treatment groups are presented in **Table 2.3**. The rate of change (Δ) in serum 25(OH)D concentration was noted to decrease over time (p<0.0001) with a significant (time*treatment) interaction (p=0.0009). Regardless of skin pigmentation of infants, those who were born with serum 25(OH)D concentration < 30 nmol/L did not respond differently than those who born with serum 25(OH)D concentration between 30-49.9 nmol/L (**Figure 2.2**).

2.7 Discussion

To the best of the authors' knowledge, this is the first study to test how infants born with low vitamin D status respond to vitamin D supplementation according to their skin pigmentation. Dark skin color was previously linked with infant vitamin D deficiency [7], yet it is unclear if there is a difference in the response to vitamin D supplementation between various skin colors, and if the dose to overcome vitamin D deficiency is also different. This study shows that skin pigmentation was not a significant determinant of infant serum 25(OH)D concentration. After 6 months of vitamin D supplementation, both the 400 IU/d and 1000 IU/d resulted in improved vitamin D status for all infants regardless of their skin color. In addition, 97% (approximating the Recommended Dietary Allowance (RDA) [170]) of the infants in the 400 IU/d achieved the 50 nmol/L cut off at 6 months, and if it is desirable for infants to reach this cut off sooner, the higher dose would be required. Both the 400 and 1000 IU vitamin D supplement dosages supported a mean serum 25(OH)D concentration above the 50 nmol/L threshold as suggested by the IOM for achievement of bone health [2]. This recommendation was not only set according to the IOM [2], but also Dietitians of Canada, Health Canada [22] and the American Academy of Pediatrics (AAP) [21]. Although the Canadian Paediatric Society (CPS) recommends twice (800 IU/d) the IOM recommendation of vitamin D supplementation for infants in communities where deficiency is common such as northern communities in the winter months [20], the results from this study show that this dosage may not be necessary as long as compliance rates are high. For public health policy recommendations, the focus should be that all breastfed infants receive the 400 IU/d of vitamin D supplementation on a routine basis.

There are several possible explanations for these results. As previously mentioned, the compliance rate in all groups was very high. The high level of compliance might be ascribed to

parents high-level of education. In addition, endogenous sources and synthesis capacity were not likely issues as most mothers reported following Health Canada recommendations that infants must kept out of direct sunlight. This result matches those observed in other studies [139, 140, 142, 143] although not all of these previous studies included an objective assessment of skin pigmentation. Alternatively, it is possible that no differences were observed among skin color groups owing to the nature of the classification (1-3 versus 4-6). In fact, there is one study conducted in Italy that classified infant skin color in three groups: (1) vary fair/fair skin, (2) light olive/light brown skin, and (3) medium brown/black skin [144]. It was demonstrated that group (1) was different from both (2) and (3) in terms of vitamin D status. However, that study only studied infants at birth using cord blood and venous blood within 3 days after birth, whereas the present study preselected only infants born with low vitamin D stores and intervened with vitamin D supplementation. Therefore, future studies should consider such classification in the assessment of vitamin D status of infants if the general population is studied.

Although there was no difference in compliance between any of the groups at 3 and 6 months ($p=0.15$), Δ serum 25(OH)D concentration was observed to decrease over time. This difference can be explained in part by the changes in body weight (IU/kg) over time ($p=0.015$). Hence, it could be conceivably hypothesized that the relationship between dosage and weight is present. This result has previously been described [28]. Nonetheless, 97% of the infants had serum 25(OH)D above 50 nmol/L at 6 months, reinforcing that the recommendation of 400 IU/d is suitable for infants from birth to 6 months.

The UL was set at 1000 IU/d for infants from birth to 6 months [2, 17]. Thus, both dosages used in this study are considered safe for the general population. In addition, the safety of vitamin D at dosages greater than 400 IU/d for infants has been tested in multiple studies [28,

128, 171]. All of the study data reflected normal growth and calcium homeostasis. No infant at any time point had serum 25(OH)D concentration above 225 nmol/L, which is considered as potentially toxic [20].

This study has many strengths including the study design; the study includes effective and practical methods for infant assessments which remained constant during all study visits such as the objective measurement of skin pigmentation using a spectrophotometer. The generalizability of these results however is subject to certain limitations. For instance, 67% of infants were recruited during the vitamin D synthesizing period, that in theory could affect 25(OH)D concentration after screening, however exposure to UVB was limited. Also, the study sample consisted of a large proportion of mothers with high income and education, limiting extrapolation to other demographic groups. Unfortunately, the sample size was not balanced in the number of infants with dark and light skin color.

The findings of this study suggest that no significant difference is observed in serum 25(OH)D concentration in response to vitamin D_3 supplementation between infants with light or dark skin. These results are in accordance with the most recent IOM vitamin D recommendation which does not differentiate between skin colors [2]. Further research is needed to support the results as the IOM in 2007 asked for more research to improve the knowledge about optimal circulating 25(OH)D concentration across the lifespan, including infants, for different race and ethnicity groups in the USA and Canada [2]. In addition, the effect of ethnicity on 25(OH)D is one of the topics that the AHRQ-Ottawa evidence-based report (2008) suggested to be studied [172].

2.8 Tables

Table 2.1: Screening characteristics of mothers and infants

Characteristic	Treatments (IU/d) 400 (n=36)	1000 (n=36)	p-value	Infant skin color Light (n=55)	Dark (n=17)	p-value
Mothers						
Mother's age at delivery, y	32.1±4.4	31.3±4.8	0.46	31.9±4.1	31.1±6.1	0.58
Income ≥ 70000 Canadian $	21 (62)	19 (58)	0.73	34 (67)	6 (38)	0.04
Non-white[1]	19 (54)	20 (56)	0.81	25 (45)	14 (82)	0.01
Mother's education, ≥ university	24 (67)	30 (83)	0.10	42 (76)	12 (71)	0.63
Pre-pregnancy BMI (< 25), kg/m^2	19 (53)	25 (69)	0.17	36 (65)	8 (47)	0.17
Pregnancy weight gain, kg	13.5±5.2	13.0±6.9	0.75	14.3±5.4	9.8±6.9	0.01
Infants						
Male	19 (53)	19 (53)	1.00	29 (53)	9 (53)	0.99
Female	17 (47)	17 (47)		26 (47)	8 (47)	
Gestation age, weeks	39.7±1.1	39.5±1.1	0.52	39.6±1.1	39.5±1.0	0.69
Birth weight, g	3.4±0.4	3.3±0.4	0.84	3.4±0.4	3.3±0.4	0.56
Birth weight-for-length z-score [2]	0.91±1.39	0.89±1.48	0.95	0.94±1.56	0.76±0.88	0.66
Birth weight-for-age z-score[2]	0.09±0.83	0.07±0.85	0.94	0.06±1.04	0.42±0.96	0.21
Birth length, cm	51.2±2.3	51.1±2.3	0.76	51.3±2.5	50.8±1.5	0.31
Birth length-for-age z-score[2]	0.80±1.14	0.77±1.19	0.90	0.84±1.24	0.61±0.85	0.48
Birth head circumference, cm	34.5±1.3	34.4±1.4	0.79	34.4±1.4	34.8±1.3	0.28
Birth head circumference-for age z-score[2]	0.16±1.01	0.13±1.06	0.92	0.61±1.04	0.42±0.96	0.21
Season of birth[3]	24 (67)	20 (56)	0.33	34(62)	10 (59)	0.82
APGAR						
1 minute, ≥7	33 (92)	34 (95)	1.00	51 (93)	16 (94)	1.00
5 minutes, ≥7	36 (100)	35 (97)	1.00	54 (98)	17 (100)	1.00
Birth (24-36 h) serum 25(OH)D[4], nmol/L	30.2±9.5	33.1±12.6	0.28	32.2±10.6	29.9±13.2	0.46

Data are mean (± SD) and number (%) for categorical variables.

[1] non-white mothers: South Asian (n=3), Chinese (n=1), Black (n=6), Latin American (n=5), Arab (n=12), Southeast Asian (n=3), West Asian (n=1), and other (n=8).
[2] Calculated using WHO software (WHO AnthroPlus, Switzerland).
[3] Born during vitamin D synthesizing period (1 April to 31 October).
[4] Infants were screened within 24-36 h after birth for low vitamin D.

Table 2.2: Breastfeeding status of infants at baseline, 3 and 6 months

Time	Breastfeeding status	Infants skin color					
		Light		p-value	Dark		p-value
		400 IU/d	1000 IU/d		400 IU/d	1000 IU/d	
Baseline[1]	Exclusively	22 (81.5)	22 (84.6)	1.00	6 (85.7)	8 (80.0)	1.00
	Mixed	5 (18.5)	4 (15.4)		1 (14.3)	2 (20.0)	
3 months[2]	Exclusively	19 (86.4)	17 (81.0)	0.69	4 (66.7)	8 (80.0)	0.60
	Mixed	3 (13.6)	4 (19.0)		2 (33.3)	2 (20.0)	
6 months[3]	Exclusively	12 (60.0)	14 (77.8)	0.30	4 (100.0)	3 (37.5)	0.08
	Mixed	8 (40.0)	4 (22.2)		0 (00.0)	5 (62.5)	

Data are presented as number (%).

Exclusively Formula Feeding, [1] n=2, [2] n=6, [3] n=11.

Exclusive breastfeeding was defined as only breast milk, but allows the infant to receive vitamins, minerals, or medicines.

Baseline (1-4 weeks) (n=72), 3 months (n=65), 6 months (n=61).

Table 2.3: Serum 25(OH)D concentration for each treatment group (400 and 1000 IU/d) across time

	Treatments (IU/d)	
Time	**400**	**1000**
Screening[1] (n=72)	30.2±9.5[a]	33.1±12.6[a]
Baseline[2] (n=58)	46.7 ±16.2[a]	43.3±12.4[a]
3 months (n=64)	77.9±24.1[b]	127.2±37.8[c]
6 months (n=61)	82.5±22.3[b]	122.0±34.6[c]

[1] Infants were screened within 24-36 h after birth for low vitamin D status.

[2] Insufficient serum volume, sample reserved for LC-MC/MS.

Values are means ± SD.

Different superscripts indicate statistically significant ($p \leq 0.05$) using mixed model ANOVA with Tukey-Kramer post hoc test accounting for mother race/ethnicity, mother pregnancy weight gain, infant born during vitamin D synthesis period (1 April to 31 October) and income.

p values: time <0.0001, treatment <0.0001, time*treatment <0.0001, infant skin color=0.14, and time*infant skin color=0.6.

Table 2.4: Biochemistry values for each time point for all study groups

Variable	Time [1]	Light skin		Dark skin		Normal rang
		400 IU/d	1000 IU/d	400 IU/d	1000 IU/d	
Urinary Ca:Cr	Baseline	1.3±0.8	1.4±0.8	0.6±0.1	1.4±0.9	< 2.2[2]
mmol: mmol	3 months	1.6±1.0	1.9±1.2	0.8±0.6	1.4±0.7	< 2.2[2]
	6 months	1.4±0.7	1.2±0.5	0.7±0.2	0.8±1.1	< 2.2[2]
Blood iCa	Baseline	1.44±0.06	1.45±0.08	1.43±0.05	1.43±0.06	1.32-1.47
mmol/L	3 months	1.42±0.03	1.41±0.04	1.41±0.03	1.40±0.06	1.31-1.46
	6 months	1.37±0.11	1.39±0.03	1.41±0.05	1.38±0.04	1.29-1.41

Values are means ± SD.

[1] Baseline (n=72), 3 months (n=65), 6 months (n=61).

[2] Value currently used at the Montreal Children's, Shriners, Children's Hospital of Eastern Ontario and General Hospitals.

Table 2.5: Changes in infant categorical variables over time

Variable	Time[1]	Light skin			Dark skin		
		400 IU/d	1000 IU/d	p-value	400 IU/d	1000 IU/d	p-value
Compliance, % of dosage taken (100-80)	Baseline	n/a	n/a	n/a	n/a	n/a	n/a
	3 months	21 (81)	16 (76)	0.70	5 (83)	9 (90)	1.00
	6 months	15 (60)	14 (64)	0.80	3 (100)	10 (100)	1.00
Compliance, every day/almost every day[2]	Baseline	25 (96)	17 (68)	0.01	7 (100)	9 (100)	1.00
	3 months	25 (93)	20 (91)	1.00	6 (100)	10 (100)	1.00
	6 months	19 (76)	20 (91)	0.17	4 (100)	10 (100)	1.00
Serum 25(OH)D ≥ 50 nmol/L	Baseline	10 (43)	5 (23)	0.14	1 (20)	3 (38)	1.00
	3 months	24 (92)	22 (100)	0.49	5 (83)	10 (100)	0.37
	6 months	25 (100)	22 (100)	1.00	3 (75)	10 (100)	0.29
Serum 25(OH)D ≥ 75 nmol/L	Baseline	1 (4)	0 (00)	1.00	00 (00)	00 (00)	1.00
	3 months	14 (54)	19 (86)	0.03	1 (17)	10 (100)	0.001
	6 months	14 (56)	19 (86)	0.03	2 (50)	9 (90)	0.18

Data are number (%).

[1] Baseline (n=72), 3 months (n=65), 6 months (n=61).

[2] Before entering the study, 93% of the mothers reported giving their infants vitamin D supplement, and around 87% (n=58) of infants met the Health Canada recommendation by using 400 IU/d.

2.9 Figures

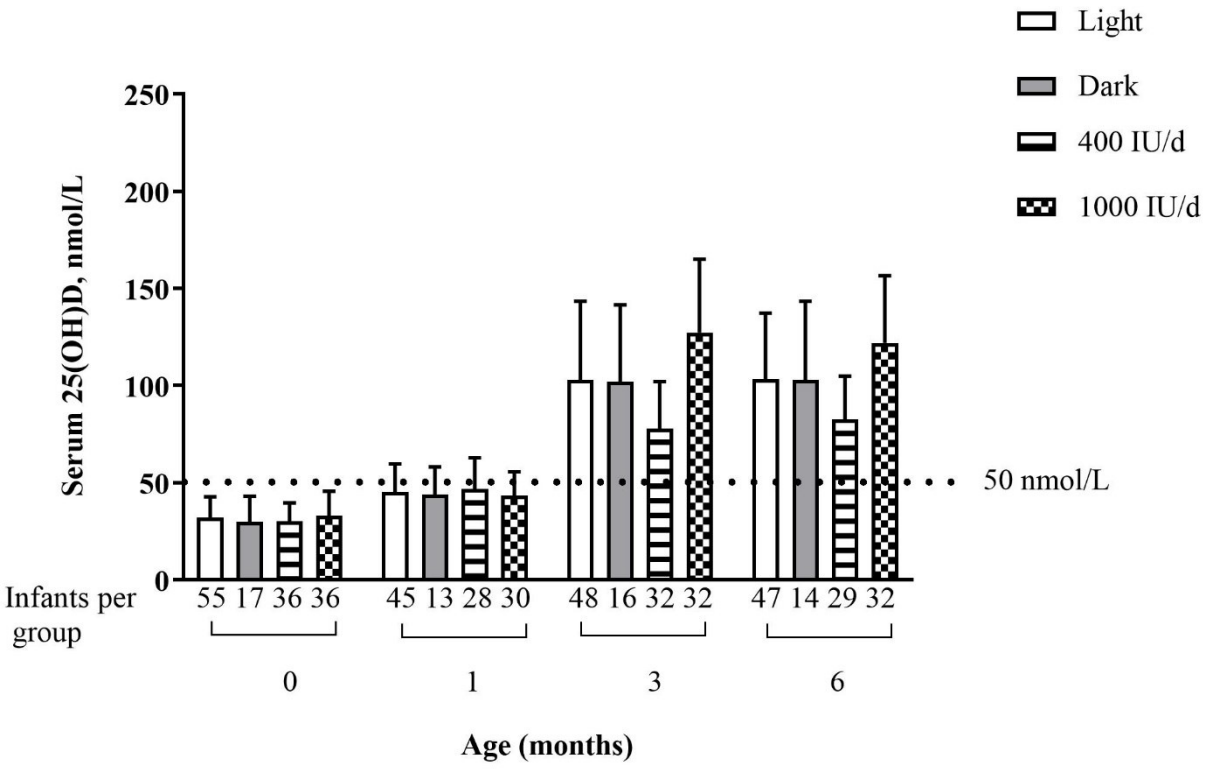

Figure 2.1: Infant serum 25(OH)D concentration from screening at 24-36 h of life to 6 months according to infant skin color and treatment groups (Mean ± SD)

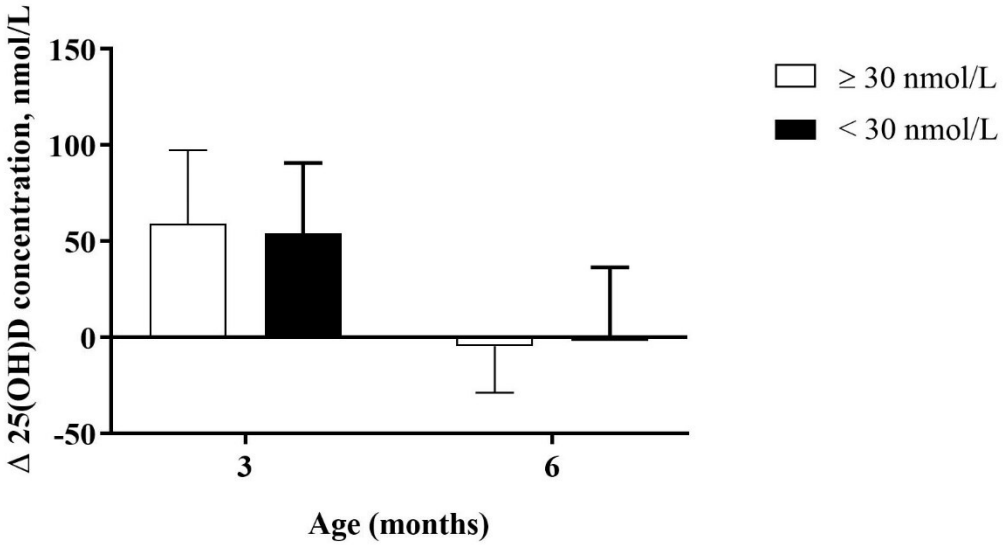

Figure 2.2: Infant serum 25(OH)D concentration from baseline to 3 months and from 3 to 6 months by screening serum 25(OH)D category (Mean change ± SD)

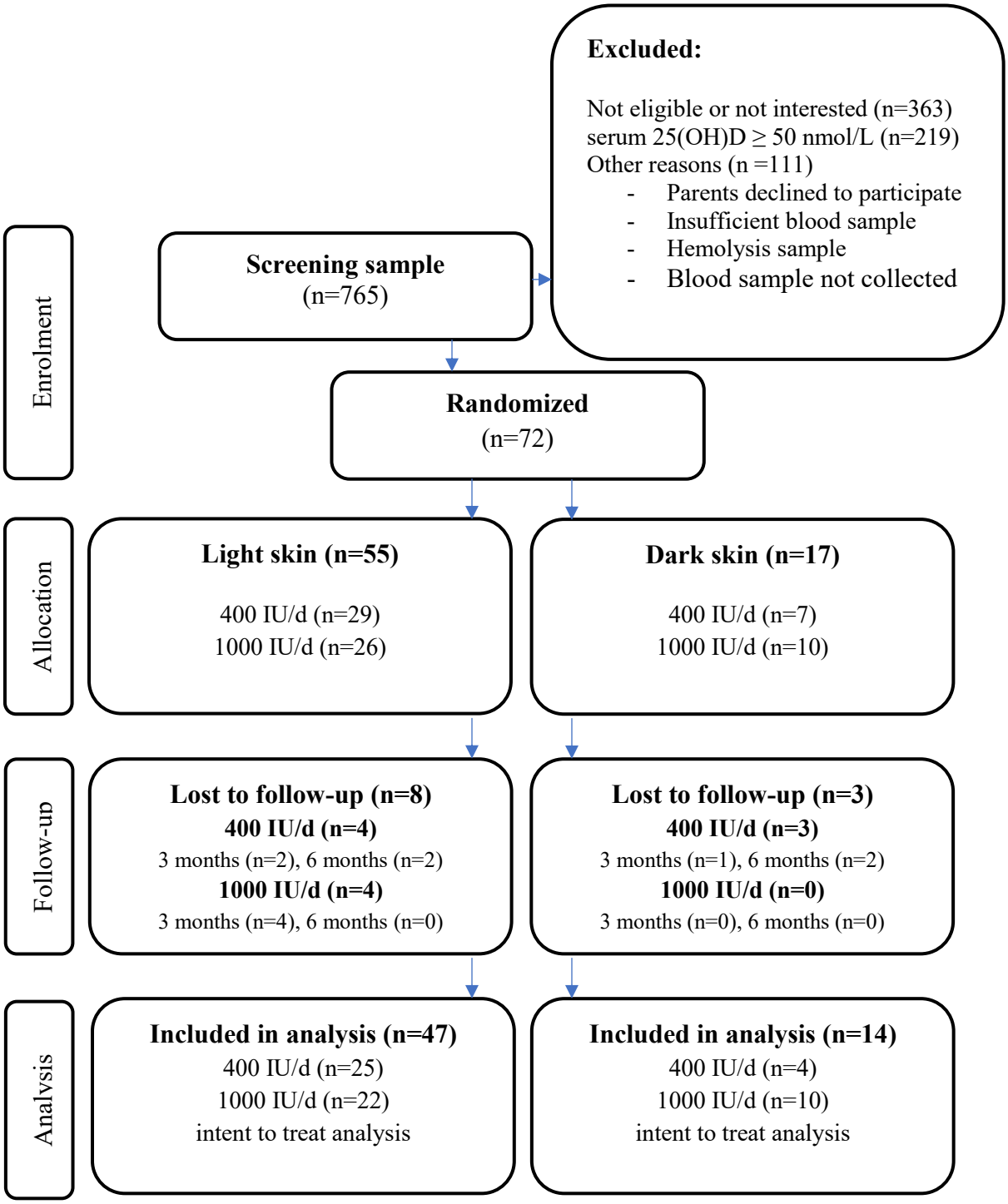

Figure 2.3: CONSORT diagram for the study

3.0 General discussion

3.1 Findings

This study was designed to test how infants born with low vitamin D status respond to vitamin D supplementation according to their skin pigmentation. Dark skin color was previously linked with infant vitamin D deficiency [7], yet it is unclear if there is a difference in the response to vitamin D supplementation among various skin colors and if the dose to overcome vitamin D deficiency is also different. It was hypothesized that the response to vitamin D supplementation would be lower in infants with dark skin pigmentation, and the infants with darker skin color would need the higher dose to support faster improvements in 25(OH)D concentration. Also, the main factor that would determine an infant's response to vitamin D supplementation would be the birth serum 25(OH)D concentration. The main observation was that skin pigmentation was not a significant determinant of infant serum 25(OH)D concentration in this study. After 6 months of vitamin D supplementation, both the 400 IU/d and 1000 IU/d resulted in greater vitamin D status for all infants regardless of their skin color or the vitamin D status at birth. Therefore, it is plausible that vitamin D status should not be a concern for all healthy, term, breastfed infants regardless of skin color or initial vitamin D status when the 400 IU/d vitamin D supplementation is used on a routine basis.

The possibility that vitamin D status at birth might be an important covariate in the response to supplementation was also not accepted based on the results of this thesis. Although only infants with low vitamin D status were studied (< 50 nmol/L of serum 25(OH)D), it was clear that screening status < 30 nmol/L did not result in a different response to supplementation compared with infants with 25(OH)D between 30 and 50 nmol/L. It seems more likely that infants with dark skin are often born with low vitamin D status as a reflection of mother's own

vitamin D status and the consequently low maternal-fetal transfer [146]. Synthesis capacity and endogenous sources of vitamin D for the infants were unlikely to contribute to a change in 25(OH)D concentration after birth as most mothers reported following Health Canada's recommend that infants must kept out of direct sunlight.

The results of this thesis are in agreement with other studies in adults and infants [139, 140, 142, 143] that also suggest vitamin D supplementation does not have to be specific for individuals with light or dark skin pigmentation. In regard to other aspects, it is difficult to compare the results of this study with others because most either have been performed with differences in 25(OH)D concentration at inception of the treatment between the study population, or the lack an objective method to measure skin color, if any. As mentioned earlier, skin pigmentation develops during the first 6 months of life [75]. So that poses challenges in randomizing infants at the beginning of the study according to their skin color. As shown in **Figure 3.1** the number of infants in each skin type changes with time to lighter or darker skin color. Additionally, if the data are categorized by ITA° values for each time point, and then examined again for how each skin type effects the serum 25(OH)D response to vitamin D supplementation, all skin type groups increased with time. However, this increase is more pronounced in skin type 1 and 2 ($p=0.033$); although this effect disappeared after adjustment for mothers' pregnancy weight gain, birth during the vitamin D synthesis period and income ($p=0.27$). Thus, it is possible that no difference was observed because the group classification was too broad with only two groups: 1-3 and 4-6. Cadario et al. classified neonates in three groups: (1) vary fair/fair skin, (2) light olive/light brown skin, and (3) medium brown/black skin [144]. The results showed that significant differences were found between group 1 and 2 ($p<0.01$); group 1 and 3 ($p<0.01$); and group 2 and 3 ($p<0.02$) for cord blood 25(OH)D.

Although the study was cross-sectional, and skin color classification was subjectively assessed, it shows a pattern in neonatal skin color classification. On the other hand, this pattern might be a reflection of maternal vitamin D status and supplementation during pregnancy or her skin color. Moreover, this division as three groups was not possible in the present thesis due to sample size limitations. Alternatively, given the limited number of infants with darker skin pigmentation, changing the classification of infants to light skin type 1 and 2, and dark skin types 3, 4, 5, and 6 may have been more informative (**Figure 3.2**). This would not only improve the sample size balance, but also would better reflect a key factor that affects endogenous synthesis capacity after 6 months of age when infants are exposed to the sun. The dark group would likely better reflect mixed ethnic/racial background and culture. Another way to improve upon the results of this thesis could be to randomize a large group of infants to the same dosage and follow them to 6 months or more and then divide infants with the same previous classification (light 1 and 2; and dark 3, 4, 5 and 6).

Various skin colors might reflect differences in ethnicity/race of the family background. It is important to realize that there is a variation in skin pigmentation within each ethnic/racial group. Of infants with dark skin, 76.5% of fathers and 70.6% of mothers were born outside of Canada, and 82.4% of mothers and 76.5% of fathers were non-white in ethnicity/race. Several publications have appeared in recent years documenting significant differences among various racial/ethical groups in vitamin D metabolites and vitamin D binding protein [156-158, 173, 174]. This could also have been an important aspect in explaining infant vitamin D status although the current knowledge about genetic factors is limited, and factors such as sun exposure and lifestyle remain important factors to determine vitamin D status [159]. In addition, since vitamin D binding protein is low in newborn infants [173], changes in this protein over time may

explain why differences were not observed in the present study of young infants compared to other studies in older children and adults [97, 158, 175].

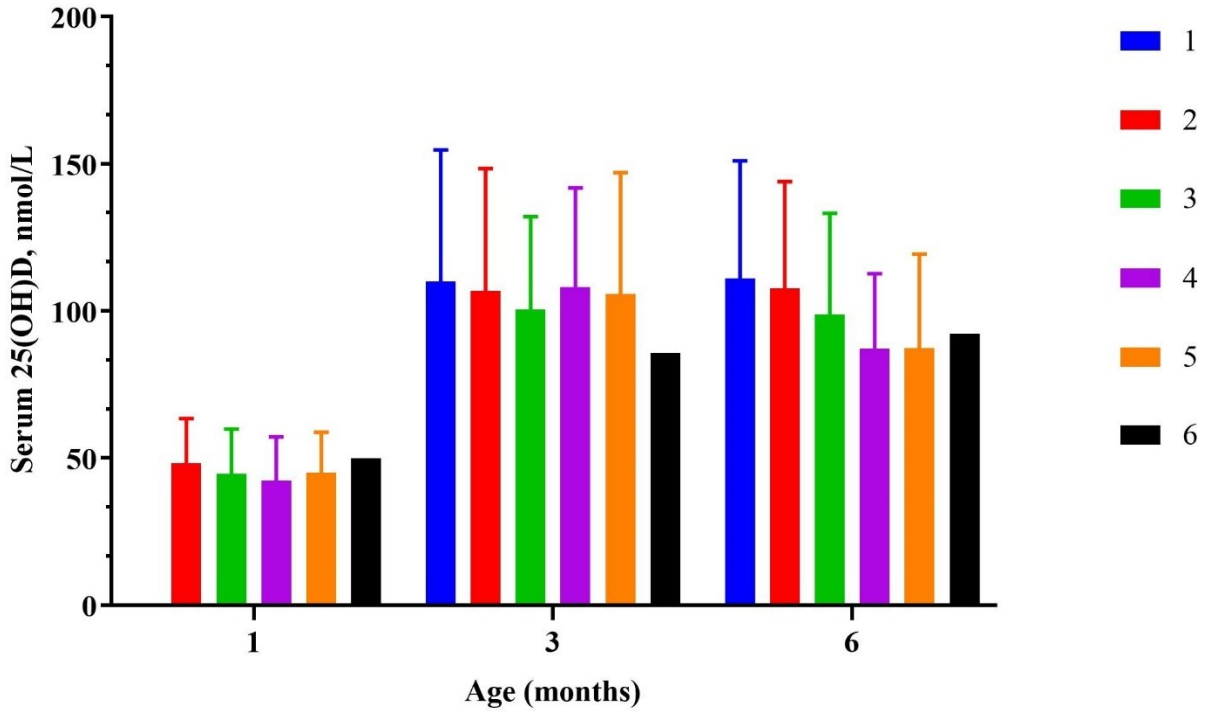

Figure 3.1: Infant serum 25(OH)D concentration for each skin type by using inner upper arm ITA° value from each visit: baseline, 3 and 6 months (Mean ± SD)

Time	**Baseline**						**3 months**						**6 months**					
Skin type	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
n	0	20	23	11	6	1	11	29	11	4	4	2	16	22	12	5	4	2

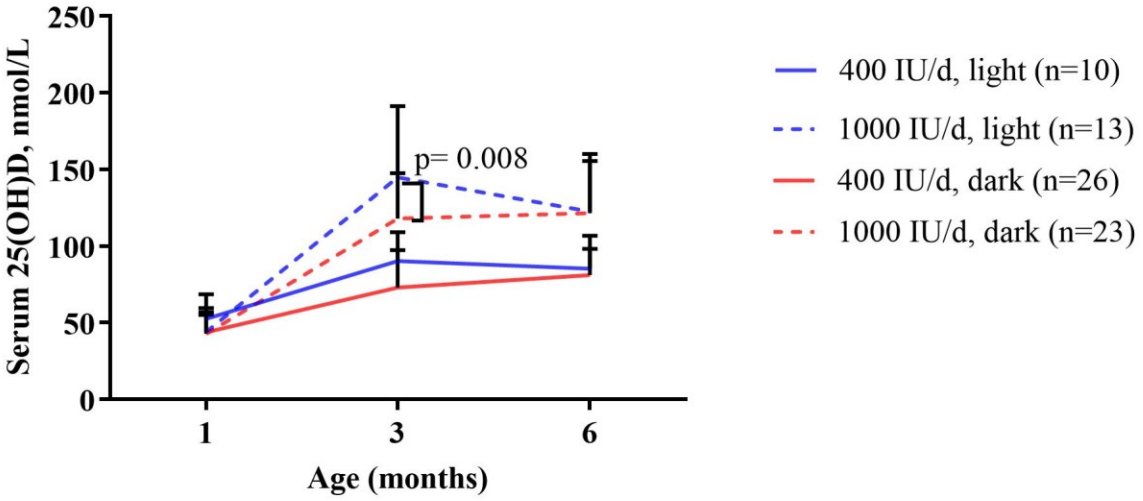

Figure 3.2: Infant serum 25(OH)D concentration from baseline to 6 months by different skin color classifications (means ± SD)

In this figure infants were reclassified as light skin type 1 and 2, and dark skin types 3,4,5, and 6. Adjusted for multiple comparison was not used in this analysis because of preliminary nature of the work. In addition, the sample size estimate was not based on adjusting for multiple comparison.

p- values: time <0.0001, dosage <0.0001, time* treatment <0.0001, infant skin color=0.054, and time*infant skin color=0.07

It is important to realize that the 400 IU/d dose of vitamin D was effective in raising serum 25(OH)D concentration ≥ 50 nmol/L for 97% of infants at 6 months. This is 3 months longer than the duration in the Gallo et al. dose response study where a dose of 400 IU/d maintained serum 25(OH)D concentration ≥ 50 nmol/L for 97% of infants at 3 months [28]. This might be related to the difference in the study design (inclusion criteria). Gallo et al. included infants regardless of baseline status which was predominantly over 50 nmol/L of serum 25(OH)D, while the present study included only infants born with low vitamin D stores with serum 25(OH)D below 50 nmol/L.

Although there was no difference in compliance between 3 and 6 months ($p=0.15$), Δ serum 25(OH)D concentration was observed to decrease over time. This difference can be explained in part by the changes in body weight (IU/kg) over time ($p=0.015$). Hence, it could be conceivably hypothesized that the relationship between dosage and weight is present. This result has previously been described [28]. However, it is not deemed necessary to have different recommendation across infancy since healthy vitamin D status was achieved by all infants.

Other covariates that were examined in this study included infant season of birth, fat and lean mass. Season of birth was found to impact serum 25(OH)D concentration as summer born infants usually have higher serum 25(OH)D concentration than winter born infants [145, 176]. In this study, 67% of infants were recruited during the vitamin D synthesizing period (1 April-31 October) resulting in higher birth serum 25(OH)D concentration ($p=0.005$). This is likely a reflection of maternal exposure to UVB and resultant higher vitamin D status and maternal-fetal transfer. For skin color, a Korean study that used a spectrophotometer to measure melanin index (a parameter for melanin content of the skin) found that there was no effect of season of birth on

melanin index [77]. Whether melanin index over time affects vitamin D status of infants is not clear.

Serum 25(OH)D concentration can capture both endogenous and exogenous vitamin D sources. It is likely that the main exogenous source was the vitamin D supplement, because breast milk is not a major source for vitamin D, and 81% of infants were exclusively breastfed to 3 months of age and 66% to 6 months of age. The rates of mothers who exclusively breastfed for 6 months in this sample were higher than Canadian statistics (25.9%) [177]. No infant in this study received cow's milk. Although it might be anticipated that formula fed infants would have higher mean serum 25(OH)D concentration than breastfed infants, for the few infants who were receiving formula, they had similar serum 25(OH)D concentration to those who were breastfed for all time points. Almost two-thirds of the mothers 64% (n=39) reported giving their infants solid food, mainly cereal, before 6 months of age, but it is not expected to have a significant impact on vitamin D status. In a study done on Canadian infants, it is suggested that from 0 to 6 months the mean daily intake of vitamin D from solid food was 16 IU [108]. In addition, in Canada infant food is not usually fortified with vitamin D [108].

Maternal nutrition during pregnancy affects infant 25(OH)D concentration at birth. For that reason, a validated FFQ was used to estimate vitamin D intake from food and supplements. Almost half of them (52%) consumed more than 600 IU/d which is recommended by the IOM [2]. There was a trend ($p=0.06$) between the FFQ results and vitamin D status at birth based on the screening serum 25(OH)D concentration (24-36 h). No significant differences were found between maternal use and frequency of prenatal supplements from the study questionnaires and infant screening 25(OH)D at birth. The dietary intake during lactation was not analyzed for this study because it is not expected to have a significant effect on vitamin D content of breast milk.

Adherence to vitamin D supplementation is one of the greatest challenges that researchers and clinicians face. The study data found good compliance with Health Canada's recommendation by using the standard care of 400 IU/d before entering the study as well as with the study supplement. The high level of compliance might be associated with parents high-level of education. Previously, researchers found that the primary reason for noncompliance with supplementation was that mothers thought that vitamin D supplemental was not needed during the transition to fortified formula [110]. In this study, there were no significant differences found between exclusively breastfed and formula fed (exclusively and mixed) in supplement adherence. Perhaps this is related to being as a part of a vitamin D study and receiving advice about the importance of vitamin D supplement from the study researchers.

The findings of this study suggest that no significant differences in serum 25(OH)D concentration in response to vitamin D supplement exists between infants with light or dark skin. Also, it seems that the 400 IU/d is a good population health strategy to support healthy vitamin D status for all infants. These results are in accordance with the new IOM vitamin D recommendation which do not differentiate among skin colors [2]. For the general population, it may be better to focus on ensuring that all infants receive the standard care of 400 IU/d dose on a routine basis as an initial step towards improved vitamin D status. This can be achieved through collaboration between several institutions and groups. For example, breastfeeding support groups in order to clarify that the introduction of vitamin D supplement with breastfeeding does not affect its being exclusive. As exclusive breastfeeding defined by the WHO as only breast milk, but allows the infant to receive vitamins, minerals and medicines [122].

3.2 Strengths and limitations

The study conducted as part of this thesis has many strengths besides being, a double-blind, randomized and a single-center study. In epidemiological studies, this design is considered the gold standard where many covariates and confounders can be controlled. Unlike an observational study, it can demonstrate causality. The study includes effective and practical methods for infant assessments, which remained constant during all study visits.

The study sample consisted of a large proportion of mothers with a high level of income and education. Because low socioeconomic status and education are risk factors for having low vitamin D status [178], the study results may not be representative to other segments of the population. Although having all infants in the study as healthy, appropriate size for gestational age, and term born might limit the generalization of the results, it helps to ensure that body pool sizes are homogeneous.

In order to reduce the chance of making a type 1 error, calculating sample size is important. Hence previously published data [143] was used to estimate the approximate sample size. A total of 23 infants for each group would be needed to detect a difference between groups, with α (two-tailed) =0.05, (1-β) =0.80, pooled standard deviation=19.6 nmol/L, and effect size=16.3 nmol/L. The study sample size is less than the target, and in view of that the study power might have been affected (**Table 3.1**). However, the power calculation using the existing study data on infants with light and dark skin suggested that 2195740 infants for each group (total sample size 4391480) to achieve a power of 80% and level of significance of 5% (two-sided). In view of that, it seems less important to focus on knowing the difference between infants with light or dark skin color and vitamin D supplementation response when 97% of them

achieve ≥ 50 nmol/L. It is important to also realize that the sample size estimate did not include multiple comparison or even the two doses within each group.

Season of birth was found to affect screening serum 25(OH)D concentration [173, 179]. The majority of the recruitment period was during the vitamin D synthesizing period (1 April-31 October). Hence, serum 25(OH)D concentration was significantly higher (p=0.005) during synthesizing compared to non-synthesizing period. For this reason, born during vitamin D synthesizing period explored as a covariance in the statistical analysis.

Table 3.1: Sample size requirement for detecting a difference in means of 16.3 nmol/L in 25(OH)D, with a pooled standard deviation of 19.6 nmol/L at different level of power and significance

Power	80%	80%	90%	67%	67%
Significant	0.05	0.01	0.01	0.05	0.01
n, per group	23	34	44	17	27

Two-sided level of significant for equal group size.

Based on [143].

The key strength of this study is measuring skin color which was embedded in the study design and is used as stratification criteria. The objective assessment by spectrophotometer is widely used in epidemiological studies not only to measure natural skin color but also skin color changes due to UVB radiation. To date, only one study on infant serum 25(OH)D concentration in Canada that used this instrument [142] whereas most other studies either used a subjective method or used infant race/ethnicity as approximation to infer skin color.

Spectrophotometer data show values that can be used to classify skin color as a continuous or categorical variable. This portable instrument is quick; it takes around one second for each measurement. This is a good feature when working with infants especially 6 months and older. However, inferences drawn from this instrument must be taken cautiously for several reasons. Firstly, because of the infant skin nature [86] and the potential for newborn jaundice [88]. For example, the inner upper arm was used to determine all infants constitutive skin color, but this site was affected by jundice in some infants. For those infants, the lower outer calf was also evaluated, although it resulted in the same skin color classification. It might be better to rely only on the inner under arm ITA° result in the classification especially that objective diagnosis was not included to ensure consistency. Alternatively, infants with jaundice should be excluded [77, 180]. For some infants, when they cry, they turn pink which might affect the measurement. So not only measurement technique but also time should be taken into account. In addition to that, infants reach young adult skin color within six months of age [75]. These factors might have influenced infants allocation into the two skin color groups at baseline visit (1-4 weeks) however at 6 months of age only one infant's color was changed by the amount that would result in change classification from one group to another. Thus assessment at 1 month may be valid for

the categories used in the present study. If more groupings are used, this needs further consideration.

To measure constitutive skin color, it may also be useful to include the buttocks area as it is a reliable site [80]. It would be of value to add the back of the hand to measure facultative pigmentation the same way used by recent Canadian Health Measures Survey (CHMS) although infants were not included [181]. Spectrophotometer assessments were used also to measure facultative pigmentation at the forehead, mid-forearm, and lower leg. At 3 and 6 months study visits, differences between ITA° for faculatative and constitutive values assisted in estimate recent degree of sun exposure although sun exposure was limited for this age group that in line with the current Canadaian recommendation. With this in mind, there is no need to use an objective method such as a dosimeter to measure sun exposure. Especially since researchers found a positive correlation ($r=0.88$, $p<0.001$) between dosimeter reading and the total minutes outside as recorded by mother [64]. Unlike questionnaire, dosimeters do not take into acount BSA exposed to sun light. Moreover, sun exposure was assessed by questionnaire that can capture sun exposure for individual with skin type I who burn but do not tan, and allowed for comparison with spectrophotometre values. This was not possible in the current study due to the fact that few infants in this study were exposed to direct sun light.

Another limitation of this thesis is lack of some baseline serum 25(OH)D concentration results (n=14, missing values) because the autoanalyzer assessment was designed for safety assessments which would not be necessary at baseline. Ultimately, liquid chromatography mass spectrometry (LC-MS/MS) will be used in the full trial to obtain serum 25(OH)D at all time-points. Screening 25(OH)D at birth results reflect season of birth and maternal status during pregnancy and therfore would not explain supplementation practices after birth and before

joining the study. For that reason, a sub group analysis was done without baseline value with no obvious differences.

For ease of infant blood sampling, serum 25(OH)D concentration was collected from a capillary blood sample although it has been demonstrated in adults to overestimate serum 25(OH)D by around 19 nmol/L in comparison to venous blood (4). Infants under one year of age have a significant concentration of C-3 epimers [13]. Because serum 25(OH)D was measured by the (Liaison, DiaSorin, Stillwater, MN 55082, USA) CLIA, which does not detect C-3 epimers, this would not explain the heigher value at 3 and 6 months [182]. However, CLIA does cross react with 24,25-dihydroxyvitamin D and calciferol. Thus, infants' vitamin D concentration might be overestimated [182, 183].

3.3 Conclusions

The finding of the study reported upon in this thesis suggests that no difference exists in serum 25(OH)D as a response to vitamin D supplementation between infants with light or dark skin. Also, it seems that the 400 IU/d is a good population health strategy to support healthy vitamin D status in infants of all skin colors. These results are in accordance with the most recent IOM vitamin D recommendation which does not differentiate between skin colors [2]. If all healthy infants who are born with low vitamin D status respond well to vitamin D supplementation, the next step is to ensure that they get it.

4.0 References

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5 0 Appendix

Table 5.1: Changes over time for continuous variables

		Light skin		Dark skin	
Variable	**Time**	**400**	**1000**	**400**	**1000**
Age, months	Baseline	0.70±0.26	0.63±0.19	0.66±0.19	0.73±0.23
	3 months	3.20±0.21	3.04±0.20	3.10±0.09	3.18±0.44
	6 months	6.13±0.23	6.21±0.32	6.19±0.23	6.37±0.33
Weight, kg	Baseline	3.9±0.6	3.8±0.4	3.9±0.5	3.9±0.5
	3 months	6.2±0.8	6.3±0.9	6.5±1.0	6.1±0.9
	6 months	7.7±0.9	7.9±1.1	7.9±1.4	8.0±1.5
Weight-for-length z-score	Baseline	-0.32±1.03	-0.49±076	0.05±1.23	-0.29±0.59
	3 months	0.03±1.01	-0.01±1.05	0.40±1.33	-0.35±0.82
	6 months	0.05±0.96	-0.01±0.99	0.50±1.20	0.06±1.26
Weight-for-age z-score	Baseline	-0.21±0.87	-0.11±0.66	-0.06±0.99	-0.28±0.61
	3 months	-0.08±0.99	0.23±1.08	0.34±1.21	-0.20±1.16
	6 months	-0.01±1.08	0.18±1.05	0.39±1.35	0.14±1.55
Length, cm	Baseline	52.8±1.9	52.7±2.4	52.3±2.3	52.5±1.6
	3 months	60.9±2.2	61.7±3.4	61.3±3.1	61.4±2.3
	6 months	67.0±2.1	67.9±3.2	67.1±3.2	68.1±3.1
Length-for-age z-score	Baseline	-0.16±0.06	0.06±1.04	-0.26±1.15	-0.22±0.65
	3 months	-0.07±1.05	0.47±1.49	0.20±1.56	0.13±1.08
	6 months	0.05±0.98	0.36±1.22	0.23±1.58	0.35±1.29
BMI, kg/m^2	Baseline	13.9±1.4	13.7±0.6	14.2±1.2	13.9±1.1
	3 months	16.7±1.4	16.6±1.5	17.3±2.0	16.2±1.4
	6 months	17.1±1.3	17.2±1.5	17.6±2.1	17.2±1.9
BMI-for-age z-score	Baseline	-0.17±0.95	-0.21±0.54	0.12±1.1	-0.24±0.53
	3 months	-0.05±0.96	-0.04±1.03	0.34±1.26	-0.38±0.95
	6 months	-0.09±0.92	-0.04±1.01	0.38±1.20	-0.06±1.25
Head circumference, cm	Baseline	36.3±1.1	36.0±1.4	36.7±0.9	36.1±1.4
	3 months	40.3±1.1	40.2±1.4	40.7±0.8	40.1±1.3
	6 months	43.1±1.1	43.0±1.4	43.5±0.7	43.0±0.9
Head circumference -for-age z-score	Baseline	0.09±1.02	0.35±2.38	0.49±0.61	-0.12±0.68
	3 months	0.07±0.92	0.15±0.93	0.48±0.67	-1.18±0.91
	6 months	0.16±0.92	0.03±0.88	0.65±0.95	-0.02±0.66

Data are mean ± SD.