

Vitamin D3

Cholecalciferol

[CAS No. 67-97-0]

Comments of DSM Nutritional Products AG on the proposed harmonized classification and labelling for vitamin D3 (cholecalciferol)

Author: DSM Nutritional Products AG
Wurmisweg 576
4303 Kaiseraugst
Switzerland

Document status: Final

Release Date: 22-February-2016

TABLE OF CONTENTS

Overall Summary	3
1. Germ cell mutagenicity	7
1.1. <i>In vitro</i> data – Instability of vitamin D3 in solvent	7
1.2. <i>In vivo</i> MNT/Comet Assay	10
1.2.1. <i>In vivo</i> MNT	10
1.2.2. Acceptability of the <i>in vivo</i> Comet	10
1.2.3. Toxicity in the target organ biased the results of the Comet assay in the liver	11
2. Carcinogenicity	12
2.1. Adequacy and Reliability of animal data	12
2.1.1. Ikezaki study	12
2.1.2. Tischler study	14
2.2. Mode of Action and Relevance of Animal Data for Human Being	16
2.2.1. Disturbed calcium homeostasis can lead to pheochromocytoma in the rat adrenal medulla	16
2.2.2. Pheochromocytoma due to disturbed calcium homeostasis is species specific to rats	21
2.3. CLH proposal versus conclusion of other competent bodies	23
2.4. Human Data	23
2.4.1. Natural human exposure to vitamin D3 from synthesis in skin	23
2.4.2. Epidemiological data	25
3. Reproductive toxicity	27
4. References	28

Overall Summary

DSM Nutritional Products AG (referred as DSM thereafter) with its headquarter in Kaiseraugst, Switzerland manufactures vitamin D3 (also known as cholecalciferol) and vitamin D3 containing formulations at its sites in the European Union and in Switzerland. DSM sells vitamin D3 into the food, the feed, and the pharma market. Thus, DSM complies with the CLP Regulation requirements regarding packaging and labelling of its products and other regulations regarding worker safety.

Vitamin D3 is an essential nutrient for human being. Insufficient exposure is known to produce serious health problems such as rickets or osteoporosis. Vitamin D3 is available for human being either by synthesis in the skin from UV-light exposure or through the diet. Intake of vitamin D3 in the modern developed Western-style of living resulted in too low exposure to vitamin D3. In fact infants are supplemented with vitamin D3 orally during their 1st year of life. Likewise, elderly are usually recommended to take vitamin D3 supplements to overcome serious health implications. Additionally, vitamin D3 is used for animal nutrition to support their production.

DSM is aware of the current intention to further expand the existing classification and labelling of vitamin D3 based on a biocide registration process. The Rapporteur Member State Sweden submitted a respective CLH report which is available from the ECHA webpage (referred as CLH report (2016)). In this CLH report the Swedish authority provides argumentation on their rationale for proposing vitamin D3 as a germ cell mutagen (category 2) and carcinogen (category 2).

DSM would like to express its serious concerns in that respect, since vitamin D3 is a biological compound present in the bodies of animals and humans to support the homeostasis of calcium, in particular for the bones formation and maintenance. Indeed, vitamin D3 is synthesized in the skin thanks to the light of sun. From a biomonitoring survey performed in African tribes having high sun exposures, it can be estimated that this production from sun exposure is equivalent to oral vitamin D3 doses of 100-250 µg/day. Insufficient exposure to the sun, motivates its supplementation in the diet of both people and animals. There is currently high concern in the countries of northern latitudes as well in countries in which part of the population has to be highly covered due to cultural practices, and the supplementation of vitamin D3 is promoted to ensure a healthy population.

Classification and labelling of vitamin D3 as a mutagen (category 2) and carcinogen (category 2) may have regulatory implications within the EU and potentially health issues for both human being and livestock: *“The Scientific Committee is of the opinion that substances which are both genotoxic and carcinogenic should not be approved for deliberate addition to foods or for use earlier in the food chain, if they leave residues with are both genotoxic and carcinogenic in food.”* (EFSA 2005). Considering this opinion, the use of vitamin D3 in animal production and for people supply could be limited.

Based on an in depth analysis of the currently available data consisting of the CLH report, DSM proprietary data, and published literature, DSM does not consider vitamin D3 as a mutagen or carcinogen fulfilling the requirements laid down in the Guidance on the Application of the CLP criteria (CLP 2015).

In this document DSM submits to ECHA and European Union Member States as well as to the public domain its consideration and scientific argumentation which allows DSM to conclude that vitamin D3 is neither a mutagen nor carcinogen. DSM provides data and additional information in this document on both endpoints:

Mutagenicity:

The proposal for considering vitamin D3 as a germ cell mutagen (category 2) is based on a recent Ames Test and a recent *in vivo* Comet assay in the liver; both studies performed with vitamin D3. These studies were considered positive in the CLH report.

Ames Test: The CLH report gives consideration that the most recent Ames Test overrules the other evidence from *in vitro* mutagenicity testing. This other evidence consists of a negative mutation test *in vitro* in mammalian cells and two earlier but publically available Ames tests with vitamin D3.

When testing natural substances in standard toxicological testing care needs to be taken into account for susceptibility of the isolated material towards oxygen and light being not protected with e.g. antioxidants. Such artificial conditions may lead to degradation of the material and consequently results from toxicological studies which are irrelevant for the real-life situation. For example it was recently published that improper handling of the carotenoid zeaxanthin produced artificial oxidation products being responsible for a positive response in the Ames Test. Using appropriate protection, a negative Ames Test was obtained (Edwards 2016).

Vitamin D can be artificially oxidized to epoxide and cyclic peroxide structures once being unprotected from light and oxygen in solvents (Min & Boff 2002). It is known that peroxides and epoxides are reactive and such artificially produced deterioration products could have caused the equivocal positive responses in one out of three Ames Tests.

DSM investigated the degradation of vitamin D3 in different solvents without protection against light and oxygen. Within 1 hour incubation oxidation products were not detected by analytical means in ethanol, methanol, and acetone. Whereas in DMSO, already after 1h, oxidative products were seen. One of them was identified as 7,8-epoxy vitamin D3 (DSM 2015).

Based on these results a further Ames test was performed using tester strains TA100 and TA1535 (the two ones with the equivocal positive results as cited in the CLH report) under GLP and OECD guideline thereby using protection against oxygen and light as much as possible. The results of this new study (DSM 2016) confirm the negative responses seen previously in the earlier published Ames Tests and put the results of this single weakly positive study into questions.

In addition, Ames Test data on 25-hydroxy vitamin D3, the metabolite of vitamin D3 formed in the liver, also showed no indication of an increased revertant frequency (DSM 2013).

Overall, the weight of evidence using the currently available reports of three negative Ames Tests and one negative *in vitro* mutation test in mammalian cells, the negative Ames Test of 25-hydroxy vitamin D3, as well as the possibility of artefacts due to improper handling indicate that vitamin D3 poses no mutagenic properties *in vitro*.

***In vivo* Comet assay:** Considering the overall weight of evidence on the mutagenic activity of vitamin D3 *in vitro* discussed above, it is difficult to understand why the DNA-damage observed in the liver should be the result of a direct DNA-interacting

activity of vitamin D3 or of vitamin D3 metabolites. Therefore, the present *in vivo* Comet assay study should be looked at in more details.

Literature data show liver damage in laboratory animals shortly after vitamin D3 intoxication consisting of hepatocyte necrosis and mitochondrial damage (partly using non-standard techniques such as electron microscopy, Gascon-Barre & Cote 1978, Kocher et al., 2010, Chavan et al., 2011). It is possible that such effects may not be seen in standard histopathological examination in such short-term studies as discussed by Speit et al. (2015).

Furthermore, the animals showed all signs of a severe hypervitaminosis D evident by hypercalcemia and hyperphosphatemia resulting in severe body weight loss. Thus, the dose levels used in the study seem to be clearly above the Maximum Tolerated Dose (MTD) and are close to dose levels causing death after single application. This severe intoxication could have resulted in the observed strand breaks.

Overall, the increased DNA-migration seen in the *in vivo* Comet assay in the liver after administration of doses being close to the LD50 are likely a secondary response to the hypercalcemia and/or liver damage.

Therefore, vitamin D3 should not be considered a germ cell mutagen (category 2) because it does not match the criteria laid down in CLP (2015).

Carcinogenicity:

The proposal for considering vitamin D3 as a carcinogen (category 2) is based on two published studies (Ikezaki et al., 1999, Tischler et al., 1999) where vitamin D3 or a downstream metabolite (24R,25-dihydroxy vitamin D3) were tested in male rats. A low incidence of adrenal medulla tumors (pheochromocytoma) was noted in the treated groups at high dose levels only.

The dose levels used in the Tischler study with vitamin D3, produced severe systemic toxicity as evident by mortality, no or only very limited body weight gain, and hypercalcemia. The applied dose levels clearly exceeded the MTD and are therefore of limited relevance for classification and labelling (CLP 2015).

Pheochromocytoma have a high background incidence in rats and the low incidence seen in this study is certainly within the normal biological variation rather than a biologically relevant increase in tumor incidence.

In the Ikezaki study, a different test substance was used. The test substance is 24R,25-dihydroxy vitamin D3, CAS 126356-63-6 a down-stream metabolite of vitamin D3. Oral dosing of this substances produces a qualitative different plasma metabolite profile as compared to the metabolite profile after dosing vitamin D3. Vitamin D3 metabolites like 25-hydroxy vitamin D3 or 1,25-dihydroxy vitamin D3 cannot be produced upon oral administration of 24,25-dihydroxy vitamin D3 (Shepard & DeLuca 1980, DeLuca 1986). We therefore believe that the use of data for this down-stream metabolite is not justified to evaluate the hazard of vitamin D3. Further the respective study does not comply to any testing guideline, is non-GLP, and thus has a low reliability.

Pheochromocytoma induction in rats due to disturbed calcium homeostasis is a species specific mode of action for rats. This mode of action has no relevance for human as already discussed

Vitamin D3 – DSM Comments to Public Consultation on CLH Report

by other competent bodies like the Joint FAO/WHO Expert Committee on Food Additives (JECFA 1997).

It has been demonstrated that vitamin D3 is non-carcinogenic in human being. Human data provide no evidence of increased tumor incidences in relation to high doses of 100 µg/d vitamin D3 supplementation even in susceptible population (Marshall et al., 2012). 100 µg/d is the agreed Upper Level of vitamin D3 in adults (EFSA, 2012). A recent meta-analysis indicates that cancer occurrence in vitamin D3 supplemented groups is identical to the non-vitamin D3 supplemented control groups (Bjelakovic et al., 2014). Likewise, human being is exposed ever since to vitamin D3 due to vitamin D3 production in skin. The produced amount is equivalent to high oral doses (≥ 100 µg/d).

We therefore conclude that the available data for vitamin D3 do not justify classification as a carcinogen category 2 according to CLP requirements (CLP 2015).

1. Germ cell mutagenicity

The proposal for considering vitamin D3 as a germ cell mutagen (category 2) is based on a weak positive Ames Test and a positive *in vivo* Comet assay in the liver. Below we will evaluate these studies in the light of further data and conclude in a weight-of-evidence approach.

1.1. *In vitro* data – Instability of vitamin D3 in solvent

The CLH report summarizes in total 3 different Ames Tests. Two of those studies showed negative outcomes i.e. no indication for mutagenic potential whereas only one of the studies was weakly positive. In addition, mutagenicity testing in mammalian cells (Mouse Lymphoma Assay, MLA) was also negative.

In the positive Ames Test, inconsistent and only marginally positive responses were seen with increases in revertant numbers when compared to the concurrent control just above the respective thresholds for a positive response: For TA 100 an increase slightly above the biologically relevant threshold of factor 2 and without a clear dose-response relationship as compared to the vehicle control (Table 1) and for TA1535 an increase of factor 3 without a clear dose-response relationship (Table 2) has been noted.

Table 1 Ames Test: Summary table of TA 100 (taken from CLH report)

Concentration [µg/plate]	Initial Mutation Test Mean number of revertants		Confirmatory Mutation Test Mean number of revertants	
	- S9	+ S9	- S9	+ S9
Untreated control	122.3	111.0	101.7	94.3
DMSO	118.3	116.3	94.0	106.3
Distilled water control	118.3	-	106.7	-
5000	211.3	305.0	99.3	245.7
3750	221.7	299.7	94.7	204.3
2500	182.7	265.3	98.7	173.3
1250	139.7	201.7	105.3	125.0
625	123.7	175.3	107.3	135.7
312.5	124.3	158.0	107.7	115.0
156.25	115.3	139.7	104.0	114.0
78.125	100.0	123.0	107.7	114.7
PC	1477.3	2330.7	1440.0	2214.7

PC (positive controls): without S9-mix SAZ; with S9-mix 2-AA

Table 2 Ames Test: Summary table of TA 1535 (taken from CLH report)

Concentration [µg/plate]	Initial Mutation Test		Confirmatory Mutation Test		Complementary Mutation Test
	Mean number of revertants		Mean number of revertants		Mean number of revertants
	- S9	+ S9	- S9	+ S9	- S9
Untreated control	11.0	15.3	12.0	9.0	9.0
DMSO	6.0	12.0	9.7	11.0	8.3
Distilled water control	8.3	-	9.3	-	8.7
5000	19.3	14.7	5.0	28.0	40.3
3750	18.7	22.0	7.0	27.3	29.3
2500	18.7	16.0	8.3	19.3	12.0
1250	12.7	15.0	8.7	12.7	12.3
625	7.7	10.3	10.7	17.0	9.0
312.5	10.3	11.7	12.3	11.7	7.7
156.25	13.0	12.0	8.7	10.0	8.7
78.125	8.3	11.3	8.7	13.7	7.7
PC	1354.7	205.3	1206.7	204.7	1280.7

PC (positive controls): without S9-mix SAZ; with S9-mix 2-AA

The limited detail of reporting of the Ames Test results in the CLH report is a pity. Without information on standard deviations which would allow to assess variability in more detail, interpretation becomes very difficult. Likewise, historical control data are very important to assess whether revertant numbers are normal i.e. within the historical control range: For example, in strain TA 1535, the concurrent negative controls show great variability from 6.0 up to 15.3 mean revertant number (Table 2). Thus, the positive response in the initial mutation test without S9-mix may simply be due to a very low number of revertants in the DMSO control as compared to the normal control variation. The results in this most recent study in TA1535 do not seem to be very reproducible.

It is further important that in another published study vitamin D3 was tested in these two strains (TA100 and TA1535) up to much higher concentrations (up to 10'000 µg/plate) and showed negative results.

Vitamin D3 but also its analogue vitamin D2 (combined referred as to vitamin D) are compounds with conjugated double-bonds or more specifically a conjugated triene system (see Figure 1). Conjugated double-bonds can be oxidized as this is known from other natural compounds such as carotenoids, unsaturated fatty acids, or cholesterol (Min & Boff 2002). It has been shown that natural substances with the structural feature of unsaturated conjugated double-bonds can degrade once being isolated and not protected from air and light. The result of this deterioration is the formation of mutagenic degradation products as nicely shown for the carotenoid zeaxanthin (Edwards 2016).

In fact already early literature shows that vitamin D can be oxidized in aqueous systems and in solvents in the presence of light, moisture, heat, and oxygen. In general, vitamin D is not very stable when exposed to light and air.

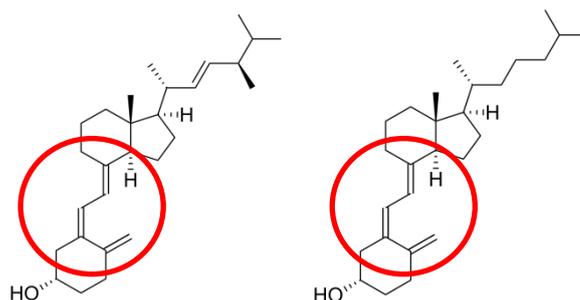


Figure 1 Chemical Structure of vitamin D2 (left) and vitamin D3 (right) with the conjugated triene system (red circle)

Amer et al. (1970) performed several experiments on the stability of vitamin D2 under different conditions such as illumination of the pure compound in combination with high humidity or in solvents. Although the analytical capabilities at that time were more limited as compared to today, the infrared spectrum showed that the conjugated triene system rapidly disappeared especially in solvents. The infrared spectra further show the occurrence of a ketonic structure.

Kozhina et al. (1971) also studied the oxidative abiotic degeneration of vitamin D2 in aqueous solution: A solution of 1g vitamin D2 in 1L of aqueous medium (50 mL ethanol plus 950 mL distilled water) was stirred at 50°C for 4h. The following degradation products were identified: a carbonyl compound, a cyclic peroxide, and pre-calciferol.

The stability of vitamin D3 in acetonitrile was investigated (Renken & Warthesen 1993). Samples held at 4°C without exposure to air exhibited no degradation. In the presence of air at 21°C, however, it degraded with a 60% loss after 10 days.

In another study by King & Min (2002), vitamin D2 storage stability was studied in a model system of 12% water and 88% acetone. In the presence of riboflavin and under light conditions, the triene system reacted with oxygen. The combined information from UV, MS, and FTIR spectra indicated that a 5,6-epoxide of vitamin D2 was formed from vitamin D2 (Figure 2).

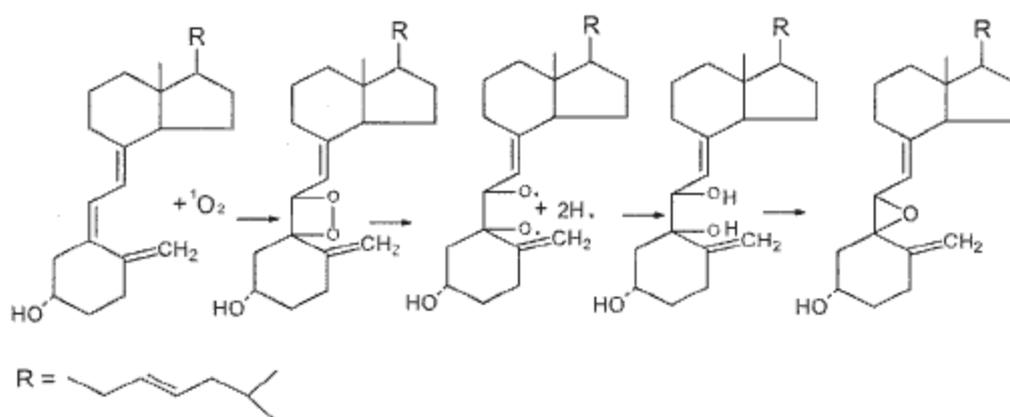


Figure 2 Proposed chemical mechanism for the formation of 5,6-epoxide from vitamin D2 (taken from King & Min (2002))

Thus, vitamin D3 in aqueous solution or in organic solvent can be oxidized in the presence of light and oxygen and form reactive degradation products such as peroxides or epoxides. Therefore, care needs to be taken in the preparation of samples and dilutions of vitamin D3 before they enter the Ames Test.

Vitamin D3 – DSM Comments to Public Consultation on CLH Report

Recently, DSM investigated the short-term behavior of vitamin D3 in different solvents at high concentrations (DSM 2015). Oxidative degradation was observed in all tested solvents (DMSO, ethanol, methanol, and acetone). However, only in DMSO, formation 7,8-epoxy vitamin D3 was seen within the first 4 h after solubilisation whereas a similar behavior in the other three solvents was not noted. Thus, the use of DMSO results in artificial formation of reactive oxygen products.

DSM performed an Ames Test in strains TA 100 and TA1535 using ethanol as solvent. The handling was performed under red-light conditions. Further weighing and solubilisation were performed in an oxygen-free cabinet (glove box). No increase in the revertant rate in both strains either in the presence or absence of metabolic activation was seen (DSM 2016).

The first down-stream metabolite of vitamin D3 (25-hydroxy vitamin D3) was not mutagenic in a state-of-the-art Ames Test (DSM 2013) which is further supportive that a single weakly positive result could be the result of improper handling.

The currently available study reports show only one weakly positive Ames Test, against all the other negative *in vitro* mutation studies (3 negative Ames Tests, a negative forward mutation study in mammalian cells, and a negative Ames test with the liver metabolite of vitamin D3 (25-hydroxy vitamin D3)). The artificial degradation of vitamin D3 in organic solvent to reactive compounds further suggests that vitamin D3 is not a mutagen *in vitro*.

1.2. *In vivo* MNT/Comet Assay

1.2.1. *In vivo* MNT

In the CLH report p. 30, the following statement is made: “*Cholecalciferol was tested in vivo in a combined Comet and micronucleus assay. The study was performed in accordance with GLP and the draft OECD TG for Comet assay (December 2013). The result of the micronucleus assay was negative. However, since no cytotoxic effect was seen in the bone marrow it is not possible to conclude that the target tissue was exposed in the study although this seems likely considering that cells such as osteoblasts, osteoclasts and chondrocytes express VDR (Vitamin D Receptor).*”

Published literature and DSM internal investigations show that vitamin D3 and its metabolites are present in plasma upon single or repeated oral dosing of rats (Shepard & DeLuca 1980, DSM 2010). The bone marrow is readily accessible to substances that are present in the blood. Thus, the target tissue bone marrow for the *in vivo* MNT was reached and the results of the *in vivo* MNT part of the study are fully valid. DSM therefore does not agree with the above cited paragraph.

1.2.2. Acceptability of the *in vivo* Comet

The combined *in vivo* MNT/Comet Assay does not fulfill the acceptance criteria for an *in vivo* Comet assay as specified in OECD 489 (2014):

Paragraph 58 on the acceptance of a test indicates the following acceptance criteria:

“... d. The criteria for the selection of highest dose are consistent with those described in paragraph 36.”

Paragraph 36 states:

“The study should aim to identify the maximum tolerated dose (MTD), defined as the dose inducing slight toxic effects relative to the duration of the study period (for example, clear clinical signs such as abnormal behaviour or reactions, minor body weight depression or target tissue cytotoxicity), but not death or evidence of pain, suffering or distress necessitating euthanasia.”

DSM considers body weight losses of 3.6% and 7.8% in the mid (7.5 mg/kg bw) and high dose level (15 mg/kg bw), respectively, within only 3 days not minor but major body weight loss. It is therefore a clear and definitive sign that the MTD was exceeded and that animals were lethally intoxicated. This view is supported by acute oral toxicity studies performed with vitamin D3 (p. 15 of the CLH report (2016)) where a dose of 25 mg/kg bw caused death in two out of ten animals. Likewise, lethally intoxicated rats usually fade away between four and five days after administration (DSM 2004). Overall, this is not in compliance with paragraphs 58 and 36 cited above and the study does not meet all the acceptance criteria defined by OECD 486 (2014). No judgment should be made on the outcome of the study as further detailed in section 1.2.3. below.

1.2.3. Toxicity in the target organ biased the results of the Comet assay in the liver

There are apparently differences in the interpretation of the results: The CLH report (p. 30-31) considers the results of the *in vivo* Comet Assay in the liver as positive whereas other experts consider the study results as irrelevant due to the high toxicity observed.

It is agreed in the CLH report (p. 31) that “*excessive toxicity may give false positive results due to induction of necrosis followed by hyperplasia*”. The question therefore is whether the absence of inflammation in the liver of the exposed rats automatically needs to result in the conclusion that “*the increased DNA migration in the liver cannot be explained by an excessive toxicity*”. This is an important aspect especially when considering that histopathological changes are more likely to become evident during longer repeat dose studies (Speit et al., 2015).

It is therefore a pity that the liver in the 28-day study was not examined by histopathology to have a broader basis of information in terms of liver toxicity after short-term treatment with vitamin D3. There are, however, data in the peer-reviewed literature which should be used and which can help to decide whether or not the borderline liver results in the Comet Assay are the result of cytotoxicity in the organ:

Upon electron-microscopic investigation of rat liver obtained from animals intoxicated with a single dose of vitamin D3 (0.6×10^6 IU/kg equivalent to 15 mg/kg¹) a dilatation of the rough endoplasmic reticulum and swelling of mitochondria was observed (Gascon-Barre & Cote 1978).

Giving single doses of 36 mg/kg bw to rats results in liver toxicity, hepatic cell degeneration, dilation of hepatic sinusoids and marked centrilobular necrosis (Kocher et al., 2010).

Likewise Chavan et al (2011) report about histopathological changes in rats treated for 10 to 19 days with 2 mg/kg bw/d: The histopathological findings in the liver included fatty/vacuolative degeneration of hepatocytes.

The severity of effects seen in the study being mainly the pronounced body weight losses likely due to impaired food consumption accompanied by hypercalcemia during dosing clearly indicates severe systemic toxicity and exceedance of the MTD. Histopathologically, this is reflected by reduced glycogen in the liver of the animals. The CLH report, however, argues that the observed glycogen depletion in the mid and high dose is not the result of the severe toxicity (and strong body weight losses) but it could be the consequence of fasting prior to necropsy (CLH report p. 31).

¹ 1IU vitamin D3 is equivalent to 0.025 µg vitamin D3

In a study of Freminet & Leclerc (1980) groups of male Sprague-Dawley rats were fasted for 48h. In comparison to the control fed ad libitum, the glycogen in the liver of the fasted rats was totally exhausted. The fasted animals lost 13% weight within the 48h fasting period. Thus, the observed body weight losses and reduced glycogen in the liver observed in the Comet assay reflects starvation of the animals rather than a simple fluctuation in the glycogen content amongst the groups.

To conclude the additional literature data support the view that increased DNA migration in isolated liver cells upon dosing of rats with 7.5 and 15 mg/kg bw/d in an *in vivo* Comet assay is not biologically relevant due to (i) exceedance of the MTD and consequently extreme systemic toxicity, (ii) liver damage and consequent possible interference with the Comet assay endpoint, and (iii) the difficulty to conclude on a direct induction of strand-breaks by vitamin D3 and/or its metabolites in the light of the results of negative vitamin D3 in *in vitro* mutagenicity testing.

2. Carcinogenicity

The proposal for considering vitamin D3 as a carcinogen (category 2) is based on two published articles where vitamin D3 (Tischler et al., 1999) or a downstream metabolite (24R,25-dihydroxy vitamin D3) were tested in male rats (Ikezaki et al., 1999). A very low incidence of pheochromocytoma was noted: in the study of Tischler 1 out of 9 male rats showed a pheochromocytome after 26 weeks of treatment with vitamin D3; 1 out of 20 male rats had a pheochromocytome after 1 year of treatment with 24R,25-dihydroxy vitamin D3 (Ikezaki et al., 1999).

2.1. Adequacy and Reliability of animal data

The CLH report references two published reports in rat to support their conclusion on carcinogenic hazard of vitamin D3 (Tischler et al., 1999; Ikezaki et al., 1999).

These two investigations are neither compliant to any guideline with the aim of studying carcinogenicity nor were they conducted under GLP. Both studies cannot be considered full carcinogenicity studies: The study design such as dose level setting, number of dose groups, or number of animals per group is not acceptable for studies to be used for classification and labelling. Additionally, in one of the studies a different test substance not being vitamin D3 was used. In the sections below, the studies are discussed in detail.

2.1.1. Ikezaki study

The study of Ikezaki et al. (1999) is not relevant for discussion about vitamin D3.

The study investigated a down-stream metabolite of vitamin D3 not vitamin D3 itself. The test substance was 24R,25-dihydroxy vitamin D3 (CAS: 126356-63-6). The hydroxylation of vitamin D3's major plasma metabolite 25-hydroxy vitamin D3 in position 24 results in 24R,25-dihydroxy vitamin D3 and is considered to be an inactivation step resulting in less biological activity (Christakos et al., 2010).

Shepard & DeLuca (1980) nicely showed the circulating metabolites formed in rats upon oral treatment of vitamin D3 and measured the concentrations of vitamin D3, 25-hydroxy vitamin D3, 25R-OH-26,23S-lactone (lactone), 24,25-dihydroxy vitamin D3, 25,26-dihydroxy vitamin D3, and 1,25-dihydroxy vitamin D3 (Figure 3, Table 3).

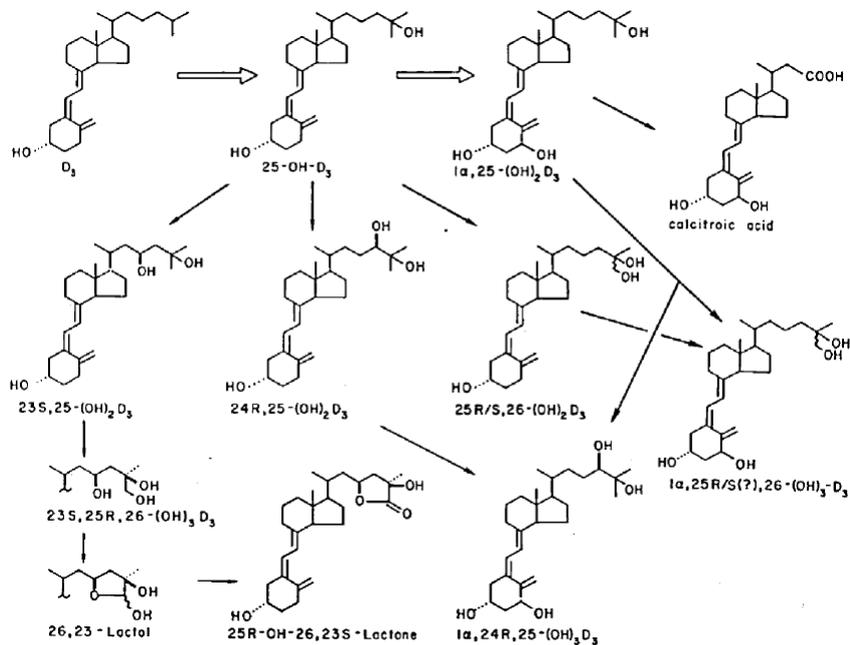


Figure 3 Suggested metabolic pathway of vitamin D3 (taken from DeLuca 1986): D3 (Vitamin D3 or cholecalciferol), 25-OH-D3 (25-hydroxy vitamin D3 or calcifediol), 1 α ,25-(OH) $_2$ D3 (1 α ,25-dihydroxy vitamin D3 or calcitriol), 24R,25-(OH) $_2$ D3 (24R,25-dihydroxy vitamin D3), 25R-OH-26,23S-lactone (lactone) and 25R/S,26-(OH) $_2$ D3 (25,26-dihydroxy vitamin D3). When giving the down-stream metabolite 24R,25-dihydroxy vitamin D3, vitamin D3, 25-hydroxy vitamin D3 and 1,25-dihydroxy vitamin D3 cannot be present.

Upon oral dosing of 24,25-dihydroxy vitamin D3 there is a different metabolite profile than after dosing of vitamin D3: When giving the down-stream metabolite 24R,25-dihydroxy vitamin D3, vitamin D3, 25-hydroxy vitamin D3 and 1,25-dihydroxy vitamin D3 cannot be present in the circulation. Indeed, the only metabolites detected after administration of 24R,25-dihydroxy vitamin D3 were 24-oxo-25-hydroxy vitamin D3 and 1,24,25-trihydroxy vitamin D3 (Jarnagin et al., 1985). Thus, vitamin D3 and 24R,25-dihydroxy vitamin D3 have qualitatively different metabolite profiles.

Table 3 Plasma concentrations of vitamin D3 and metabolites in rats given various amounts of vitamin D3 (taken from Shepard & DeLuca 1980)

Amount (nmol/day)	Vitamin D ₃ (ng/ml)	25-OH-D ₃ (ng/ml)	Lactone (ng/ml)	24,25-(OH) ₂ D ₃ (ng/ml)	25,26-(OH) ₂ D ₃ (ng/ml)	1,25-(OH) ₂ D ₃ (pg/ml)	Plasma calcium (mg/100 ml)
0.65	11.3 ± 6.1	2.3 ± 1.9	<0.06	0.56 ± 0.13	<0.2	80 ± 60	9.0 ± 0.1
6.5	110 ± 43	14.7 ± 8.6	0.35 ± 0.12	3.98 ± 1.90	0.20 ± 0.36	77 ± 64	9.4 ± 0.4
65	368 ± 121	74.2 ± 14.5	10.3 ± 3.9	25.5 ± 5.2	7.60 ± 2.78	88 ± 9	9.7 ± 0.3
650	1339 ± 329 ^b	643 ± 93 ^b	64.5 ± 19.1 ^c	73.5 ± 29.6 ^d	16.4 ± 4.7 ^c	51 ± 11 ^c	12.4 ± 1.0 ^b
6500	3108	1111	43.6	86.5	8.4	37	13.8

^a Rats were orally dosed daily for 14 days with indicated amounts of vitamin D₃. The data were expressed as the mean of 5 rats ± SD.

^b Differ from control group (0.65 nmol/day) and from group receiving 65 nmol/day at $P < 0.001$.

^c Differ from group receiving 65 nmol/day at $P < 0.001$.

^d Differs from control group (0.65 nmol/day) at $P < 0.001$ and from group receiving 65 nmol/day at $P < 0.010$.

In the Ikezaki study only 1 dose group was used rather than 3 dose groups (as required in any repeated dose toxicity study guideline), there were only 20 male animals per group (instead of the usually required 50 animal per sex per group in a carcinogenicity study) resulting in deviations from all guidelines about repeated dose toxicity testing.

At the end of the study, a pheochromocytoma incidence of 1 animal in 20 animals was noted in the treated group as compared to zero in the control group. This is of limited relevance considering that the study was performed in Wistar rats which have a considerable high background incidence for pheochromocytoma of up to 82% (Tischler et al., 2015, Lynch et al., 1996).

We conclude that the study is not appropriate to assess vitamin D3 in terms of carcinogenicity because of the poor quality of the study design. Administration of 24,25-dihydroxy vitamin D3 results in differences in terms of metabolite profile when compared to vitamin D3 and results of this study are not to be used.

2.1.2. Tischler study

The study was also not conducted in compliance with OECD guidelines for repeated dose toxicity testing and not in compliance with GLPs. The administered dose levels (0, 5000, 10000, or 20000 IU/kg bw/d equivalent to 0, 125, 250, or 500 µg/kg bw/d) were extremely high and exceeded the MTD. Originally, it was scheduled that 12 animals per group should be available after 26 weeks for examination. At the end of the treatment period, however, 11, 12, 10, or 9 animals were available for histopathology in the control, low, mid and high dose group, respectively.

At the end of treatment at 10000 IU/kg bw/d (250 µg/kg bw/d), 22% lower body weights as compared to controls and at 20000 IU/kg bw/d (500 µg/kg bw/d) 69% lower body weight as compared to controls were observed (see Figure 4). In fact, the 20000 IU/kg bw/d dose group gained almost no weight.

Vitamin D3 – DSM Comments to Public Consultation on CLH Report

Severe systemic toxicity was evident by mortality, severely impaired body weight gain, hypercalcemia and hypercalcuria together with nephrotoxicity. At 20000 IU/kg bw/d mild to moderate nephrocalcinosis, patchy tubular atrophy and scarring, and at 10000 IU/kg bw/d mild nephrocalcinosis were noted.

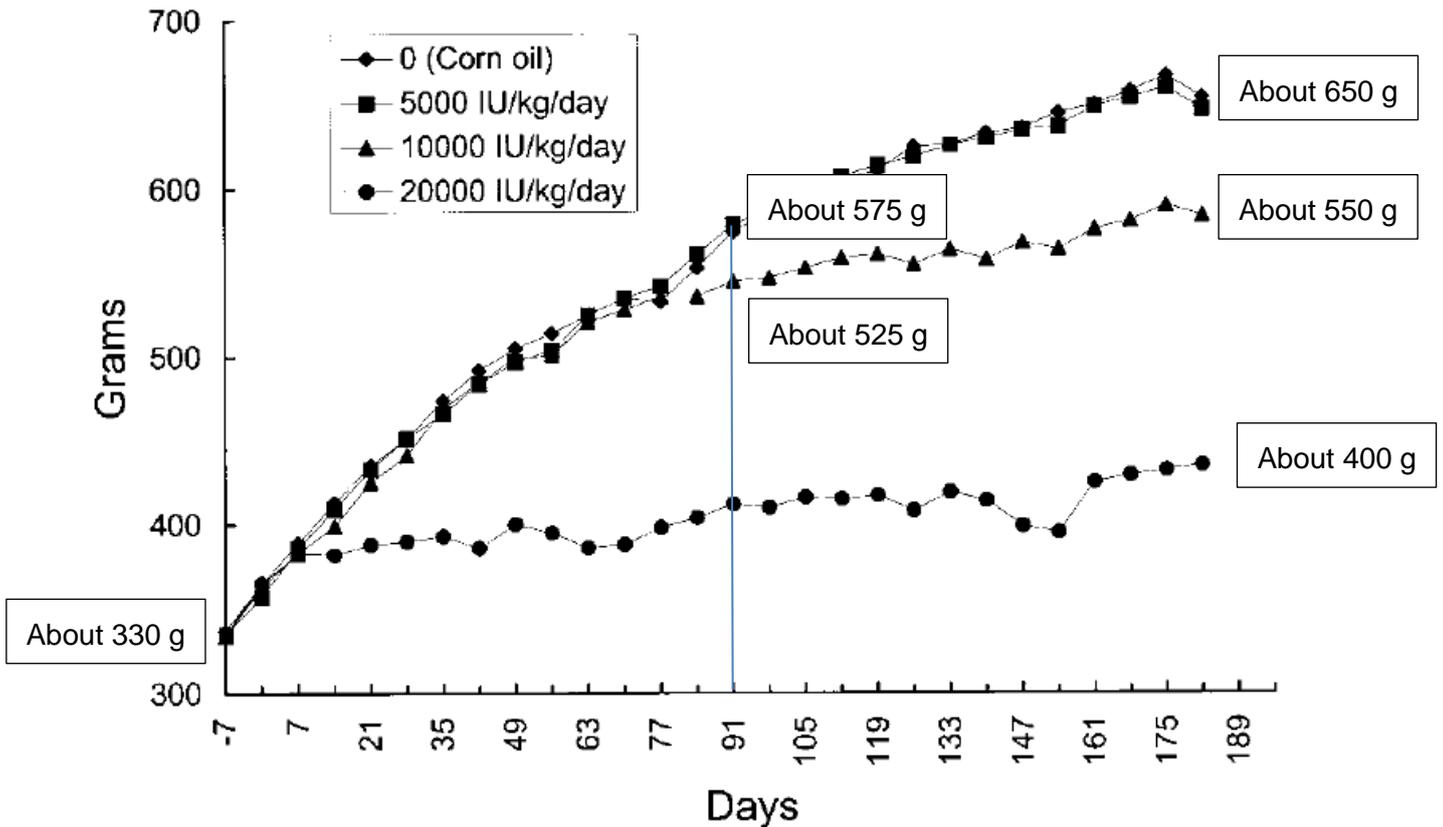


Figure 4 Effects of vitamin D3 treatment on body weight development upon oral dosing with 0, 5000, 10000, or 20000 IU/kg/d (0, 125, 250, 500 µg/kg/d) in the study of Tischler et al. (1999): modified from Figure 8 of the publication.

It is agreed that for assessing carcinogenicity a 10% decreased body weight gain should not be exceeded and the longevity of the animals should not be impaired (CLP 2015). In the Tischler publication (Tischler et al., 1999), reduced body weight gains of 20-70% of control were noted (Table 4). In addition, animal losses were reported being 3 animals out of 12 at the high dose. Thus, pheochromocytomas were only seen in severely intoxicated rats and at doses clearly exceeding the MTD. We therefore consider this study to be heavily biased by the severe toxicity and non-acceptable for judgment on carcinogenic activity.

Table 4 Summary of body weight gain observed in the Tischler study (Tischler et al., 1999). Values were taken from Figure 4 above.

	control	mid dose: (250 µg/kg bw/d)	high dose: (500 µg/kg bw/d)
start weight (g)	330	330	330
weight after 13 weeks (g)	575	525	400
bw gain start to week 13 (g (% of control))	245	195 (79)	70 (29)
weight after 26 weeks (g)	650	550	400
bw gain start to week 26 (g (% of control))	320	220 (69)	70 (22)

The observation of pheochromocytoma in the Tischler study can be either within the normal background variation of a tumor with well-known high background incidence or can be a secondary effect related to excessive toxicity as demonstrated by stagnation of body weight development, hypercalcemia and nephrocalcinosis. It was published by the National Toxicology Program (Nyska et al., 1999) that nephrocalcinosis can lead to the formation of pheochromocytoma in rats as a secondary effect due to increased circulating calcium concentrations.

Overall, we do not consider the study protocol and results robust enough to conclude that vitamin D3 should be classified as a category 2 carcinogen.

2.2. Mode of Action and Relevance of Animal Data for Human Being

2.2.1. Disturbed calcium homeostasis can lead to pheochromocytoma in the rat adrenal medulla

Vitamin D3 as an endogenous substance in humans being produced from UV-light in exposed skin serves to ensure adequate calcium homeostasis. High oral doses exceeding the normal vitamin D3 requirements can produce disturbance of the calcium homeostasis visible by hypercalcuria, hypercalcemia, organ mineralization, and/or nephrotoxicity. Intravenously administered calcium produced hypercalcemia and adrenal chromaffin cell proliferation in rats (Isobe et al., 2012). Thus, impaired calcium homeostasis in rats results in proliferative changes in the adrenal medulla.

Nephrotoxicity - a well-known effect of high vitamin D3 doses - is also a condition known to result in secondary pheochromocytoma in the rat (Nyska et al. 1999).

Overall, disturbed calcium homeostasis producing pheochromocytoma is described for several substances like several poorly absorbed sugars or vitamin D3 (Lynch et al., 1996; Tischler et al., 1999, Greim et al., 2009). Table 5 gives an overview on the relation between disturbed calcium

Vitamin D3 – DSM Comments to Public Consultation on CLH Report

homeostasis and adrenal chromaffin cell proliferation after administration of vitamin D3 or excess calcium.

Vitamin D3 – DSM Comments to Public Consultation on CLH Report

Table 5 Association between disturbed calcium homeostasis, effects on adrenal medulla and kidney toxicity due to disturbance of calcium homeostasis

Substance	Study Duration	Sex	Dose (mg/kg bw/d or in concentration water or diet)	serum calcium concentration	serum phosphorus concentration	urinary calcium concentration	effects on adrenal medulla		kidney toxicity	Reference
							proliferative lesion (incidence)	incidence pheochromocytoma		
vitamin D3	90-day gavage study in Wistar rats	M	0.012	↑		--	--	--	--	CLH report
		M	0.06	↑		--	--	--	x1	
		M	0.3	↑	↑	--	chromaffin cell hyperplasia (3/10)	--	x1	
		F	0.012			--	--	--	--	
		F	0.06	↑		--	--	--	x1	
		F	0.3	↑	↑	--	chromaffin cell hyperplasia (4/10)	--	x1	
vitamin D3	26-week study feeding study in Crl:CD BR rats	M	0.125	↑	↑	↑	--	--	x2	Tischler et al., 1999
		M	0.25	↑	↑	↑	hyperplastic nodules (5/10)	1/10	x2	
		M	0.5	↑	↑	↑	hyperplastic nodules (7/9)	1/9	x2	
calcium gluconate	7-day infusion study in SD rats	M	1920	↑	n.m.	↑	proliferation of chromaffin cells*	--	--	Isobe et al., 2012
			3840	↑	n.m.	↑	proliferation of chromaffin cells*	--	x2	

Vitamin D3 – DSM Comments to Public Consultation on CLH Report

Substance	Study Duration	Sex	Dose (mg/kg bw/d or concentration in water or diet)	serum calcium concentration	serum phosphorus concentration	urinary calcium concentration	effects on adrenal medulla		kidney toxicity	Reference
							proliferative lesion (incidence)	incidence pheochromocytoma		
lactitol	130-week feeding study	M	0 %	n.a.	n.a.	n.a.	no data	benign: 10/45 malignant: 3/45	--	Sinkeldam et al., 1992
		M	2%	--	n.m.	--	no data	benign: 18/50 malignant: 4/50	--	
		M	5%	--	n.m.	--	no data	benign: 8/44 malignant: 3/44	--	
		M	10%	--	n.m.	↑ (trend)	no data	benign: 18/48 malignant: 2/48	x3	
lactose		M	25%	--	n.m.	↑ (trend)	no data	benign: 21/50 malignant: 9/50	x3	
lactitol		F	0%	n.a.	n.a.	n.a.	no data	benign: 1/50 malignant: 0/50	--	
		F	2%	--	n.m.	↑ (dose-response)	no data	benign: 3/50 malignant: 0/50	--	
		F	5%	--	n.m.	↑ (dose-response)	no data	benign: 2/49 malignant: 1/49	x3	
		F	10%x4	--	n.m.	↑ (dose-response)	no data	benign: 3/50 malignant: 2/50	x3	

Vitamin D3 – DSM Comments to Public Consultation on CLH Report

Substance	Study Duration	Sex	Dose (mg/kg bw/d or concentration in water or diet)	serum calcium concentration	serum phosphorus concentration	urinary calcium concentration	effects on adrenal medulla		kidney toxicity	Reference
							proliferative lesion (incidence)	incidence pheochromocytoma		
lactose		F	25%x4	--	n.m.	↑ (dose-response)	no data	benign: 2/50 malignant: 1/50	x3	

↑ statistically significant higher than control
 X present
 x1 tubular mineralization, degeneration/regeneration, tubular dilatation
 x2 nephrocalcinosis
 x3 pelvic nephrocalcinosis
 x4 decrease in femur calcium content
 * seen only by BrdU-labelling technique but not by standard histopathology
 -- not detected, not changed
 n.m. not measured
 n.a. not applicable

2.2.2. Pheochromocytoma due to disturbed calcium homeostasis is species specific to rats

Adrenal medullary pheochromocytoma are often observed in rat studies. However, pheochromocytomas in rats differ from those in all other species in several important aspects: they are common, they are often bilateral and multicentric, and they are induced by many agents whereas it is usually not seen in mice cancer studies. Pheochromocytoma in humans are rare and usually solitary, except in patients with hereditary disorders that predispose to their development. Moreover, there are no data suggesting that they may be inducible. These differences have increasingly led regulatory agencies to discount the importance of rat pheochromocytomas for purposes of risk assessment because they are considered to be species specific and irrelevant for human (Tischler et al., 2015). For example ECHA concluded that the occurrence of pheochromocytoma as a secondary response to exposure to a particulate compound is not relevant for human (RAC 2011). The JECFA considered the occurrence of adrenal pheochromocytoma as secondary due to disturbed calcium homeostasis as non-relevant for human being (JECFA 1997).

Pheochromocytomas are tumors originating from chromaffin cells and are common in aging rats but rare in mice and human being (Chandra et al., 2013). As discussed in detail by Greim et al. (2009), chemically induced pheochromocytoma in rats can occur due to several conditions such as hypoxia, uncoupling of oxidative phosphorylation, disturbance of calcium homeostasis (see above), and disturbance of the hypothalamic endocrine axis. Thus, they are usually secondary.

An in depth analysis in terms of species differences was reviewed by Lynch et al. (1996) and is briefly summarized in Table 6 for the sake of completeness.

To conclude, disturbed calcium homeostasis can result in rats in the formation of adrenal pheochromocytoma as a secondary reaction. This phenomenon is species specific to rats and not relevant for human being. Therefore, it is not appropriate to classify vitamin D3 as a carcinogen.

Table 6 Adrenal Medulla: Species differences between rat and human (based on Lynch et al., 1996)

		Rat	Human
Anatomy of the adrenal gland	types of chromaffin cells	distinct chromaffin cells for epinephrine and norepinephrine	chromaffin cells contain both epinephrine and norepinephrine within a single cell
	small granule containing cells	existing	not clearly defined counterpart identified
	content of acidic proteins (chromogranins) within granules of chromaffin cells	chromogranin A predominates	chromogranin A and B present in about equal amounts
Characteristics of chromaffin cells	basal proliferation of chromaffin cells	proliferation throughout life	no proliferation observed in normal adult human adrenal medullary cells
	response of chromaffin cells to stimuli	proliferation in response to mitogenic factors	inherently less responsive to mitogenic stimuli than adult rat chromaffin cells
Characteristics of proliferative lesions	Incidence	spontaneous high incidence with strong dependence on the strain: Wistar up to 86%, Sprague-Dawley 31%	hyperplasia in the adrenal medulla occurs rarely and tend to be observed in humans with a familial disposition to the development of pheochromocytoma as part of multiple endocrine neoplasia syndrome. incidence in the general human population is estimated to range from 0.005 to 0.1%
	Morphology	proliferative lesion contains cells that are smaller than normal chromaffin cells, have small secretory granules, show little staining for the presence of epinephrine synthesizing ability	cells within proliferative lesions can be either smaller or larger than normal chromaffin cells, generally have no consistent granule morphology and often contain abundant stores of epinephrine
	Function	proliferative lesions normally are not hyperfunctional	patients often show symptoms of catecholamine excess

2.3. CLH proposal versus conclusion of other competent bodies

The CLH report further mentions on page 42 in section 10.9.1, relevance to humans, c) that *“information is lacking with respect to any association of high dose (i.e. doses outside the supplement range) exposure to vitamin D in humans and cancer. The human data described above only support lack of association with cancer in the dose range 10 to 27.5 µg/day.”*

Vitamin D3 is a naturally occurring constituent of human body and it is synthesized via UV-light exposure in our skin. Thus, human being was always exposed to vitamin D3 mainly via exposure to sunlight.

Two important authoritative bodies concluded on a safe maximum supplementation dose (Upper Level (UL)) being 4000 IU²/d (100 µg vitamin D3/d) for adults. This UL is 4 to 10 times higher than the dose level range given in the CLH report not being associated with cancer. Details on the UL of vitamin D3 are given in the report of the Institute of Medicine (IOM 2011) and in the scientific opinion of the European Food Safety Authority (EFSA 2012).

2.4. Human Data

2.4.1. Natural human exposure to vitamin D3 from synthesis in skin

25-hydroxy vitamin D3 is the circulating metabolite of vitamin D3. The vitamin D3 status of a person is determined by measuring the plasma or serum 25-hydroxy vitamin D3 concentration, the product of the first activation step (IOM, 2011; EFSA 2012) and the measurement of circulating 25-hydroxy vitamin D3 concentration is used in clinical surveillance and surveys in the general public.

The IOM (2011) considers persons with serum 25-hydroxy vitamin D3 level below 30 nmol/L as deficient and above 50 nmol/L as sufficient. In a systematic review of the vitamin D status in populations worldwide based on published literature, 37.3% of the studies reported 25-hydroxy vitamin D3 levels below 50 nmol/L (Hilger et al., 2014).

An interesting population is the traditionally living tribes near the equator in Africa because they get their vitamin D3 exclusively from sun like our ancestors. Bio-monitoring showed surprisingly high circulating 25-hydroxy vitamin D3 concentrations.

Luxwolda et al. (2012a & 2012b) measured in members of these African tribes (Hadzabe, Maasai, Sengerema, Same, and Ukerewe) the circulating concentration of 25-hydroxy vitamin D3.

In their first investigation, adults from Maasai and Hadzabe tribes donated blood (as shown in Table 7). The overall mean 25-hydroxy vitamin D concentration was 115 nmol/L with a range between 58 and 171 nmol/L.

² IU = international Unit, 1 IU corresponds to 0.025 µg vitamin D3

Table 7 Serum 25-hydroxy vitamin D concentrations (nmol/L) in Maasai and Hadzabe (taken from Luxwolda et al., 2012b)

(Mean values, standard deviations and ranges)

	Maasai (n 35)			Hadzabe (n 25)		
	Mean	SD	Range	Mean	SD	Range
Age (years)	34	10	17–65	35	12	16–57
Sex (% male)	43 ^b			84 ^a		
Weight (kg)	59	11.5	36–100	58	6.7	41–72
Height (m)	1.67 ^a	0.08	1.49–1.85	1.62 ^b	0.08	1.45–1.74
BMI (kg/m ²)	20.9	3.7	15.4–33.5	22.2	2.2	17.1–26.8
25(OH)D (nmol/l)	119.0	26.0	58–167	109.0	28	71–171
25(OH)D ₂ (%)*	nd		nd–11.2	5.1		nd–23.4

nd, Not detectable.

^{a,b} Mean values with unlike superscript letters were significantly different ($P < 0.05$).

* Median percentage of 25(OH)D₂.

Further data of Luxwolda et al. (2012a) covered also pregnant women and infants. In total the blood of 367 adults and 82 infants were investigated. In all groups, the concentration of 25-hydroxy vitamin D3 was remarkably high (up to 262.4 nmol/L):

Table 8 Serum 25-hydroxy vitamin D concentrations (nmol/L) in Hadzabe, Maasai, Sengerema, Same and Ukerewe tribe members (taken from Luxwolda et al., 2012a)

	Mean +/- SD (n)	range
nonpregnant adults	106.8 ± 28.4 (88)	31.1 - 171.1
pregnant	138.5 ± 35.0 (138)	45.6 - 262.4
mothers at delivery	135.9 ± 31.8 (25)	87.2 - 195.7
Infants at delivery	90.6 ± 28.2 (25)	57.6 - 176.0

These tribes live like our ancestors and have their vitamin D from sun exposure. It is unlikely that they take supplements. To conclude: Humans are used to high exposures of vitamin D3.

To estimate what amount of orally vitamin D3 intake would be necessary to achieve comparably high serum 25-hydroxy vitamin D3 concentration, dose-response data which relate the oral external supplemented dose to the systemic exposure (25-hydroxy vitamin D3 serum concentration) are helpful:

Vitamin D3 at dose levels of 0, 25, 125, and 250 µg/d was given to healthy male volunteers for up to 20 weeks (Heaney et al., 2003). At steady state, the 25-hydroxy vitamin D3 concentrations were measured as shown in Figure 5. An oral dose of 250 µg/d results in a 25-hydroxy vitamin D3 concentration of about 200 nmol/L; with 125 µg/day, approximately 150 nmol/L can be achieved.

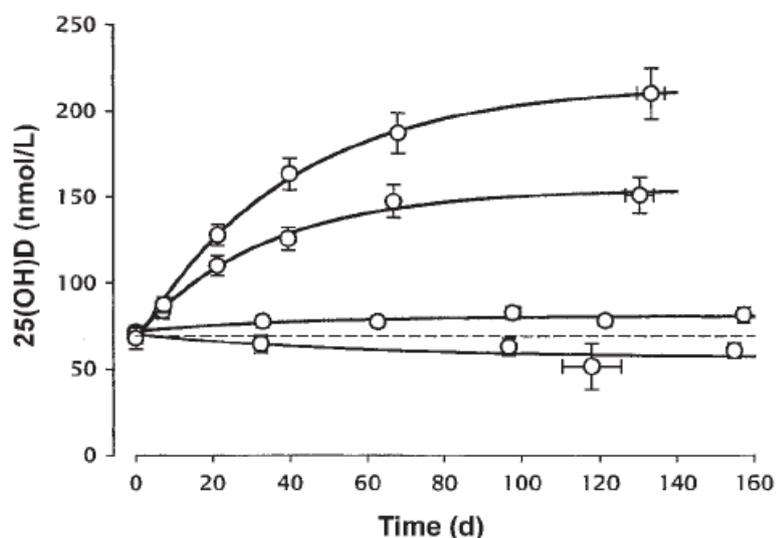


Figure 5 Steady-state 25-hydroxy vitamin D concentrations (nmol/L) after 20 weeks of treatment with 0, 25, 125, or 250 µg vitamin D3/d (taken from Figure 1 in Heaney et al., 2003): The curves, from the lowest upward, are for 0, 25, 125, and 250 µg cholecalciferol (labeled dose)/d. The horizontal dashed line reflects zero change from baseline.

A comparable result was obtained in a 1-year study, where volunteers were given vitamin D3 at a dose of 4000 IU/d (100 µg/d). After 12 months the mean 25-hydroxy vitamin D3 concentration was 67 ng/mL, equivalent to 167 nmol/L (Garret-Mayer et al., 2012).

In a placebo-controlled randomized trial, human volunteers were given vitamin D3 at the following doses: 0, 1000, 2000, or 4000 IU/d (equivalent to 0, 25, 50, or 100 µg vitamin D3/d) and the resulting 25-hydroxy vitamin D3 plasma concentration was measured (Ng et al., 2014). With the highest supplementation level (100 µg/d) a median concentration of 115 nmol/L 25-hydroxy vitamin D3 was achieved with a 25th percentile of 98 nmol/L and the 75th percentile of 138 nmol/L.

Thus, the mean measured serum concentrations in the sampled members of African tribes of 90-120 nmol/L correspond to a daily oral dose of 100-125 µg vitamin D3 per day. The upper ranges (>170 nmol/L) of the measured concentrations correspond to oral doses above 100 µg/d up to 250 µg vitamin D3/day.

In the light of the existing safe Upper Level of 100 µg/d (EFSA 2012) and considering human studies supplemented with doses of 100 µg/d or above (e.g. Ng et al., 2014, Heaney et al., 2003; Garret-Mayer et al., 2012 and others), it seems appropriate to conclude that exposure to vitamin D3 is well above 27.5 µg/d which the CLH report considers to be the higher end of the dose range being not associated with cancer. Natural Vitamin D3 production from sun exposure is equivalent to oral doses of around 100 up to 250 µg/d.

2.4.2. Epidemiological data

Recent reviews on the topic of anti-carcinogenicity of vitamin D supplementation should be considered as well (Feldman et al., 2014; Bikle, 2014). The authors summarize evidence of anti-carcinogenic activity derived from *in vitro* experiments, *in vivo* animal models, and epidemiology

studies. *In vitro*, Vitamin D³ exerts anti-proliferative effects, induces apoptosis, stimulates differentiation, has anti-inflammatory effects, inhibits invasion and metastasis, and inhibits angiogenesis. In animal models of breast, colon, and prostate cancers consisting of xenografts of human cancers, chemical induced or carcinogen induced cancers, and genetically engineered cancer models, vitamin D showed anticancer activity. Finally, the available human trials suggested a statistically significant benefit of vitamin D in reducing mortality due to cancer.

Bjelakovic et al. (2014) assessed the beneficial and harmful effects of vitamin D supplementation for prevention of cancer in adults based on randomized trials that compared vitamin D at any dose, duration, and route of administration versus placebo or no intervention in adults. Cancer occurrence was observed in 1918/24,908 (7.7%) recipients of vitamin D3 versus 1933/24,983 (7.7%) in recipients of control. Thus, there was no indication of an increase in tumor formation in vitamin D3 supplemented persons as compared to non-supplemented groups.

An interesting human study is the investigation done by Marshall et al. (2012). In this study vitamin D3 supplementation at 4000 IU/d (100 µg/d) was given for 1 year to patients with a diagnosis of low-risk prostate cancer. No adverse events were associated with vitamin D3 supplementation. 55% of the patients showed a benefit by means of a reduced number of positive biopsy cores or a decrease in Gleason score of the cancer-containing biopsies. Historical controls were statistically more likely to show progression (Feldman et al., 2014).

Fedirko et al. (2014) investigated the association between the risk of hepatocellular carcinoma in European populations and pre-diagnostic circulating serum 25-hydroxy vitamin D3 levels. In this prospective, nested case-control study among 52,000 participants higher 25-hydroxy vitamin D3 levels (equal and above 75 nmol/L, a concentration which is achieved with approximately 50-100 µg/d considering the data from Heaney et al., 2003 or Ng et al., 2014) were associated with a 49% reduction in the risk of hepatocellular carcinoma.

In conclusion, the data show the availability of epidemiological studies without association to cancer at dose levels above 27.5 µg vitamin D3/d.

³ Note: vitamin D is the sum of several vitamin D forms and covers vitamin D3, vitamin D2 as well as metabolites of both substances

3. Reproductive toxicity

DSM agrees to the conclusion of non-classification provided in the CLH report with respect to this endpoint. There is even more human data on pregnancy outcomes upon supplementation with vitamin D3 which supports the non-classification:

- In a double-blind trial, pregnant Iranian women were safely supplemented with 100'000 IU/month (2500 µg vitamin D3/month, Sabet et al., 2012).
- Likewise, no adverse effects were seen in three randomized controlled trials in pregnant women with treatment up to 4000 IU/day (100 µg vitamin D3/day, Dawodu et al., 2013, Hollis et al., 2011, and Wagner et al., 2013).
- Supplementation of breast feeding mothers with up to 6400 IU/day (160 µg vitamin D3/day) showed no indication of adverse effects in the mothers as well as their infants (Wagner et al., 2006)

4. References

Amer MM, Ahmad AKS, Varda SP (1970) On the Autoxidation of vitamin D preparations II: The autoxidation of ergocalciferol, *Fette Seifen Anstrichmittel* 72(12): 1040-1045.

Bikle DD (2014) Vitamin D and cancer: the promise not yet fulfilled, *Endocrine* 46: 29-38.

Bjelakovic G, Gluud LL, Nikolova D, Whitfield K, Krstic G, Wetterslev J, Gluud C. Vitamin D supplementation for prevention of cancer in adults. *Cochrane Database of Systematic Reviews* 2014, Issue 6. Art. No.: CD007469. DOI: 10.1002/14651858.CD007469.pub2.

Chandra S, Hoenerhoff MJ, Peterson R (2013) *Endocrine Glands*, Book Chapter in: *Toxicologic Pathology* edited by Sahota PS, Popp JA, Hardisty JF, Gopinath C, ISBN: 978-1-4398-7210-9.

Chavan SG, Brar RS, Banga HS, Sandhu HS, Sodhi S, Gadhav PD, Kothule VR, Kammon AM (2011) Clinicopathological Studies on Vitamin D3 Toxicity and Therapeutic Evaluation of Aloe vera in Rats, *Toxicol Int* 18(1): 35-43.

Christakos S, Ajibade DV, Dhawan P, Fechner AJ, Mady LJ (2010) Vitamin D: Metabolism, *Endocrinol Metab Clin North Am*, 39(2): 243–253, doi:10.1016/j.ecl.2010.02.002.

CLH report (2016) CLH report Proposal for Harmonised Classification and Labelling Based on Regulation (EC) No 1272/2008 (CLP Regulation), Annex VI, Part 2, International Chemical Identification: Colecalciferol, Vitamin D3, Version number 4, dated January 2016, available at: <http://echa.europa.eu/documents/10162/376b620f-0c9a-446e-a66a-db97974b32d5>, accessed 27th January 2015

CLP (2015) Guidance on the Application of the CLP Criteria, Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, Version 4.1, June 2015, available at: https://echa.europa.eu/documents/10162/13562/clp_en.pdf, accessed July 2015.

Dawodu A, Saadi HF, Bekdache G, Javed Y, Altaye M, Hollis BW (2013) Randomized Controlled Trial (RCT) of Vitamin D Supplementation in Pregnancy in a Population with Endemic Vitamin D Deficiency, *J Clin Endocrin Metab*, doi: 10.1210/jc.2013-1154

DeLuca HF (1986) The metabolism and functions of vitamin D, *Adv Exp Med Biol* 196, 361-375.

DSM (2004) Cholecalciferol: Acute Oral Toxicity Study in Rats, DSM Internal Document No. 1015386, dated 19-March-2004.

DSM (2010) Comparative Plasma Kinetics after single oral administration of 60 µg/kg bw vitamin D3 and 25OHD3 to male rats, DSM Internal Document No. 0006321, dated 16-Aug-2010.

DSM (2013) DSM047117: Salmonella typhimurium and Escherichia coli reverse mutation assay, DSM Internal Document No 00016967, dated 08-April-2013.

DSM (2015) Study to investigate potential oxidative degradation of vitamin D3 in solvents, DSM Internal Document 00052196, dated 10-Dec-2015

DSM (2016) Evaluation of the mutagenic activity of Ro 04-2361, batch UW01504004, dissolved in ethanol in the Salmonella typhimurium reverse mutation assay, DSM Internal Document 00052658, dated 01-Feb-2016

Edwards JA (2016), Zeaxanthin: Review of Toxicological Data and Acceptable Daily Intake, *Journal of Ophthalmology*, <http://dx.doi.org/10.1155/2016/3690140>.

EFSA (2005) Opinion of the Scientific Committee on a request from EFSA related to A Harmonised Approach for Risk Assessment of Substances Which are both Genotoxic and

Carcinogenic, The EFSA Journal 282: 1-31, available at http://www.efsa.europa.eu/sites/default/files/scientific_output/files/main_documents/282.pdf, accessed 05-Jan-2016.

EFSA (2012) EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA): Scientific Opinion on the Tolerable Upper Intake Level of Vitamin D, EFSA Journal 2012:10(7): 2813 available at: <http://www.efsa.europa.eu/en/efsajournal/doc/2813.pdf> accessed 08-January-2012

Fedirko V, Duarte-Salles T, Bamia C, Trichopoulou A, Aleksandrova K, Trichopoulos D, Trepo E, Tjønneland A, Olsen A, Overvad K, Boutron-Ruault MC, Clavel-Chapelon F, Kvaskoff M, Kühn T, Lukanova A, Boeing H, Buijsse B, Klinaki E, Tsimakidi C, Naccarati A, Tagliabue G, Panico S, Tumino R, Palli D, Bueno-de-Mesquita HB, Siersema PD, Peters PH, Lund E, Brustad M, Olsen KS, Weiderpass E, Zamora-Ros R, Sánchez MJ, Ardanaz E, Amiano P, Navarro C, Quirós JR, Werner M, Sund M, Lindkvist B, Malm J, Travis RC, Khaw KT, Stepien M, Scalbert A, Romieu I, Lagiou P, Riboli E, Jenab M (2014) Prediagnostic circulating vitamin D levels and risk of hepatocellular carcinoma in European populations: a nested case-control study, *Hepatology* 60(4): 1222-30

Feldman D, Krishnan AV, Swami S, Giovannucci E, Felman BJ (2014) The role of vitamin D in reducing cancer risk and progression, *Nature Reviews Cancer* 14(5): 342-357

Freminet A, Leclerc L (1980) Effect of fasting on liver and muscle glycogen in rats and guinea pigs, *J Physiol Paris* 76: 877-880.

Garret-Mayer E, Wagner CL, Hollis BW, Kindy MS, Gattoni-Celli S (2012) Vitamin D3 supplementation (4000 iu/d for 1y) eliminates differences in circulating 25-hydroxy vitamin D between African American and white men, *Am J Clin Nutr* 96:332–6.

Gascon-Barre M, Cote MG (1978) Effects of phenobarbital and Diphenylhydantoin on acute vitamin D3 toxicity in the rat, *Toxicology and Applied Pharmacology* 43: 125-135.

Greim H, Hartwig A, Reuter U, Richter-Reichhelm HB, Thielmann HW (2009) Chemically induced pheochromocytomas in rats: mechanisms and relevance for human risk assessment, *Crit Rev Toxicol* 39(8): 695-718.

Heaney RP, Davies KM, Chen TC, Holick MF, Barger-Lux MJ (2003) Human serum 25-hydroxycholecalciferol response to extended oral dosing with cholecalciferol, *Am J Clin Nutr* 77:204-210.

Hilger J, Friedel A, Herr R, Rausch T, Roos F, Wahl DA, Pierroz DD, Weber P, Hoffmann K (2014) A systematic review of vitamin D status in populations worldwide, *British Journal of Nutrition* 111:23–45, doi:10.1017/S0007114513001840.

Hollis BW, Johnson D, Hulsey TC, Ebeling M, Wagner CL (2011) Vitamin D Supplementation During Pregnancy: Double-Blind, Randomized Clinical Trial of Safety and Effectiveness, *Journal of Bone and Mineral Research* 26 (10): 2341–2357, DOI: 10.1002/jbmr.463

Ikezaki S, Nishikawa A, Furukawa F, Tanakamaru Z, Nakamura H, Mori H, Hirose M (1999) Influence of long-term administration of 24R,25-dihydroxyvitamin D3, a vitamin D3 derivative, in rats, *The Journal of Toxicological Sciences* 24 (2), 133-139.

IOM (2011) Dietary Reference Intakes for Calcium and vitamin D, ISBN 978-0-309-16394-1, available at http://www.nap.edu/catalog.php?record_id=13050.

Isobe K, Ito T, Komatsu S, Asanuma K, Fujii E, Kato C, Adachi K, Kato A, Sugimoto T, Suzuki M (2012) Stimulation of Adrenal Chromaffin Cell Proliferation by Hypercalcemia Induced by Intravenous Infusion of Calcium Gluconate in Rats, *J Toxicol Pathol* 25: 281-285.

Jarnagin K, Zeng SY, Phelps M, DeLuca HF (1985) Metabolism and Pharmacokinetics of 24,25-Dihydroxy vitamin D3 in the Vitamin D3-replete rat, *The Journal of Biological Chemistry*, 260 (25): 13625-13630.

JECFA (1997) Evaluation of certain food additives and contaminants, WHO Technical Report Series 868, http://apps.who.int/iris/bitstream/10665/41962/1/WHO_TRS_868.pdf, accessed 31-August-2015

King JM, Min DB (2002) Riboflavin-Photosensitized singlet oxygen oxidation product of vitamin D2, *JAOCS* 79(10): 983-987.

Kocher DK, Kaur G, Banga HS, Brar RS (2010) Histopathological changes in vital organs of house rats given lethal dose of cholecalciferol (vitamin D3), *Indian J Anim Res* 44(3): 193-196.

Kozhina ZP, Bogoslovskii NA, Spirichev VB, Kabanova EV (1971) A study on the products of the autooxidation of vitamin D I. nonpolar products of the oxidative degradation of vitamin D2, translated from *Khimiya Primrodnikh Soedinenii* No 4, pp 480-484, July-August 1971, original article submitted April 23, 1971

Luxwolda MF, Kuipers RS, Kema IP, Dijck-Brouwer DAJ, Muskiet FAJ (2012b) Traditionally living populations in East Africa have a mean serum 25-hydroxy vitamin D concentration of 115 nmol/l, *British Journal of Nutrition*, 2012, doi:10.1017/S0007114511007161

Luxwolda M, Kuipers R, Kema I, van del Veer E, Dijck-Brouwer J, Muskiet F (2012a) Vitamin D status indicators in indigenous populations in East Africa, *Eur J Nutr* 2012, DOI 10.1007/s00394-012-0421-6

Lynch BS, Tischler AS, Capen C, Munro IC, McGirr LM, McClain RM (1996) Low digestible carbohydrates (polyols and lactose): Significance of adrenal medullary proliferative lesions in the rat, *Regulatory Toxicology and Pharmacology* 23: 256-297.

Marschall DT, Savage SJ, Garrett-Mayer E, Keane TE, Hollis BW, Horst RL, Ambrose LH, Kindy MS, Gattoni-Celli S (2012) Vitamin D3 Supplementation at 4000 international units per day for one year results in a decrease of positive cores at repeat biopsy in subjects with low-risk prostate cancer under active surveillance, *J Clin Endocrinol Metab* 97(7):2315-2324.

Min DB, Boff JM (2002) Chemistry and Reaction of Singlet Oxygen in Food, *Comprehensive Reviews in Food Science & Food Safety*, 1(2): 58-72.

Ng K, Scott JB, Drake BF, Chan AT, Hollis BW, Chandler PD, Bennett GG, Giovannucci EL, Gonzalez-Suarez E, Meyerhardt JA, Emmons KM, Fuchs CS (2014) Dose response to vitamin D supplementation in African Americans: results of a 4-arm, randomized, placebo-controlled trial, *Am J Clin Nutr* 99: 587-598.

Nyska A, Haseman JK, Hailey JR, Smetana S, Maronpot RR (1999) The Association between severe nephropathy and pheochromocytoma in the male F344 rat – the national toxicology program experience, *Toxicologic pathology* 27(4): 456-462.

OECD 489 (2014) OECD guideline for the testing of chemicals *in vivo* mammalian alkaline comet assay, adopted 26-September-2014.

RAC (2011) Committee for Risk Assessment (RAC) Opinion Proposing harmonised classification and labelling at Community level in relation to carcinogenicity of gallium arsenide, ECHA/RAC/A77-O-0000001412-86-05/F, adopted 01-December-2011, available at https://echa.europa.eu/documents/10162/13641/gallium_arsenide_opinion_en.pdf, accessed 31-August-2015.

Renken SA, Warthesen JJ (1993) Vitamin D Stability in Milk, *Journal of Food Science* 58(3):553-557

Sabet Z, Ghazi A, Tohidi M, Oladi B (2012) "Vitamin D supplementation in pregnant Iranian women: effects on maternal and neonatal vitamin D and parathyroid hormone status, *Acta Endocrinologica* 8(1): 59-66.

Shepard RM, DeLuca HF (1980) Plasma concentration of vitamin D3 and its metabolites in the rat as influenced by vitamin D3 or 25-hydroxyvitamin D3 intakes, *Archives of Biochemistry and Biophysics* 202 (1), 43-53.

Sinkeldam EJ, Woutersen RA, Hollanders VMH, Til HP, van Garderen-Hoetmer A, Bär A (1992) Subchronic and chronic toxicity / carcinogenicity feeding studies with lactitol in rats, *Journal of the American College of Toxicology* 11(2): 165-188.

Speit G, Kojima H, Burlinson B, Collins AR, Kasper P, Plappert-Helbig U, Uno Y, Vasquez M, Beevers C, De Boeck M, Escobar PA, Kitamoto S, Pant K, Pfuhler S, Tanaka J, Levy DD (2015) Critical issues with the *in vivo* comet assay: A report of the comet assay working group in the 6th International Workshop on Genotoxicity Testing (IWGT), *Mutation Research* 783: 6-12.

Tischler AS, Powers JF, Pignatello M, Tsokas P, Downing JC, McClain RM (1999) Vitamin D3-Induced Proliferative Lesions in the Rat Adrenal Medulla, *Toxicological Science* 51, 9-18.

Tischler AT, Nyska A, Elmore SA (2015) Toxic Responses of the Adrenal Medulla, Book Chapter In: *Reference Module in Biochemical Research 2015*, ISBN: 978-0-12-801238-3, <http://dx.doi.org/10.1016/B978-0-12-801238-3.02146-2>

Wagner CL, Hulsey TC, Fanning D, Ebeling M, Hollis BW (2006) High-Dose Vitamin D3 Supplementation in a Cohort of Breastfeeding Mothers and Their Infants: A 6-Month Follow-Up Pilot Study, *Breastfeeding Medicine* 1(2): 59-70

Wagner CL, McNeil R, Hamilton SA, Winkler J, Rodriguez C, Warner G, Bivens B, Davis DJ, Smith PG, Murphy M, Shary JR, Hollis BW (2013) A randomized trial of vitamin D supplementation in 2 community health center networks in South Carolina, *Am J Obstet Gynecol* 208:137.e1-13.