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Annex A – Protocol for the Scientific Opinion on Tolerable Upper Intake Level for preformed vitamin A and β-carotene

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1. Introduction

Directive 2002/46/EC[1] on food supplements and Regulation (EC) No 1925/2006[2] on fortified foods delegate the power to the European Commission (EC) to adopt maximum amounts of vitamins and minerals that may be used in food supplements or added to foods. In this context, the Commission asked EFSA to update the guidelines of the Scientific Committee on Food (SCF) for the development of tolerable upper intake levels (ULs) for vitamins and minerals (SCF, 2000a) and to review existing scientific evidence and provide advice on ULs for the European populations of the following nutrients: iron, folic acid/folate, manganese, vitamin A, vitamin B6, vitamin D, vitamin E and β-carotene.[3]

The term vitamin A comprises all-trans-retinol (also called retinol) and the family of naturally occurring molecules associated with the biological activity of retinol (such as retinal, retinoic acid and retinyl esters), as well as the group of provitamin A carotenoids (such as β-carotene, α-carotene and β-cryptoxanthin) that are dietary precursors of retinol. Provitamin A carotenoids are subject to specific routes regarding their absorption and metabolism and their vitamin A activity is lower than that of preformed vitamin A, i.e. retinol and retinyl esters (EFSA NDA Panel, 2015). Considering these differences, the SCF addressed β-carotene and preformed vitamin A separately in its previous evaluations of UL (SCF, 2000b, 2002). The present protocol aims at updating the risk assessment of vitamin A, including both preformed vitamin A and β-carotene.

EFSA's process to address this mandate is illustrated in Figure 1. For each nutrient, a protocol shall be developed for planning the scientific assessment (EFSA et al., 2020). The protocol clarifies the aim and scope of the assessment (problem formulation) and defines the methods for addressing the problem. The principles outlined in the draft NDA Panel guidance for establishing and applying tolerable upper intake levels for vitamins and essential minerals are applied (EFSA NDA Panel, 2022).

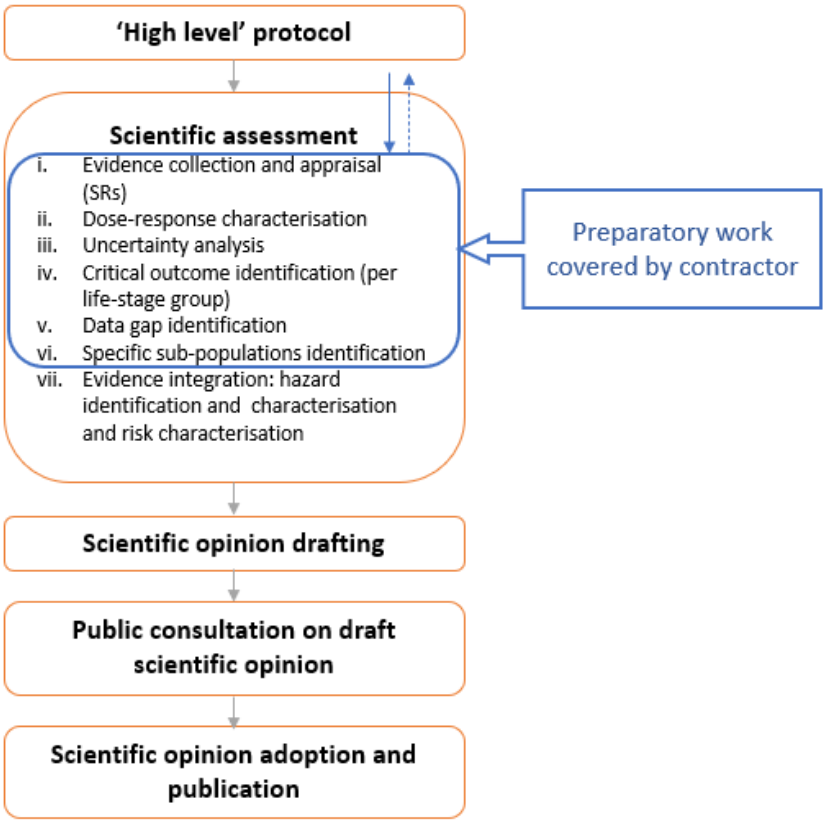

Figure 1. EFSA process to deliver scientific opinions on UL for vitamins and minerals

[1] Regulation (EC) No 1925/2006 of the European Parliament and of the Council of 20 December 2006 on the addition of vitamins and minerals and of certain other substances to foods. *OJ L 404, 30.12.2006, p. 26–38*
[2] Directive 2002/46/EC of the European Parliament and of the Council of 10 June 2002 on the approximation of the laws of the Member States relating to food supplements. *OJ L 183, 12.7.2002, p. 51–57*
[3] EFSA Mandate No M-2021-00058 of 7 June 2021

EFSA launched a call for tenders to subcontract preparatory work for the assessment (Figure 1).[4] The objectives of the contract are specified in Box 1. This protocol shall guide the Awarded Organisations in conducting the preparatory work.

Box 1. Objectives of the contract with the Awarded Organisations (OC/EFSA/NUTRI/2021/01)

Objective 1 Where needed, to further specify the parts of the protocol that will be implemented, in consultation with EFSA. This may include, for instance, further specification of the literature search strategy(ies), specification of data extraction processes, specification of the analytical plan for the statistical synthesis of the evidence, tailoring of risk of bias tools.

Objective 2 To collect and appraise scientific evidence that could be used to derive a UL for each micronutrient. The data collection and appraisal process should follow the requirements of the "high-level" protocol agreed with EFSA. It will include systematic review(s) of the literature (SR) on the relationship between the dietary intake of the awarded micronutrient and health outcomes identified in the protocol. This entails literature screening, data extraction, evidence appraisal (i.e. risk of bias assessment) and evidence (statistical) synthesis (e.g. meta-analysis, dose-response modelling, where appropriate) (EFSA, 2010). Narrative reviews may be required to gather contextual evidence relevant to the interpretation of the main body of evidence (e.g. absorption, distribution, metabolism, elimination, of the micronutrient; mechanistic data and modes of action).

Objective 3 Preparatory work for Hazard identification & for Hazard characterisation using the scientific evidence retrieved and in accordance with EFSA's updated Guidance for establishing UL for vitamins and minerals. In particular:

- To critically summarise the evidence concerning the capacity of the micronutrient to cause one or more types of adverse effects in humans. This includes an analysis of the uncertainties in the body of evidence according to the framework that will be provided by EFSA. Critical outcomes, for various life-stage groups within the population, that could be used to derive UL for the micronutrient should be identified as the result of this step.
- To characterise the dose–response relationship between micronutrient intake (dose) and identified adverse effects, where applicable.

Objective 4 To characterise sub-groups of the general populations having distinct and exceptional sensitivities to the adverse effects of the micronutrient, where relevant.

Objective 5 To identify data gaps

2. Context of the assessment

The SCF evaluated the UL for preformed vitamin A (retinol and retinyl esters) in 2002 (SCF, 2002). The SCF used the teratogenic potential of vitamin A as critical endpoint to derive the UL and established an UL of 3,000 µg RE[5]/day for women of child-bearing age. The SCF considered that the UL of 3,000 µg RE/day is also appropriate for men, and for infants and children after correction for differences in metabolic rate, because it is 2.5-fold lower than the lowest daily intake that has been associated with hepatotoxicity during chronic intake. Further, the UL applies to intakes during pregnancy and lactation, as it is also protective for hepatotoxicity and developmental toxicity. However, the SCF considered that the value does not apply to postmenopausal women, who represent the group at greatest risk of bone fracture, because it may not provide an adequate margin of safety in relation to the possible decrease in bone density and the risk of bone fracture; for this group, the SCF concluded that it would be advisable to restrict intake to 1,500 µg RE/day (SCF, 2002).

The SCF evaluated the UL for β-carotene in 2000. The SCF considered that human trials had shown that supplemental ß-carotene increases both lung-cancer incidence and mortality in human smokers and that mechanisms which offer likely explanations of these adverse effects have been derived from experimental studies in appropriate animal models. The SCF concluded that existing evidence from human trials indicated that supplemental β-carotene (20 mg/day or more) was contraindicated for use in heavy smokers. However, there was insufficient scientific basis to set a UL as no dose-response relationship for β-carotene effects was available either from the intervention trials in humans or from

[4] Tender reference number OC/EFSA/NUTRI/2021/01, available at: https://etendering.ted.europa.eu/cft/cft-display.html?cftId=8872
[5] 1 RE = 1 µg retinol

appropriate animal models. It was also not possible to distinguishing between the different isomeric forms of ß-carotene or specific formulations (SCF, 2000b).

In 2012, EFSA's ANS Panel evaluated the safety of β-carotene and concluded that exposure to β-carotene from its use as food additive and as food supplement at a level below 15 mg/day does not give rise to concerns about adverse health effects in the general population, including heavy smokers (EFSA ANS Panel, 2012).

A summary of evaluations by other risk assessments bodies is tabulated in Appendix A.

3. Problem formulation

The mandate tasks EFSA to review existing scientific evidence and provide advice on ULs for vitamin A, including its currently authorized forms for the addition to fortified foods and food supplements for the general population and, as appropriate, for vulnerable subgroups of the population.

Throughout this protocol, total vitamin A refers to, specifically preformed vitamin A (retinol and retinyl esters) and the provitamin A β-carotene. Although it is acknowledged that other provitamin A carotenoids (α-carotene and β-cryptoxanthin) may contribute to total vitamin A intake, their contribution to the overall toxicity of vitamin A is expected to be marginal. Thus, no specific considerations are made in this protocol regarding these compounds.

The assessment questions underlying the UL evaluation are formulated as follows:

I. **What is the maximum level of total chronic daily intake of vitamin A (including the preformed vitamin A and β-carotene) from all sources which is not expected to pose a risk of adverse health effects to humans?** ***(Hazard identification and characterisation)***

II. **What is the daily intake of vitamin A (including preformed vitamin A and β-carotene) from all dietary sources in EU populations?** ***(Intake assessment)***

III. **What is the risk of adverse effects related to the intake of vitamin A in EU populations, including attendant uncertainties?** ***(Risk characterisation)***

The mandate specifies that "for nutrients for which there are no, or insufficient, data on which to base the establishment of an UL, an indication should be given on the highest level of intake where there is reasonable confidence in data on the absence of adverse effects."

The problem formulation requires to specify the target population (Section 3.1), the exposure of interest (Section 3.2) and to identify relevant endpoints (Section 3.3) (EFSA NDA Panel, 2022).

The present document focuses on the assessment question I. The assessment question is broken down into sub-questions that are specific to vitamin A. The methods used to address each sub-question are defined in sections 4 and 5.

3.1. Target population

ULs should be protective for all members of the general population, including sensitive individuals, throughout their lifetime. The mandate specifies that "Tolerable Upper Intake Levels should be presented separately for the age group from 4/6 months onwards until 3 years of age and the general population group from 3 years onwards, taking into account, as appropriate, the varying degrees of sensitivity of different consumer groups."

Even within relatively homogenous life-stage groups, there is a range of sensitivities to adverse effects. The derivation of ULs accounts for the expected variability in sensitivity among individuals (e.g., heavy smokers). In principle, individuals under medical care are not excluded unless: a) there is an expected interaction between the medical condition and the occurrence of possible adverse effects of a nutrient, or b) they are under medical treatment with the nutrient under assessment.

The UL may exclude sub-populations with extreme and distinct vulnerabilities to adverse effects of the nutrient due to specific genetic predisposition or other factors. In case relevant genetic predisposition, pathological states or other conditions influencing the effect of excess intake of vitamin A are identified during the course of the assessment, this will be flagged as part of the risk characterisation.

3.2. Definition of the exposure of interest

3.2.1. Chemical forms of vitamin A

The assessment will address vitamin A and β-carotene from all dietary sources, i.e. foods (including fortified foods), beverages (including water), and food supplements (EFSA NDA Panel, 2022).

In the diet, vitamin A is found in products of animal origin, mainly as retinyl esters (primarily retinyl palmitate, with smaller amounts of retinyl oleate, retinyl stearate, retinyl myristate) and retinol. Provitamin A carotenoids are dietary retinol precursors that can be converted to retinol in the body and are mainly found in plant foods. In comparison with other carotenoids, β-carotene is the most important one in terms of its relative provitamin A activity, being the most potent retinol precursor, and also being the most abundant one in the diet (Harrison, 2012; Rodriguez-Amaya, 2015).

Authorised forms of preformed vitamin A for addition to foods[6] and for use in food supplements[7] in the EU are reported in Table 1. These vitamin A compounds, together with their metabolites, and synthetic derivatives that exhibit the same properties, are called retinoids. β-carotene is also authorised for addition to foods and for use in food supplements.

Table 1. Forms of vitamin A authorised as nutrient sources in the EU

	Addition to foods *Regulation (EC) 1925/2006*	**Food supplements** *Directive 2002/46/EC*
Retinol	x	x
Retinyl acetate	x	x
Retinyl palmitate	x	x
β-carotene	x	x

The physical forms of vitamin A should also be considered since water-miscible, emulsified preparations may be more likely to induce adverse effects than oil-based preparations and vitamin A in foods owing potentially to the higher bioavailability of vitamin A (see e.g. Myhre et al. (2003)).

3.2.2. ADME of the different forms of vitamin A

The absorption, distribution, metabolism and elimination (ADME) of vitamin A (retinol and retinyl esters; provitamin A carotenoids) were previously reviewed by the NDA Panel in its opinion on DRVs for vitamin A (EFSA NDA Panel, 2015). A summary is provided below.

Preformed vitamin A is efficiently absorbed in the small intestine over a wide range of intake (~ 70–90 %). Before entering the intestinal mucosa, dietary retinyl esters must be hydrolysed by retinyl ester hydrolases. Free retinol is taken up into the intestinal cells by protein-mediated facilitated diffusion and passive diffusion mechanisms via the action of membrane-bound lipid transporters involved in fatty acid and cholesterol uptake. Retinol then undergoes esterification with long-chain fatty acids, particularly with palmitic acid. Retinyl esters are packed, along with dietary fat and cholesterol, into nascent chylomicrons, which are secreted into the lymphatic system for delivery to the blood.

Dietary provitamin A carotenoids are absorbed via passive diffusion or taken up by the enterocyte through facilitated transport via SR-B1 and CD36. Inside the enterocyte, the majority (more than 60%) of the absorbed provitamin A carotenoids are cleaved at their central bond into all-*trans*-retinal. All-*trans*-retinal binds to CRBPII, is incorporated intact with fatty acids and cholesterol into nascent chylomicrons, or is further oxidised irreversibly to retinoic acid or reduced reversibly to retinol. The remaining ~40% of absorbed provitamin A carotenoids that are not cleaved in the intestine are absorbed intact. The absorption of β-carotene appears to be highly variable (~5–65 %), depending on food- and diet-related factors, genetic characteristics and the health and vitamin A status of the subject. This has significant implications on the bioequivalence of β-carotene to retinol.

β-carotene is mainly converted to retinol within the intestinal mucosal cells and the liver, while unaltered β-carotene is transported via the lymph to the blood where it is, after uptake of chylomicron remnants by the liver, repartitioned between lipoproteins. Although the uptake and distribution of β-carotene is

[6] *Regulation (EC) 1925/2006*
[7] *Directive 2002/46/EC*

not well understood, it presumably follows the same uptake pathways of retinoids. The maximum capacity for β-carotene cleavage into vitamin A active compounds by the intestine and the liver combined was estimated to be 12 mg β-carotene/day in a human adult.

A number of different forms of vitamin A are found in the circulation, and these differ in the fasting and postprandial states. They include retinyl esters in chylomicrons, chylomicron remnants, very low-density lipoprotein (VLDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL); retinol bound to retinol-binding protein (RBP4); retinoic acid bound to albumin; and the water-soluble β-glucuronides of retinol and retinoic acid. Approximately two-thirds of absorbed retinol is delivered to the blood via the lymph in esterified form as retinyl palmitate and other retinyl esters present in chylomicrons. Around one-third is secreted directly into the portal circulation, probably as free retinol.

Retinol and retinyl esters are the most abundant forms of vitamin A in the body. Retinol is a transport form and a precursor of the transcriptionally active metabolite all-trans-retinoic acid (ATRA). ATRA can be isomerised through a non-enzymatic process to 9-cis- or 13-cis-retinoic acid isomers. The isomer 13-cis-retinoic acid is less transcriptionally active than the all-trans and the 9-cis isomers. Retinyl esters serve as substrate for the formation of the visual chromophore 11-cis-retinal and are retinol storage forms, preliminary in the liver, where they are concentrated in the lipid droplets of hepatic stellate cells. Adipocytes are also able to accumulate significant retinyl ester stores.

The rate of retinol catabolism (i.e. rate at which retinol is irreversibly utilised each day) is related to body stores and the absolute catabolic rate appears to increase with vitamin A body stores. Overall, retinol catabolism represents a relatively low fraction of the total body pool, owing to the important storage capacity of the body and efficient recycling processes. The fractional catabolic rate may be influenced by physiological conditions (such as growth, presence of inflammation or other non-identified factors) and may be higher in children than in adults, in relation to a higher retinol utilisation for growth needs and, possibly, to relatively lower body stores than in adults.

The majority of retinol metabolites are excreted in the urine, but they are also excreted in faeces and breath. Retinol is metabolised in the liver to numerous products, some of which are conjugated with glucuronic acid or taurine for excretion in bile. Animal data indicates that the amount of retinol metabolites excreted in bile increases as the liver retinol exceeds a critical concentration, which may serve as a protective mechanism for reducing the risk of excess storage of vitamin A.

In its DRV opinion on vitamin A in 2015, the NDA Panel noted there was high variability in the β-carotene/retinol equivalency ratios estimated from the available literature, and this widely depended on the food matrix, the subjects' vitamin A status and the dose administered. Therefore, given the large uncertainties in establishing equivalency ratios from the whole diet of large populations, the Panel considered that there was insufficient new evidence to support a change from the conversion factors proposed by the SCF for European populations. Namely, 1 µg RE equals 1 µg of retinol, 6 µg of β-carotene and 12 µg of other carotenoids with provitamin A activity (EFSA NDA Panel, 2015).

Relevant assessment sub-questions regarding the ADME of the different forms of vitamin A are presented in Table 2.

Table 2. Formulation of sub-questions 1a and 1b and methods

Sub-questions	**Method to address the sub-questions**
1a. What is the ADME of different forms of vitamin A[(a)] in humans?	Narrative review[(b)]
1b. What is the extent to which β-carotene in fortified foods or supplements can contribute to "excess" vitamin A? (i.e., bioavailability/bioconversion of β-carotene in the "high" range of intake in individuals with adequate vitamin A status)	Narrative review[(b)]
1c. Are there differences related to age or other individual factors, e.g., genetic polymorphisms of vitamin A[(a)] metabolism?	Narrative review[(b)]

(a): preformed vitamin A and provitamin A β-carotene
(b): This should complement the information gathered in the NDA Panel opinion on DRVs for vitamin A (EFSA NDA Panel, 2015) with newly available information or information relevant to the interpretation and use of the data collected in the context of the UL assessment.

3.2.3. Biomarkers of exposure to vitamin A, including β-carotene

Liver content of retinol

The liver is the major tissue sites of retinol storage, where it is mostly present as retinyl esters. The liver content of retinol (free and esterified) is considered the gold standard to determine vitamin A status.

In 2016, the BOND expert panel on biomarkers for vitamin A concluded that evidence for setting the cut-off for 'excess' liver retinol concentrations is limited (Tanumihardjo et al., 2016). Until more data emerge, the panel proposed to use the terms "hypervitaminosis A" for liver concentrations >1 µmol/g liver and "toxic" for liver concentrations > 10 µmol/g liver. The panel noted the need for future research "to determine if there are deleterious effects at liver retinol concentrations > 1 µmol/g liver".

The current cut-off value for 'hypervitaminosis A' (>1 µmol/g liver) was proposed by Olson in 1984 based on the hypothetical relationship between liver and plasma concentrations of vitamin A, and was subsequently accompanied with a cut-off value for total vitamin A plasma concentration of >3.5 µmol/L (Olson, 1984, 1990). There is a lack of pathophysiological findings indicative of vitamin A toxicity in animals or humans with liver concentrations around or above 1 µmol/g liver.

A more recent publication suggested hepatoxicity at about 3 µmol retinol/g liver, as characterised by hypertrophy of stellate cells (1 subject) and perisinusoidal space enlargement and the formation of visible lipid droplets (3 subjects) (Olsen et al., 2018). The study lacked measurement of potential confounding factors such as hepatitis, chronic biliary disease and metabolic liver disease, which have been shown to cause hypertrophied stellate cells (Mounajjed et al., 2014; Hoffmann et al., 2020).

Overall, there is currently no consensus regarding a cut-off point for liver retinol concentrations associated with liver toxicity and data to define such cut-off are scarce. There is insufficient data on the relationships between preformed vitamin A intake and hepatic retinol concentration on the one hand, and hepatic retinol concentration and adverse effects on the other hand, to use hepatic retinol concentration as an endpoint for setting ULs for vitamin A.

Plasma/serum retinol concentration

The concentration of plasma/serum retinol is under tight homeostatic control (EFSA NDA Panel, 2015). In the usual range, plasma/serum retinol concentration is neither related to observed habitual vitamin A intake from dietary preformed vitamin A nor responsive to supplement use. In addition, plasma/serum retinol concentration is affected by a number of factors unrelated to vitamin A status, including infection and inflammation, which makes the interpretation of this biomarker difficult.

Overall, although serum/plasma retinol concentration has been used as a biomarker of intake, serum/plasma retinol concentration is under homeostatic control and, in the usual range, is not related to observed levels of habitual vitamin A intake. Therefore, it is not considered a reliable marker of (preformed) vitamin A intake.

Plasma/serum retinyl esters concentration

Vitamin A supplement use has been associated with an increase in fasting plasma retinyl esters (Krasinski et al., 1989; Stauber et al., 1991).

High circulating concentrations of retinyl esters have been proposed as markers of vitamin A toxicity, especially hepatic toxicity (Olson, 1984; Krasinski et al., 1989). High circulating levels of retinyl esters have been observed in clinically confirmed cases of vitamin A toxicity, with plasma concentrations ranging from 1.6-8.7 µmol/L (Smith and Goodman, 1976; Ellis et al., 1986). In subjects aged ≥ 60 years, higher prevalence of elevated AST and ALT activities were found in the group with fasting plasma retinyl esters ≥ 0.38 µmol/L compared with the group with fasting plasma retinyl esters < 0.38 µmol/L (Krasinski et al., 1989).

Cut-offs of 10% total serum vitamin A as REs in adults (Krasinski et al., 1989; Tanumihardjo et al., 2016) and 5% in children (Mondloch et al., 2015; Williams et al., 2021) have been suggested to be indicative of "excess" vitamin A intake. A recent publication suggested that the cut-off for adults may be lower (7.5%) based on observations of hepatic content (>3 µmol/g liver) associated with abnormal liver histology in autopsy samples and matched serum samples (Olsen et al., 2018).

Among 6,547 adults who participated in the National Health and Nutrition Examination Survey (NHANES III), 37% had fasting serum retinyl ester concentrations >10% of total serum vitamin A and 10% had concentrations >15%. Serum retinyl ester concentrations > 10% were not associated with abnormal liver function (Ballew et al., 2001).

Liver disease and hypertriglyceridemia can result in high circulating retinyl ester values even when vitamin A status is normal. Also, old age alone can result in impaired clearance of retinyl esters from chylomicron circulation after a meal, and thus result in higher blood retinyl ester concentrations for longer periods of time after eating than in young adults.

Fasting retinyl esters concentration is a useful marker of 'high' vitamin A intake. Regarding its use as a marker of vitamin A toxicity, the proposed cut-offs are based on limited data and there is currently no consensus regarding levels indicative of vitamin A "excess intake" or hepatic toxicity. At this stage, fasting retinyl esters concentration lacks sufficient validation to be used for setting ULs for vitamin A in isolation.

Relevant assessment sub-questions regarding vitamin A biomarkers of exposure are presented in Table 3.

Table 3. Formulation of sub-questions 2a, 2b and 2c and methods

Sub-questions	Method to address the sub-question
2a. How does hepatic retinol content reflect 'high' vitamin A intake? What is the relevance of this marker as biomarker of vitamin A toxicity?	Narrative review
2b. How does circulating fasting retinyl esters reflect 'high' vitamin A intake? What is the relevance of this marker as biomarker of vitamin A toxicity?	Narrative review(a)
2c. What are other markers of 'high' vitamin A intake and toxicity?	Narrative review(b)

(a) Relevant information will also be collected in the context of sub-questions 3a, 4a and 5a

(b) Notes in relation to question 2c: Since ATRA is likely the vitamin A species that underlies most of the toxic effects of vitamin A, it may be possible to identify altered circulating levels of ATRA-responsive gene products in blood that can serve as sensitive and specific predictive biomarkers. Alternatively, elevated blood or urine levels of conjugates of ATRA and its metabolites might be found to serve as a useful biomarker for excessive vitamin A intake. However, this would require a significant research effort to establish the utility of these possible markers; one that is not currently being undertaken. Additionally, some experiments -omics type of investigation (animal and human) suggest that some fat metabolites may also be explored as markers of 'high' vitamin A exposure/toxicity.

3.3. Identification of relevant endpoints

3.3.1. Priority adverse health effects

Priority adverse health effects were identified in consultation with a panel of qualified experts on vitamin A[8]. Priority adverse health effects are those which are expected to play a critical role for establishing an UL. These will be addressed through systematic reviews of the literature (section 4.3.3).

The term 'hypervitaminosis A' is used in the literature to refer to 'excess vitamin A intake', with inconsistent definitions. The older literature uses 'hypervitaminosis A' for describing vitamin A toxicity with clear pathophysiological indicators such as perisinusoidal fibrosis, hyperplasia and hypertrophy of stellate cells. In more recent years, this term has been used to qualify vitamin A status characterised by hepatic retinol concentrations between 1-10 µmol/g liver, i.e. below concentrations considered as 'toxic' (Tanumihardjo et al., 2016); Section 3.3.1.2). Because of the lack of consensus regarding this terminology, it will be used between quotation marks with a reference to the definition applied in the relevant publication.

[8] The expert panel was composed of William Blaner (Columbia University, USA), Georg Lietz (Newcastle University, UK), Sherry Tanumihardjo (University of Wisconsin-Madison, USA) and Johannes von Lintig (Case Western Reserve University, USA). A hearing of the expert panel was held on 21 December 2021.

3.3.1.1. Teratogenicity

The teratogenic potential of vitamin A is well established, based on animal and human evidence (IOM, 2001; SCF, 2002). The teratogenic outcome in offspring include craniofacial (cleft lip/palate), cardiac (transposition of the great vessels), thymic, and central nervous system (microcephaly, hydrocephalus) abnormalities. The critical period for susceptibility appears to be the first trimester of pregnancy. In recent years, ATRA has be shown to be involved in many critical processes in patterning and organ development (Kumar and Duester, 2011; Rhinn and Dolle, 2012; Shannon et al., 2017; Knudsen et al., 2021).

The SCF considered this effect as a critical endpoint to derive an UL of 3,000 µg RE[9]/day for women of child-bearing age (SCF, 2002). The value was based on a prospective study in which a 3.5 times higher risk of giving birth to a child with cranial-neural-crest defects was found for women taking daily more than 4,500 µg RE of total vitamin A (from food and supplement) compared to mothers ingesting less than 1,500 µg RE/day (Rothman et al., 1995). When the analysis was restricted to the supplemental intake of vitamin A only, the relative risk for mothers ingesting more than 3,000 µg RE/day was 4.8 higher than those ingesting 1,500 µg RE/day. The authors fitted a regression curve to their data, which indicated a rise in the ratio of prevalence of birth defects associated with the cranial-neural crest at doses greater than 3,000 µg RE/day of vitamin A (food and supplement).

In contrast, no association had been found in case-control studies between daily doses of vitamin A of 3,000 µg RE or less and foetal malformation (Dudas and Czeizel, 1992; Khoury et al., 1996; Czeizel and Rockenbauer, 1998)(reviewed by (IOM, 2001; SCF, 2002)).

At the time of its assessment, the IOM noted that most of the human data on teratogenicity of vitamin A involved doses equal to or greater than 7,800 µg/day (Bernhardt and Dorsey, 1974; Bauernfeind, 1980; Martínez-Frías and Salvador, 1990) and the lack of epidemiological data to define a dose-response relationship in the dose range of 3,000 to 7,800 µg/day (IOM, 2001).

These endpoints are relevant for pregnant women in the general population.

The assessment sub-questions are presented in Table 4 below. For all sub-questions, evidence on differences between the various forms of vitamin A (preformed vitamin A and provitamin A β-carotene) will be considered. The relationship between different sub-questions is illustrated in Figure 2.

Table 4. Formulation of sub-questions 3a and 3b and methods

Sub-questions	Method to address the sub-questions
3a. What is the dose-response relationship between 'high' vitamin A[(a)] intake and teratogenicity?	Systematic review
3b. What are the potential mechanisms/mode(s) of action underlying the relationship between vitamin A intake and this endpoint?	Narrative review

(a) Preformed vitamin A and provitamin A β-carotene

[9] 1 RE = 1 µg retinol

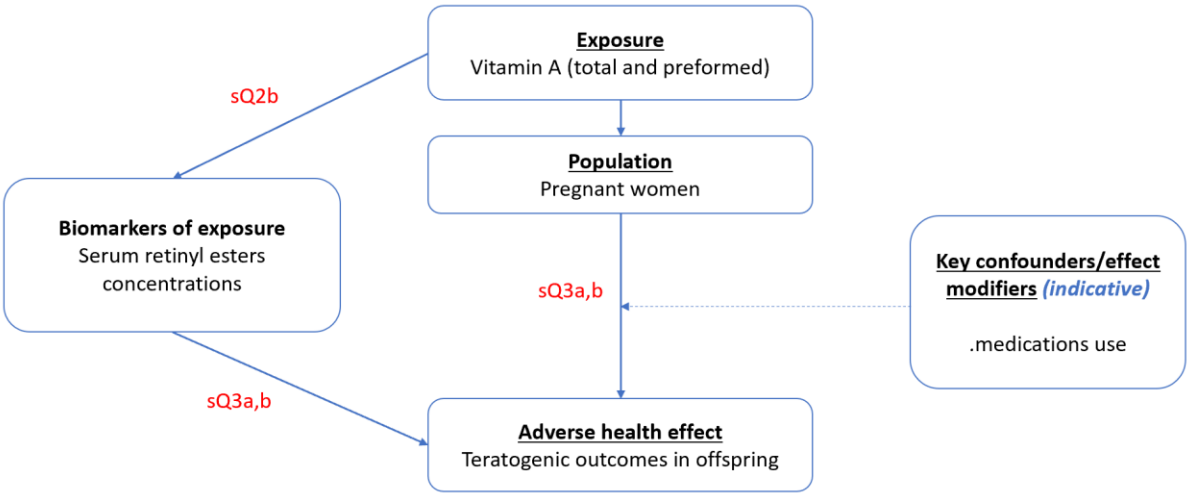

Figure 2. Relationship between assessment sub-questions relevant to teratogenicity.

Eligibility criteria for human studies to address sub-questions 3a are presented in Table 5.

Table 5. Eligibility criteria for human studies to address sub-question 3a

Design	Out	No restriction
Study duration	Out	No restriction
Study location	Out	No restriction
Population	In	Pregnant women
Exposure	In	Studies reporting quantitative estimates of: ▪ Vitamin A intake (as preformed vitamin A with or without β-carotene; from diet and/or supplements; any method, e.g. self-reported or recorded) ▪ Note: In studies identified by the eligibility criteria, data on fasting (≥ 6 hours) serum retinyl ester concentration as biomarker of exposure should also be evaluated (sQ2b)
	Out	Studies using only serum retinol and retinyl esters as biomarker of exposure
Endpoints of interest	In	Teratogenic outcomes in offspring, including craniofacial, cardiac, thymic, and central nervous system abnormalities
	Out	Studies not including at least one of the endpoints listed above
Language	In	Full-text document in English
	Out	Articles with the full text in another language
Publication year	In	2001 - 2022
	Out	Before 2001[(a)]
Publication type	In	▪ Primary research studies reported in full-text articles ▪ "Brief communication" articles reporting original data ▪ Systematic reviews and meta-analyses [(b)]
	Out	▪ Narrative reviews, expert opinions ▪ Conference abstracts and letters to editors (not reporting on original data) ▪ PhD theses ▪ Grey literature

(a): SCF (2002) will be used a source of data for studies published before 2001

(b): Systematic reviews, including meta-analyses, on this topic that will be identified during the process of literature screening will be collected for the purpose of reviewing the reference list but will not be considered to contribute to the final number of studies considered eligible unless they also contain original data.

3.3.1.2. Hepatotoxicity

Liver is the main storage site and target organ for vitamin A toxicity. Human and animal data show a causal relationship between excess vitamin A intake and liver abnormalities. Excessive vitamin A intake is associated with hepatic fibrosis arising through the activation of hepatic stellate cells. Vitamin A-induced liver abnormalities ranges from reversibly elevated liver enzymes to widespread fibrosis, cirrhosis, and sometimes death.

Available evidence on the vitamin A-induced hepatoxicity was reviewed in previous assessments of UL for vitamin A (IOM, 2001; SCF, 2002; EVM, 2003). Human data essentially came from case reports of individuals consuming high doses of vitamin A for several years.

In its evaluation of the dose-response between vitamin A intake and hepatotoxicity, the IOM (2001) considered two case reports which reported hypertrophy of hepatic stellate cells after vitamin A intake of 14,000 µg RE/day for 10 years and 15,000 µg/day for 12 years, respectively (Zafrani et al., 1984; Minuk et al., 1988). Neither of these reports appeared to be confounded by hepatitis A or B viral infections or concomitant exposure to other hepatotoxic agents including alcohol. Reports of vitamin A-induced hepatotoxicity at doses less than 14,000 µg/day (Eaton, 1978; Hatoff et al., 1982; Oren and Ilan, 1992; Kowalski et al., 1994) were not considered by the committee as these studies failed to provide information on other predisposing or confounding factors such as alcohol intake, drugs and medications used, and history of viral hepatitis infection. The IOM concluded on a lowest-observed-adverse-effect level (LOAEL) of 14,000 µg/day for hepatoxicity in adults.

From its review of the evidence, SCF (2002) concluded that an intake of 7,500 µg RE/day taken over 6 years was the lowest dose reported to cause hepatotoxicity in humans (Geubel et al., 1991; Kowalski et al., 1994) and noted that it was not known if a dose lower than 7,500 µg RE/day could induce hepatotoxicity if taken for more than 6 years.

These endpoints are relevant for all groups of the general population.

The assessment sub-questions are presented in Table 6 below. For all sub-questions, evidence on differences between the various forms of vitamin A (preformed vitamin A and provitamin A β-carotene) will be considered. The relationship between different sub-questions is illustrated in Figure 3.

The assessment will firstly aim at characterizing the dose-response relationship between 'high' vitamin A intake and hepatotoxicity. For this purpose, eligibility criteria are restricted to trials in subjects treated with vitamin A. In case that eligible trials report on fasting concentration of serum retinyl esters, the data will be extracted for the purpose of exploring the value of this biomarker as a marker of vitamin A toxicity (sQ2b).

Table 6. Formulation of sub-questions 4a and 4b and methods

Sub-questions	Method to address the sub-questions
4a. What is the dose-response relationship between 'high' vitamin A[(a)] intake and hepatotoxicity?	Systematic review of literature published since 2001
4b. What are the potential mechanisms/mode(s) of action underlying the relationship between vitamin A intake and this endpoint?	Narrative review

(a): Preformed vitamin A and provitamin A β-carotene

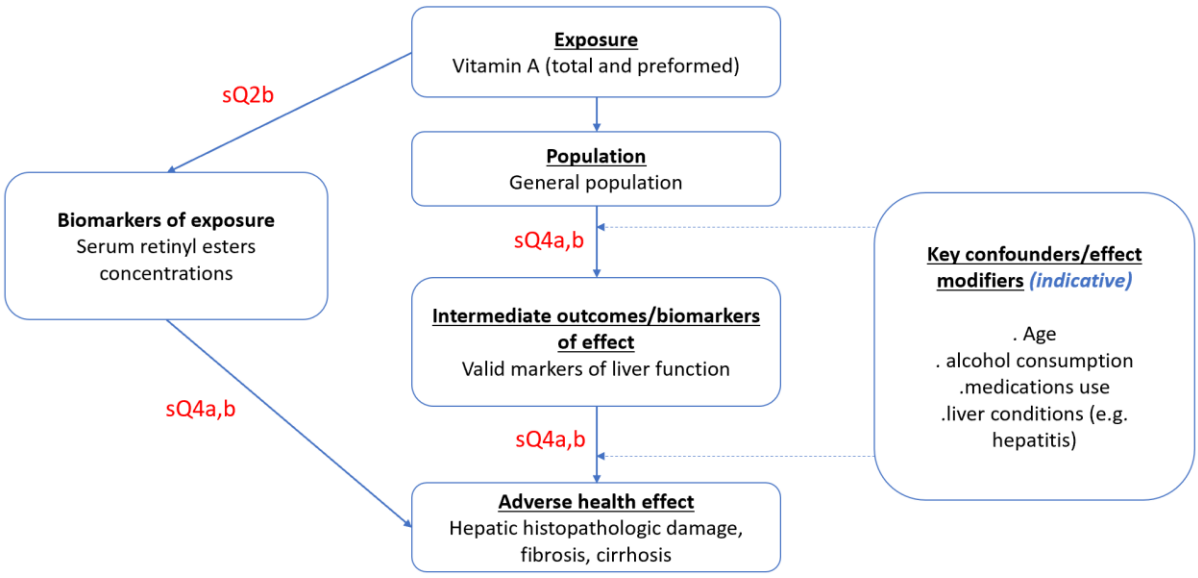

Figure 3. Relationship between assessment sub-questions relevant to hepatotoxicity.

Eligibility criteria for human studies to address sub-questions 4a are presented in Table 7.

Table 7. Eligibility criteria for human studies to address sub-questions 4a

Design	In	RCTs, including trials in subjects treated with vit A (e.g. retinitis pigmentosa)
Design	Out	Human studies: all other designs Animal studies Other studies
Study duration	Out	Less than 3 months
Study location	Out	No restriction
Population	In	No restriction NB: studies in people with liver conditions are not excluded a priori (relevance for the general population to be addressed on a case by case basis)
Exposure	In	*Supplementation:* ▪ Vitamin A supplementation (as preformed vitamin A with or without β-carotene) vs placebo or lower vitamin A doses ▪ Vitamin A supplementation (as preformed vitamin A with or without β-carotene) + a co-intervention while controlling for the co-intervention *Supplementation pattern:* ▪ Dosing at least once a week for at least 3 months *Supplementation route:* ▪ Oral ▪ In studies identified by the eligibility criteria, data on fasting (≥ 6 hours) serum retinyl ester concentration as biomarker of exposure should also be evaluated (sQ2b) ***Note:*** *Studies on parenteral supplementation or giving vitamin A less frequently than weekly (e.g. monthly, bolus) will be kept as supporting evidence (narrative review) but excluded from the primary assessment to draw conclusions on the UL*
	Out	*Supplementation:* ▪ Vitamin A supplementation (as preformed vitamin A with or without β-carotene) with a cointervention not controlling for the cointervention (e.g. multivitamin supplements vs placebo) *Supplementation pattern:* ▪ Less frequent than weekly *Supplementation route:* ▪ Parenteral

Endpoints of interest	In	• Valid markers of liver function, including liver enzymes • Liver steatosis assessed by ultrasound or MRI • Transient elastography (fibroscan) • Histopathological signs of hepatotoxicity assessed by liver biopsy • Clinically diagnosed liver cirrhosis • Clinically diagnosed portal hypertension, with or without cirrhosis
	Out	Studies not including at least one of the endpoints listed above
Language	In	Full-text document in English
	Out	Articles with the full text in another language
Publication year	In	2001 - 2022
	Out	Before 2001[(a)]
Publication type	In	▪ Primary research studies reported in full-text articles ▪ "Brief communication" articles reporting original data ▪ Systematic reviews and meta-analyses [(b)]
	Out	▪ Narrative reviews, expert opinions ▪ Conference abstracts and letters to editors (not reporting on original data) ▪ PhD theses ▪ Grey literature

(a): SCF (2002) will be used a source of data for studies published before 2001

(b): Systematic reviews, including meta-analyses, on this topic that will be identified during the process of literature screening will be collected for the purpose of reviewing the reference list but will not be considered to contribute to the final number of studies considered eligible unless they also contain original data

3.3.1.3. Bone health

In 2002, the SCF noted that an increased risk of bone fracture was reported for an intake of 1,500 µg RE/day or higher (SCF, 2002). Based on available evidence, the SCF considered that the available data did not provide sufficient evidence of causality, owing to the possibility of residual confounding, and were not appropriate for establishing a UL. The SCF noted that "because the tolerable upper level may not adequately address the possible risk of bone fracture in particularly vulnerable groups, it would be advisable for postmenopausal women, who are at greater risk of osteoporosis, to restrict their intake to 1,500 µg RE/day".

In a subsequent assessment which considered studies published until 2004, the Scientific Advisory Committee on Nutrition (SACN, 2005) concluded that the evidence of an association between high intake of retinol and poor bone health was inconsistent. The Committee noted that some epidemiological data suggest that a retinol intake of 1,500 µg/day and above is associated with an increased risk of bone fracture; the evidence was considered not robust enough to set a Safe Upper Level, and a Guidance Level for retinol intake of 1,500 µg/day was set for adults.

Several systematic reviews of the relationship between vitamin A intake and risk of bone fracture have been conducted since then. Based on PCs, Zhang et al reported an inverse association between dietary intake of retinol and total vitamin A and total fracture risk, while a positive association was found in relation to hip fracture risk (Zhang et al., 2017). Based on PCs and case-control studies, (Knapik and Hoedebecke, 2021) found that risk of hip fracture was increased by high dietary intake of total vitamin A or retinol.

Some evidence from animal studies (Lind et al., 2012; Lionikaite et al., 2018; Lionikaite et al., 2019) and human studies (Melhus et al., 1998; Binkley and Krueger, 2000; Jackson and Sheehan, 2005; Yee et al., 2021) point to a deleterious effect of high vitamin A on cortical bone and bone mineral density.

The risk of osteoporotic fractures is relevant for post-menopausal women and older adults. Adverse effects on bone mineral density and bone strength are relevant for all population groups.

The assessment sub-questions are presented in Table 8 below. For all sub-questions, evidence on differences between the various forms of vitamin A (preformed vitamin A and provitamin A β-carotene) will be considered. The review will consider whether there are critical life stages or other timing effects of exposure. The relationship between different sub-questions is illustrated in Figure 4.

Table 8. Formulation of sub-questions 5a, 5b and 5c and methods

Sub-questions	Methods to address the sub-questions
5a. Does 'high' vitamin A[(a)] intake increase the risk of bone fractures in humans? If so, could a dose-response be characterised?	Systematic review
5b. Does 'high' vitamin A[(a)] intake affect **BMD/BMC and**/or **indices of bone strength** in humans? If so, could a dose-response be characterised?	Systematic review
5c. What are the potential mechanisms/mode(s) of action underlying the relationships between vitamin A intake and these endpoints?	Narrative review

(a): Preformed vitamin A and provitamin A β-carotene

Note: In relation to question 5a and 5b, existing systematic reviews and meta-analyses could be considered and possibly be updated/expanded.

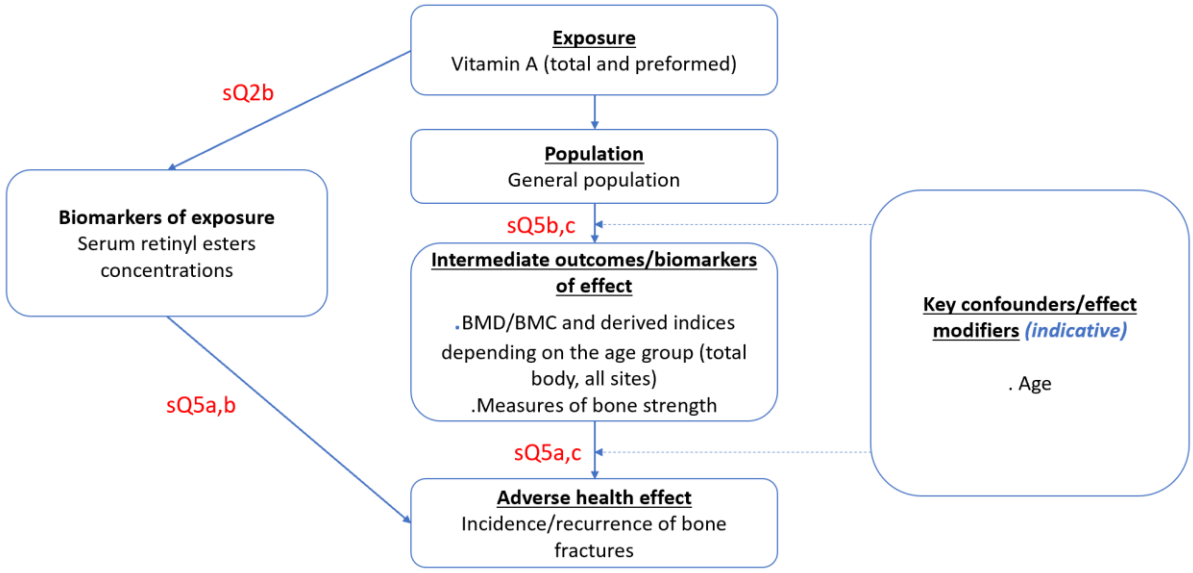

Figure 4. Relationship between assessment sub-questions relevant to bone health.

Eligibility criteria for human studies to address sub-questions 5 a and b are presented in Table 9.

Table 9. Eligibility criteria for human studies to address sub-question 5a and 5b

Design	In	Human studies: ▪ RCTs and non-randomised comparative studies of interventions ▪ Prospective (cohort, case-cohort, and nested case-control) studies
	Out	Human studies: all other designs Animal studies Other studies
Study duration	In	≥ 12 months
	Out	< 12 months
Study location	Out	No restriction
Population	In	All age groups
	Out	▪ Individuals at risk of/with vitamin A deficiency receiving therapeutical doses of (preformed) vitamin A ▪ Individuals under medical therapy with topic synthetic retinoids ▪ Individuals with primary hyperparathyroidism or other disorders affecting bone health
Exposure	In	Intervention studies

		Supplementation: ▪ Vitamin A supplementation (as preformed vitamin A with or without β-carotene) vs placebo or lower vitamin A doses ▪ Vitamin A supplementation (as preformed vitamin A with or without β-carotene) + a co-intervention while controlling for the co-intervention *Supplementation pattern:* ▪ Dosing at least once a week for at least 12 months *Supplementation route:* ▪ Oral ▪ In studies identified by the eligibility criteria, data on serum retinyl ester concentration as biomarker of exposure should also be evaluated ▪ ***Note:*** *Studies on parenteral supplementation or giving vitamin A less frequently than weekly (e.g. monthy, bolus) will be kept as supporting evidence (narrative review) but excluded from the primary assessment to draw conclusions on the UL* <u>Prospective (cohort, case cohort and nested case control) studies</u> Studies reporting quantitative estimates of: ▪ Vitamin A intake (as preformed vitamin A with or without β-carotene; from diet and/or supplements; any method, e.g. self-reported or recorded) ▪ Serum retinyl ester concentration as biomarker of exposure
	Out	<u>Intervention studies</u> *Supplementation:* ▪ Vitamin A supplementation (as preformed vitamin A with or without β-carotene) with a cointervention not controlling for the cointervention (e.g. multivitamin supplements vs placebo) *Supplementation pattern:* ▪ Less frequent than weekly *Supplementation route:* ▪ Parenteral ▪ Serum retinol concentration as biomarker of exposure
Endpoints of interest	In	▪ Bone fractures diagnosed by a physician (all sites) or self-reported ▪ Measures of BMD/BMC and measures of bone strength assessed by DXA or pCT (total body, all sites)
	Out	▪ Measures of BMD/BMC assessed by QUS (any site) ▪ Studies not including at least one of the endpoints above
Language	In	Full-text document in English
	Out	Articles with the full text in another language
Publication year	In	Existing SRs with eligibility criteria as inclusive as those spelled out in the present protocol can be used as starting point for the identification of eligible studies. In this case, the literature search will include 2001-2022.
	Out	N/A
Publication type	In	▪ Primary research studies reported in full-text articles ▪ "Brief communication" articles reporting original data ▪ Systematic reviews and meta-analyses [(a)]
	Out	▪ Narrative reviews, expert opinions ▪ Conference abstracts and letters to editors (not reporting on original data) ▪ PhD theses ▪ Grey literature

(a): Systematic reviews, including meta-analyses, on this topic that will be identified during the process of literature screening will be collected for the purpose of reviewing the reference list but will not be considered to contribute to the final number of studies considered eligible unless they also contain original data.

3.3.2. Other adverse health effects

Other adverse health effects that have been associated with excess preformed vitamin A intake are presented in the Table 10 below, including a rationale for not considering them among priority effects.

The endpoints listed in the table below were not considered priorities for the purpose of setting ULs based on the discussion with the hearing experts.

Table 10. Overview of adverse health effects that are not prioritized for a systematic review of the literature

Adverse health effects	Description	Evidence base	Rationale for not considering among priority adverse health effects
Preformed vitamin A			
Bulging fontanelle in infants	Increased risk seen with 25,000 IU (7,500 RE µg) ~ monthly dose. Dose depending on age.	Observational case-reports summarised in: Myhre et al. (2003) RCTs: (West et al., 1992; Defrancisco et al., 1993; Baqui et al., 1995; Mazumder et al., 2015) SRs: Gannon et al. (2021) and Imdad et al. (2020)	Adverse effect observed at doses above UL
Impaired growth in children	Some recent evidence seen in animal studies of reduced growth/lower birth weight with single doses of ≥25,000 IU of retinyl palmitate. Adverse outcomes were observed in South African preschool children (Sheftel et al., in press).	Animal studies: (Gannon et al., 2017; Mondloch et al., 2018)	Limited evidence in humans Low relevance to the target (European) population
Lipid metabolism	Intervention study linking vitamin A toxicity to lipid metabolism recently confirmed in animal studies.	RCTs: (Pastorino et al., 1993; Omenn et al., 1994; Cartmel et al., 1999; Sibulesky et al., 1999) Animal studies: (Finney et al., 2019; Lietz et al., 2019)	Limited evidence Adverse effect observed at doses above UL
Immune function	Impairment of the intestinal barrier because of excessive retinoic acid production in ISX-deficient mice. These ISX knockout mice displayed inflammatory responses in the gut in response to elevated retinoic acid production and also developed pancreatitis as they aged (animal models-unclear mechanism)	Animal studies: Widjaja-Adhi et al. (2017)	Limited evidence
Inflammatory bowel disease	Isotretinoin use (for the treatment of acne) and cases of IBD (case-reports, case-control). On the other hand, high doses of vitamin A are used in the treatment of Chron's Disease under medical supervision	Barbalho et al. (2019); (Crockett et al., 2010); (Lee et al., 2016)	Medical use of vitamin A Conflicting evidence
β-carotene			

Lung cancer	At the time of the SCF UL assessment of β-carotene, a number of trials identified had shown an increased risk for lung cancer among smokers and asbestos workers receiving β-carotene supplements at high doses. These findings were corroborated in EFSA's safety evaluation of β-carotene, where a meta-analysis of RCTs by Druesne-Pecollo et al. (2010) indicated an increased risk of lung cancers in individuals supplemented with β-carotene at dose levels equal to or greater than 20 mg/day (EFSA ANS Panel, 2012). In their respective risk assessments of β-carotene supplementation, both the EVM and VKM considered lung cancer as a critical endpoint to derive a UL and concluded on a LOAEL of 20 mg/day based on evidence from the ATBC study (EVM, 2003; VKM, 2015) (see Appendix A). In a recent systematic review conducted by AHRQ, evidence from 4 RCTs indicated that β-carotene, with or without vitamin A, was associated with an increased risk of lung cancer (O'Connor et al., 2022).	ATBC study in smokers, supplemented with β-carotene doses of 20 mg/d for up to 8 y (ATBC Study Group, 1994) CARET study in smokers and workers exposed to asbestos supplemented with β-carotene (30 mg/d) + retinol (25,000 IU/d) for up to 4 y (Omenn et al., 1996) Women's Health Study (WHS) in women aged ≥45 y supplemented with β-carotene doses of 50 mg/alternate days for a median treatment duration of 2.1y (Lee et al., 1999). Physicians' Health Study (PHS) in male physicians supplemented with doses of 50 mg/alternate day, with an average follow-up of 12 y (Hennekens et al., 1996) Polyp Prevention Study (PPS) in individuals at high risk of lung cancer supplemented with 25 mg/d for 4-y (Greenberg et al., 1994) SR reviews by Druesne-Pecollo et al. (2010), and O'Connor et al. (2022).	No new supplementation trials with β-carotene above doses of 20 mg/d would have been carried out since the mid-2000s due to ethical reasons. The very recent O'Connor et al. (2022) review most likely identified all relevant trials available and is a good starting point for summarising the evidence.
Other cancers	Some evidence of increased risk of other cancers besides lung cancer, such as gastric cancers and bladder cancers.	SRs by Druesne-Pecollo et al. (2010), Jeon et al. (2011), and Bjelakovic Bjelakovic et al. (2006)	Limited/conflicting evidence
Cardiovascular diseases	Some evidence available for an increased risk of CVD, and particularly CVD mortality, with higher intakes of β-carotene.	Composite CVD event (Lee et al., 1999) CVD mortality (ATBC Study Group, 1994) (Lee et al., 1999) (Hennekens et al., 1996) (Greenberg et al., 1990) (Green et al., 1990) SR by O'Connor et al. (2022).	Limited/conflicting evidence
All-cause mortality	Evidence for increased risk of mortality with higher intakes of β-carotene.	SRs by O'Connor et al. (2022), Bjelakovic et al. (2013) and Bjelakovic et al.	Limited/conflicting evidence

		(2012), and Vivekananthan et al. (2003)	

These effects (Table 10) will be addressed narratively in the opinion (Table 11).

Table 11. Formulation of sub-question 6 and method

Sub-question	Method to address the sub-question
6. What other adverse health effects have been reported to be associated with 'high' intake of vitamin A[(a)]?	Narrative review

(a): Preformed vitamin A and provitamin A β-carotene

4. Methods for answering sub-questions addressed through systematic reviews

4.1. Literature search and screening

Bibliographic databases will be searched according to a predefined literature search strategy. Systematic literature searches will be performed by research librarians from e.g. Karolinska Institutet and peer reviewed by Oslo university research librarians (or vice versa) and by EFSA or *vice versa*. Searches will be performed in MEDLINE (Ovid), Embase (Ovid), Cochrane Central Register of Controlled Trials, covering the relevant time period. Scopus will not be used. Reference lists of relevant retrieved articles and recent systematic reviews (SR) will be screened. Forward citation search from included studies will be performed. The search will be restricted to papers published in the English language. Grey literature and unpublished studies will not be searched. Study authors may if necessary be contacted to clarify questions about eligibility, outcomes, or other unpublished data. The search processes and strategies will be documented and reported, i.e. the date of the search, sources of information, search string for each bibliographic database and additional sources, and the number of records before and after de-duplication. Articles identified through the search will be screened for relevance and eligibility using DistillerSR. The results of the different steps of the study selection process will be reported in the scientific opinion using a flowchart as recommended in the PRISMA statement on preferred reporting items for systematic reviews and meta-analyses. The list of studies excluded after full-text screening will be documented, along with the reasons for excluding them.

4.2. Data extraction

Data from studies meeting the eligibility criteria will be extracted in structured forms which will include the characteristics of the studies (e.g. study design), their key-elements (e.g. population, exposure, outcomes (endpoints), setting and duration), results, and aspects related to their internal validity (e.g. confounders, randomisation). The data will be extracted in the original units of measurement, which will be subsequently harmonised to allow data analysis. The data extraction forms will be pilot tested on a subset of studies to perform quality checks and validate the process. The piloting will also be used to identify sources of contextual (i.e. related to the key elements of the studies) heterogeneity. The forms and extraction instructions will be refined if needed. Standardised evidence tables in Word will be produced according to EFSA's template.

Studies for which the information provided in the publication(s) does not allow a full scientific evaluation (e.g. studies with missing or ambiguous information) will be excluded at this step. The list of studies excluded at the data extraction stage will be documented, along with the reasons for exclusion.

4.3. Risk of bias appraisal

The internal validity of eligible studies will be appraised using OHAT risk of bias (RoB) tools (OHAT/NTP, 2015), by two independent reviewers. By default, this applies to all eligible studies, including those which may have been identified through pre-existing systematic reviews. The risk of bias appraisal may be restricted or not applied, if justified by the nature of the eligible body of evidence. The decision will be taken on a case-by-case basis upon completion of the data extraction (evidence tables).

The OHAT tools allow a parallel approach to evaluating risk of bias from human and non-human animal studies (OHAT/NTP, 2019). Specific RoB questions and instructions to reviewers are provided for each type of study design.

For each study, the appraisal will be done at outcome level. Possible discrepancies between reviewers will be discussed. If, upon further discussion, the reviewers cannot reach an agreement on a risk of bias rating for a particular domain, the more conservative judgment (the highest risk of bias) will be selected. Based on the appraisal of each risk-of-bias domain, an overall risk-of-bias judgement will be attributed to each study and outcome according to pre-defined criteria.

The risk-of-bias criteria and rating instructions provided by OHAT will be tailored to the specific sub-questions. In particular, the criteria for the three following RoB domains will be customized: 1) consideration of potential confounders, 2) confidence in the exposure characterisation, and 3) confidence in the outcome assessment. Critical elements regarding the assessment of confounding and biases related to exposure and outcome characterisation will be identified a priori. This includes a list of potential factors that could confound the relationship between vitamin A and the relevant endpoints, which will be identified on the basis of published literature and domain expertise. When assessing risk of bias in observational studies, the reviewers will consider, for each study, whether these factors can confound the association on a case-by-case basis. Additional confounders may be identified by the reviewers. The reviewers will consider whether the confounding variables were measured reliably and consistently within each study and whether the design and/or the data analysis adequately accounted for potential confounding (e.g. multivariable analysis, stratification).

The outcome of the appraisal, i.e. judgements on each risk-of-bias domain and overall, will be tabulated, by study and outcome.

4.4. Evidence synthesis

If several studies address the same outcome and are sufficiently comparable to be combined, the evidence may be synthesized through meta-analysis. If less than 3 studies are available on the same outcome, a narrative qualitative synthesis will be performed; with 3 or more studies, descriptive forest plots will be used to compare the results across studies and in relation to key characteristics. The heterogeneity of the effect size across studies will be tested by the Q statistic and quantified by estimating the I^2 statistic. Sub-group analyses will be carried out to explore potential sources of heterogeneity (methodological and contextual), where appropriate. Sensitivity analyses will be conducted to examine the influence of specific assumptions on the overall effect size. Publication bias will be assessed (e.g. by visual inspection of the funnel and by performing the Egger's test for funnel plot asymmetry). Results from existing meta-analyses may be used if their eligibility criteria are comparable to those established in this protocol and no additional eligible studies are identified through the literature search.

4.5. Characterisation of dose-responses, including data modelling

Characterisation of the dose-response consists in providing qualitative and quantitative descriptions of the levels and duration of the intake causing the adverse effect. This includes a description of:

- the nature and size of the populations studied;
- the magnitude, frequency, and duration of intake;
- relevant information on the diet history of the subjects and/or the vitamin A status;
- the methods for measuring the intake and endpoint;
- where available, data on the total intake of the nutrient substance (i.e. not restricted to the 'test' or 'supplemental' doses).

Data modelling should be used for characterising the dose-response, where possible. Dose–response meta-analyses can be valuable in describing the shape of the relationship (e.g. linear or non-linear; monotonic or not) and for the quantification of any relationship between the nutrient intake and the occurrence/level of the endpoint of interest. Mechanistic data can help interpreting the biological plausibility of the dose-response shape. The possibility of modelling the dose–response will depend on the nature and extent of available data. An array of modelling approaches may be used and/or adapted to address the relevant assessment questions. The choice of the approach requires technical support and expertise, and should consider methodological developments in the field.

Methodological and contextual sources of heterogeneity should be discussed. Where feasible, heterogeneity should be quantified and formally evaluated (e.g. through subgroup analyses or multivariable meta-regression).

Factors which could contribute to inter-individual differences in responses to 'high' vitamin A intake should be addressed and characterised, where possible.

4.6. Uncertainty analysis

Uncertainty in the body of evidence will be described, considering the following factors: risk of bias, unexplained inconsistency, indirectness, imprecision, publication bias, magnitude of the effect, dose-response, residual confounding, consistency (across animal models or species; across dissimilar populations; across study design types) (OHAT/NTP, 2019).

5. Methods for answering sub-questions addressed through narrative reviews

Information will be gathered through a narrative review of the literature. Recent textbooks, authoritative reviews and research papers retrieved through searches in bibliographic databases, and selected on the basis of their relevance, will be used as sources of information.

Appendix A. Overview of risk assessments and health-based guidance values for vitamin A

Author	Objective of the risk assessment	Indicators/ health outcomes reviewed	Reference for the HBGV used	Critical endpoint and reference point selected to derive HBGV	HBGVs (µg/day)
Preformed vitamin A					
IOM (2001)	UL for retinol	**Human data**: bone mineral density, teratogenicity, and liver abnormalities; intracranial pressure (bulging fontanel) and skeletal abnormalities in infants	**Women of childbearing age**: Rothman et al., 1995 **Other adults:** Minuk et al., 1988 Zafrani et al., 1984 **Infants**: Persson et al., 1965	**Women of childbearing age: teratogenicity** NOAEL = 4,500 µg/day UF = 1.5 selected on the basis of inter-individual variability in susceptibility; because there are substantial data showing no adverse effects at doses up to 3,000 µg/day of vitamin A supplements, a higher UF was not justified. **Other adults: liver abnormalities** LOAEL = 14,000 µg/day UF = 5.0 selected to account for the severe, irreversible nature of the adverse effect, extrapolation from a LOAEL to a NOAEL, and interindividual variation in sensitivity **Infants: bulging fontanel** LOAEL = 6,000 µg/day UF = 10 selected to account for the uncertainty of extrapolating a LOAEL to a NOAEL for a non-severe and reversible effect (i.e., bulging fontanel) and the interindividual variability in sensitivity. **Children:** Given the dearth of information and the need for conservativism, the UL values for children and adolescents are extrapolated from those established for adults, adjusted for children and adolescents on the basis of relative body weight. **Special Considerations**	**UL for women of childbearing age** $UL_{14\text{-}18yr}$ = 2,800[(a)] $UL_{19\text{-}50yr}$ = 3,000[(a)] **UL for men** UL_{19+yr} = 3,000 **UL for women** UL_{51+yr} = 3,000 **UL infants and children** $UL_{0\text{-}12mo}$ = 600 $UL_{1\text{-}3yr}$ = 600 $UL_{4\text{-}8yr}$ = 900 $UL_{9\text{-}13yr}$ = 1,700 $UL_{14\text{-}18yr}$ = 2,800 (a) including pregnant and lactating women

Author	Objective of the risk assessment	Indicators/ health outcomes reviewed	Reference for the HBGV used	Critical endpoint and reference point selected to derive HBGV	HBGVs (µg/day)
				Individuals with high alcohol intake, pre-existing liver disease, hyperlipidemia, or severe protein malnutrition may be distinctly susceptible to the adverse effects of excess preformed vitamin A intake (Ellis et al., 1986; Hathcock et al., 1990; Leo and Lieber, 1999). These individuals may not be protected by the UL for vitamin A for the general population.	
SCF (2002)	UL for retinol and retinyl esters	**Human data:** Hepatoxicity, teratogenicity, bulging fontanelle in infants/Intracranial hypertension, bone density/fracture, lipid metabolism	**Adults:** Rothman et al, 1995; Mastroiacovo et al., 1999;	**Hepatoxicity** **Teratogenicity** NOAEL = 3,000 µg/day UF = An uncertainty factor is not considered necessary, because the data from other studies indicated that the true threshold for an effect could be higher than this value *Although teratogenicity is only relevant to women of child-bearing age, the UL is appropriate for men, and for infants and children after correction for differences in metabolic rate (using scaling according to body surface area (body weight$^{0.75}$)), because it is 2.5-fold lower than the lowest daily intake that has been associated with hepatotoxicity during chronic intake. This UL does not apply to postmenopausal women, who represent the group at greatest risk of bone fracture, because it may not provide an adequate margin of safety in relation to the possible decrease in bone density and the risk of bone fracture.*	µg RE/day $UL_{1\text{-}3yr}$ = 800 $UL_{4\text{-}6yr}$ = 1,100 $UL_{7\text{-}10yr}$ = 1,500 $UL_{11\text{-}14yr}$ = 2,000 $UL_{15\text{-}17yr}$ = 2,600 UL_{18+yr} = 3,000* * Women of child-bearing age (including pregnant and lactating women) and men Safe level of intake for post-menapausal women = 1,500
EVM (2003)	UL for Vitamin A (retinol)	**Human data:** developmental toxicity, general toxicity, bone toxicity (bone fracture)	**Adults:** Martinez-Frias and Salvador, 1990; **Rothman et al., 1995;** Werler et al., 1990; Khoury et al., 1996; Shaw	**Bone fracture** Intakes above 1,500 µg/day may increase the risk of bone fracture in adults	Not possible to establish a Safe Upper Level for vitamin A.

Author	Objective of the risk assessment	Indicators/ health outcomes reviewed	Reference for the HBGV used	Critical endpoint and reference point selected to derive HBGV	HBGVs (µg/day)
		Animal data: developmental toxicity, bone toxicity,	et al., 1996; Mills et al., 1997; Mastroiacovo et al., 1999; Wald et al., 1985; Hathcock et al., 1990; Freudenheim et al., 1986; Sowers and Wallace, 1990; Houtkooper et al., 1995; **Melhus et al., 1998**; Ballew et al., 2001; Feskanich et al., 2002; Promislow et al., 2002		**Guidance Level** for retinol intake of 1,500 µg/day for adults. Insufficient data to set a Guidance Level for children
NNR (2014)	UL for retinol and retinyl esters	**Human data:** Vitamin D antagonism, hypervitaminosis A, teratogenicity, osteoporosis, bone fractures	**Adults**Michaelsson et al., 2003, Caire-Juvera et al., 2009	**Hepatoxicity** **LOAEL =** 7,500 µg RE/d A UL that is 2.5 times below the level which may cause hepatotoxicity was set. **Osteoporosis**	3,000 µg/day of retinol supplements for whole-population and women of childbearing age without further age definition 1,500 µg/day of retinol supplements for postmenopausal women
SACN (2005)	Review evidence on retinol and bone health	**Human and animal data:** Bone health	**Adults:** Sowers and Wallace, 1990; Melhus et al., 1998; Feskanich et al., 2002; Michaelsson et al., 2003; Lim et al., 2004; Ballew et al., 2001; Sigurdsson et al., 2001; Freudenheim et al., 1986; Houtkooper et al., 1995; Promislow et al., 2002; Kawara et al., 2002;	**Bone health** Insufficient evidence on the association between bone health and retinol intakes above 1500 µg/day to justify a change in dietary advice to all consumers regarding consumption of foods or supplements containing retinol	Maintains the Guidance level set by EVM (2003)
BfR (2021)	Maximum amounts for preformed			**Considered the DRVs:** SCF, 2002 D-A-CH, 2020	As the safety margin between the UL and 95th percentile of intake and the

Author	Objective of the risk assessment	Indicators/ health outcomes reviewed	Reference for the HBGV used	Critical endpoint and reference point selected to derive HBGV	HBGVs (µg/day)
	vitamin A for fortification of foods and supplements			EFSA, 2015	DRI is very small, two options were proposed for food supplements: Option 1: no addition to food supplements Option 2: Addition of up to 0.4 milligrams (mg) retinol equivalents2 (RE) per day. This corresponds to a maximum of 0.2 mg RE/d for a food supplement after applying UF of 2 (for possible uses of multiple vitamin A-containing food supplements + other uncertainties) Vitamin A should not be used to fortify conventional foods, except for margarine or mixed fat products where a maximum of 1 mg RE/100 g of food is recommended
			β-carotene		
SCF (2000b)	UL for β-carotene	**Human data:** CVD, cancer **Animal data:** acute toxicity, chronic toxicity, carcinogenicity, teratogenicity, reproductive toxicity	**Adults:** Greenberg et al, 1990; Blot et al, 1993; ATBC Study Group, 1994; Greenberg et al, 1994; Hennekens et al, 1996; Omenn et al, 1996a	No dose-response relationship for ß-carotene effects is available from the intervention trials in humans or from animal models Evidence from human trials (ATBC and CARET) indicates that supplemental ß-carotene (20 mg/day or more) is contraindicated for use in current, heavy smokers	Insufficient scientific basis to set a precise figure for a UL as no dose-response relationship for β-carotene effects was available
EFSA ANS Panel (2012)	Safety assessment of β-carotene for	**Human data**: incidence of cancer, including lung cancer	**Adults:** Mayne, 1996; Ziegler et al., 1996; ATBC Study group, 1994; Omenn	**Cancer risk**	The Panel concluded that exposure to β-carotene from its use as food

Author	Objective of the risk assessment	Indicators/ health outcomes reviewed	Reference for the HBGV used	Critical endpoint and reference point selected to derive HBGV	HBGVs (µg/day)
	use as food additive and in food supplements		et al., 1996a; 1996b; Omenn, 1998; Druesne-Pecollo et al., 2010.	Increased risk of lung cancer observed in heavy smokers with long-term supplementation of 20mg/d [ATBC study]	additive and as food supplement at a level **below 15 mg/day** do not give rise to concerns about adverse health effects in the **general population, including heavy smokers.**
EVM (2003)	UL for supplemental β-carotene	**Human data:** lung tumours	**Adults:** The α-Tocopherol and β-Carotene Cancer Prevention Study Group (ATBC), 1994.	**LOAEL from ATBC study:** 20 mg/day. **Uncertainty factor:** 3 (for LOAEL to NOAEL extrapolation). **Special considerations:** Epidemiological studies have shown an association between supplementation with ß-carotene and an increase in lung cancers in smokers and in individuals who have been heavily exposed to asbestos. **The Safe Upper Level applies only to the general population, i.e. non-smokers** and those not exposed to asbestos. There is no evidence that ß-carotene supplementation has any effect on non-smokers. However, until the mechanism for the promotion of lung tumours is established it remains uncertain whether other co-exposures could have the same effect as observed in smokers.	**UL for adults** 7 mg/day supplemental ß-carotene This Safe Upper Level applies to supplements only, as there is no evidence to suggest that current levels of ß-carotene intake from food results in adverse effects. Recommends that smokers should not use ß-carotene supplements
VKM (2015)	Tentative UL for supplemental β-carotene	**Human data:** lung cancer, all-cause mortality	**Adults:** ATBC, 1994	**LOAEL:** 20 mg/day. **Safety factor**: 5	**Tentative UL for adult** 4 mg/day Discourage the use of supplements for smokers and people with chronic inflammatory conditions in

Author	Objective of the risk assessment	Indicators/ health outcomes reviewed	Reference for the HBGV used	Critical endpoint and reference point selected to derive HBGV	HBGVs (µg/day)
					the lungs (such groups include asthmatics and COPD-patients)
IOM (2000)	UL for β-carotene and/or other carotenoids	**Human data:** Carotenoderma, lung cancer, lycopenodermia	-	-	Due to inconsistent data on adverse effects of β-carotene, a UL could not be established Supplements ≥30 mg/day of β-carotene may be associated with carotenoderma, but this effect is more cosmetic than adverse and is harmless and readily reversible. β-carotene supplements are not advisable, other than as a provitamin A source and for the prevention and control of vitamin A deficiency in at-risk populations.
BfR (2021)	Maximum amounts for β-carotene for fortification of foods and supplements			Based on the daily intake value of below 15 mg proposed by EFSA (2012) for supplemental intake of β-carotene. Taking into account residual amount for food supplements and fortification of conventional foods. Residual amount = 15 – 1.5 = 13.5/2 = 6.75 mg/d UF = 2, for use of multiple β-carotene-containing food supplements (which cannot be excluded), among other scientific uncertainties	Maximum level for food supplements (per daily recommended dose of an individual product) = 3.5 mg Options for fortification:

Appendix B. PI/ECOTSS statements for the systematic reviews

3 a) What is the dose-response relationship between 'high' vitamin A intake[(a)] and teratogenicity?

POPULATION	INTERVENTION/EXPOSURE	COMPARATOR(s)	OUTCOME	TIMING	SETTING	STUDY DESIGN
Pregnant women	Quantitative estimates, from diet and/or supplements of 1) Preformed vitamin A intake (retinol and retinyl esters) 2) Preformed vitamin A (retinol, retinyl esters) and β-carotene intake Intake of retinoic acid and synthetic retinoids are excluded	Lower intakes of 1) and 2) Placebo/no supplement	Teratogenetic outcomes in offspring • Congenital malformations including craniofacial, cardiac, thymic, and central nervous system abnormalities	No restriction	No restriction	Primary research studies, no restriction. Published ≥2001. English language. Not systematic reviews and meta-analysis (except for references).

[(a)] Preformed vitamin A and provitamin A β-carotene

4 a) What is the dose-response relationship between 'high' vitamin A intake[(a)] and hepatotoxicity?

POPULATION	INTERVENTION/EXPOSURE	COMPARATOR(s)	OUTCOME	TIMING	SETTING	STUDY DESIGN
Humans: no restriction	1) Oral Vitamin A supplementation as preformed vitamin A (retinol and retinyl esters) with or without β-carotene ➢ Dosing at least once a week for at least 3 months 2) Oral Vitamin A supplementation as preformed vitamin A (retinol and retinyl esters) with or without β-carotene plus co-intervention while controlling for the co-intervention ➢ Dosing at least once a week for at least 3 months	Placebo/no supplement/lower doses	**Hepatotoxicity**, assessed by: • Valid markers of liver damage or function, including liver enzymes in serum or plasma (ALAT, ASAT, LD), markers of liver excretory function/cholestasis (ALP, GT, bilirubin), markers of liver synthesis function (albumin, coagulation (INR), prealbumin, transferrin/TIBC, HDL-C, apoA-I, and others). • Transient elastography (fibroscan) • Histopathological signs of hepatotoxicity assessed by liver biopsy	Dosing at least once a week for at least 3 months	No restriction	Randomized controlled trials. Published ≥2001. English language. Not systematic reviews and meta-analysis (except for references).

	Fasting (≥ 6 hours) serum retinyl ester concentration as biomarker of exposure will be evaluated in eligible studies		• Clinically diagnosed liver cirrhosis • Clinically diagnosed portal hypertension, with or without cirrhosis			

(a) Preformed vitamin A and provitamin A β-carotene

5 a+b) Does 'high' vitamin A(a) intake increase the risk of bone fractures or bone mineral density in humans? If so, could a dose-response be characterised?

POPULATION	INTERVENTION/EXPOSURE	COMPARATOR(s)	OUTCOME	TIMING	SETTING	STUDY DESIGN
Humans Excluded: Individuals with - vitamin A deficiency receiving therapeutical doses of preformed vitamin A - medical therapy with topical synthetic retinols, - primary hyperparathyroidism or other disorders affecting bone health	*For interventional studies:* 1) Oral Vitamin A supplementation as preformed vitamin A (retinol and retinyl esters) with or without β-carotene ➢ Dosing at least once a week for at least 2 months 2) Oral Vitamin A supplementation as preformed vitamin A (retinol and retinyl esters) with or without β-carotene plus co-intervention while controlling for the co-intervention ➢ Dosing at least once a week for at least 12 months Fasting (≥ 6 hours) serum retinyl ester concentration as biomarker of exposure will be evaluated in eligible studies Note: Studies on parenteral supplementation or giving	Placebo/no supplementation or lower doses/lower levels of intake	Bone fractures diagnosed by a physician (any site) or self-reported Measures of bone mineral density (BMD), bone mineral content (BMC) and measures of bone strength assessed by DXA or HC-pCT (total body, all sites)	³12 months follow-up	No restriction	1) RCTs and non-randomised comparative studies of interventions 2) Prospective (cohort, case-cohort, and nested case-control) studies Published ≥2001. English language. Not systematic reviews and meta-analysis (except for references).

	vitamin A less frequently than weekly (e.g. monthly, bolus) will be kept as supporting evidence (narrative review) but excluded from the primary assessment to draw conclusions on the UL *For prospective studies:* 1) Oral Vitamin A supplementation as preformed vitamin A (retinol and retinyl esters) with or without β-carotene 2) Serum retinyl ester concentration as biomarker of exposure					

(a) Preformed vitamin A and provitamin A β-carotene

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