ANNEX 1

The Joint Health Claims Initiative

The JHCI is a unique collaboration between the food industry, consumer groups and enforcement authorities to establish a self-regulatory approach to the use of health claims on foods in the light of growing interest in the links between diet and health. The JHCI Code of Practice was launched in December 2000 in the absence of specific EU legislation for health claims and was developed to:

- define a health claim;
- outline the legal framework within which a claim can be made;
- set criteria and general nutrition principles for making a claim;
- identify the ways in which new and existing claims must be scientifically substantiated; and
- set out requirements for labelling and consumer information about the health benefits of a product.

JHCI Expert Committee Members
(The "independent experts" for the purposes of this project)

Carol Stevens, Chairman   (Worcester Scientific Services)
Dr Judy Buttriss   (British Nutrition Foundation, London)
Dr Susan Jebb   (MRC Human Nutrition Research, Cambridge)
Prof Michael Lean   (Queen Elizabeth University, Glasgow)
Prof Tom Sanders   (Kings College London)
Prof Sean Strain   (University of Ulster at Coleraine, Northern Ireland)
Prof Martin Wiseman   (Independent Nutrition Consultant, London)

The Terms of Reference of the Expert Committee are as follows:

'The Expert Committee exists to underpin the Joint Health Claims Initiative by providing an objective and credible expert opinion on the scientific validity of a health claim under the JHCI Code.'

JHCI Council Members
(The "second independent party" for the purposes of this project)

Member: Representative of:
Roger Manley, OBE   (JHCI Chairman, ex-Chief Trading Standards Officer)
Sheila Kelly   (Proprietary Association of Great Britain)
Mike Buchanan   (British Retail Consortium)
Valerie Saint   (Food and Drink Federation)
Mike O'Neill   (National Consumer Council)
Dr Mike Rayner   (Sustain, The National Alliance for Better Food and Farming)
Kate Lees   (British Dietetic Association)

David Walker   (Local Authorities Coordinators of Regulatory Services)
Kevin Wardle   (Local Authorities Coordinators of Regulatory Services)
David Pickering   (Local Authorities Coordinators of Regulatory Services)

The Council is the primary policy and control forum for the Joint Health Claims Initiative and, together with the Executive Director, it directs and manages the affairs of the Joint Health Claims Initiative.

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ANNEX 2

Evaluation report of a pilot study

The following evaluation report of a pilot study was a working document in the early stages of this project. References to JHCI documents (denoted (JHCI/xx/xx) are not included in this report but are publicly available on request to the JHCI Secretariat.

EVALUATION REPORT

of a pilot study to test the JHCI process for identifying well-established health statements.

Prepared by
JHCI Executive Director

13th March 2003

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(Numbers in parenthesis relate to the actual page number in the main report)

INTRODUCTION

The Joint Health Claims Initiative has been sponsored by the UK Food Standards Agency to develop:

> i)   A process that defines and identifies well-established health statements
>
> ii)   A list of well-established nutrient-function statements for the nutrients listed in the Annex 1 to the Food Supplements Directive (2002/46/EC).

A proposed process to identify and define well-established health claims was originally presented in paper JHCI/108d/02. Since this draft was prepared it has been agreed that the process will identify and define well-established health 'statements', rather than claims. The rationale for this change is presented in the main body of this document, along with other recommended amendments to the process.

JHCI will use the draft final process to produce an initial list of well-established nutrient function statements, however it is intended that the process itself is also applicable to enhanced function health statements and disease risk reduction health statements for food. This could also provide the European Commission with a useful mechanism for identifying well-established claims currently on the market that could be added to its Register of generally accepted health claims.

A pilot study using vitamin C was undertaken by the JHCI Secretariat to ensure that the proposed process was workable and to identify if any further changes were necessary. Members of the JHCI Council and Expert Committee have considered the results of the pilot study (refer JHCI/11/03, draft 2). Their recommendations have been reported in the following paper and will be incorporated in the draft Final Process that will be used to produce the list of well-established nutrient-function statements, described in (ii) above.

Therefore the purpose of this paper is to report the:

1) Proposed process to identify and define well-established health statements

2) Pilot study of the proposed process to identify well-established nutrient function statements for vitamin C

3) Refinements to the proposed process as recommended by the JHCI Expert Committee and Council.

THE PROPOSED PROCESS

The proposed process, as set out in JHCI/108d/02, is reported below and is split into two stages:

Stage 1 - The proposed process for approving well-established health claims
Stage 2 - Using the proposed process to identify well-established nutrient function claims for vitamin C.

STAGE 1:

Proposed process for approving well-established health claims

Step 1.   Clearly define 'well-established'.   Well-established implies 'agreed by authoritative sources'. For the purpose of this project and to provide a transparent process there needs to be a clear definition of well-established.

Step 2.   Determine priority order for groups of nutrients, food components or claims to be considered.

Step 3.   Establish working definitions as necessary, such as types of health claims to be considered.

Step 4.   Agree credible source documents to draw up a list of possible functions for nutrients and food components and to identify which of the possible functions are 'well established'.

Step 5.   Develop phraseology as necessary to provide a model for acceptable wording for claims.

Step 6.   Draw up comprehensive list of functional claims. This step will involve consideration of claims in relation to current legislation and consumer perception principles.

STAGE 2:

Using the proposed process to produce a list of well-established nutrient function claims

1.   JHCI agreed definition of 'well-established'

Functions will be considered to be 'well-established' when the source documents are consistent in their reporting of the relevant functions.

2. Priority order for consideration of nutrients

The FSA and JHCI have agreed that the nutrients in the Food Supplements Directive (2002/46/EC) will be considered to produce an initial list of claims (and that vitamin C would be used to test the process).

3. Other definitions

To test the process it has been agreed that JHCI will consider nutrient-function claims at this stage. For these purpose the Codex definition of 'nutrient function claim' will be adopted:
'...a claim that describes the physiological role of the nutrient in growth, development and normal functions of the body'. (Codex Guidelines for the Use of Nutrition Claims 1997).

4. Source documents

The JHCI Expert Committee has suggested that information provided by the USA's Institute of Medicine (and published on the National Academy of Science/IOM website) should be used as a starting point for drawing up a list of possible functions, as this information is based on systematic reviews of evidence and is internationally recognised. This list will be cross-checked with similar material from the UK and Europe, both to demonstrate consistency in the functions reported (ie, to confirm that the information is well-established) and to anglicise the list for the UK population, as follows:

- Report of the COMA Committee on Dietary Reference Values (1991)
- Draft Report of the Expert Group on Vitamins and Minerals on Safe Upper Levels for Vitamins and Minerals
- Report of the Scientific Committee for Food (Thirty-first series) on 'Nutrient and energy intakes for the European Community' (1992).
- 'Introduction to Human Nutrition'. Edited on behalf of The Nutrition Society by Michael J. Gibney, Hester H. Vorster and Frans J. Kok. Blackwell Science, September 2002.
- The Merck Index

5. Proposed phraseology

It is proposed that, for the purpose of developing a list of well-established nutrient function claims, the claims should be phrased in the following way:

x is essential for / required for / helps in the normal development of y
x is essential for / required for / helps in the normal growth of y
x is essential for / required for / helps in the normal function of y
x is essential for / required for / helps in z

Where:
- **x** is a nutrient where a nutrient is defined as energy, protein, carbohydrate, fat (or sub-fractions thereof); fibre, vitamins and minerals (adapted from the 1990 Nutrition Labelling Directive)

- **y** is the whole body, a bodily system (the cardiovascular system), organ (e.g. the heart,), a tissue (e.g. the blood), or a component of a tissue (e.g. red blood cells)
- **z** is a normal function of the body (e.g. the metabolism) or a specific function (e.g. oxidative processes).

When appearing in food labelling then the wording of the well-established nutrient function claim may be altered, as long as the spirit of the claim remains.

The JHCI Council agreed, at its meeting on 13th December, that the distinctions between 'essential for / required for / helps in the normal…' may not be helpful, meaningful or easily understood by consumers. The wording of the claims will be considered during the pilot to determine whether JHCI should recommend to the FSA that consumer research be undertaken.

6.   Draw up comprehensive list claims

6a. *Pilot the process*. The JHCI Council has agreed that vitamin A, vitamin C and a mineral should be used to test that the process works. A report of the pilot will be prepared for consideration by the Expert Committee and Council (and available to the FSA) before the process is approved for use to develop the final list. Refinements to the process will be made if necessary during the pilot process. At this stage, it is envisaged that addition of claims to the list will occur via the following process:

i.   Undertake and audit of functional claims, for the relevant nutrient, currently on the market in major supermarkets and health food shops

ii.   Prepare a brief monograph for each nutrient, presenting functions cited in the source documents and including claims currently on the market as an Annex

iii.   Submit each monograph to the Expert Committee for it to determine whether the functions are well-established

iv.   Generate a 'model claim' using the proposed phraseology as a guide and after consideration of how the claim could be presented on food products

v.   Consult with nutrition scientists with expertise in consumer perception to help ensure that the model claims are likely to be understand and meaningful to consumers.

vi.   Add to final list.

6b. *The final list.* This will be limited to well-established nutrient-function claims for dietary and synthetic forms of nutrients listed in the Annex 1 of the Food Supplements Directive 2002. Members of the JHCI Council, who's expertise includes food law, enforcement and consumer perception of claims, will agree the list before the final version is submitted to the Food Standards Agency.

PILOT STUDY & RECOMMENDATIONS

1. Selection of source documents:

A detailed review of the source documents recommended in Stage 2, (4) above, found that, apart from the Institute of Medicine's Dietary Reference Intakes publication, the documents largely reported information in relation to the determination of safe upper limits, or dietary reference values, rather than a detailed account of the functions of the vitamin. The JHCI Expert Committee therefore recommended the following pool of alternative source documents:

- Reports of the Expert Group on Vitamins and Minerals (EVM)
- British Nutrition Foundation Task Force reports
- CRC Press handbooks
- Report of the Scientific Committee for Food (Thirty-first series) on 'Nutrient and energy intakes for the European Community' (1992)
- International Life Sciences Institute documents
- Other papers relevant to each nutrient as identified by the JHCI Expert Committee

It was agreed by the Expert Committee that the following source documents would be used for the review of vitamin C, whereby functions that were reported in Reference 1 would need to be supported by either Reference 2 or Reference 3 in order to meet the requirement of 'well-established':

Reference 1:
Institute of Medicine Dietary Reference Intakes for Vitamin C, E, selenium and carotenoids. Washington D.C. National Academy Press, 2000.

   AND

Reference 2 :
Review of Vitamin C. Expert Group on Vitamins and Minerals. November 1999.

   AND/OR

Reference 3:
Report of the Scientific Committee for Food (Thirty-first series) on 'Nutrient and energy intakes for the European Community' (1992).

Recommendation:

A tailored mix of source documents will be used to support functions reported in IOM documents, whereby the Expert Committee will provide advice on the most appropriate sources for specific nutrients.

2. Selection of quotes from source documents

The quotes reported for vitamin C were selected to present the broadest range of functions for the nutrient. To minimise repetition, comments that were reported more than once within each source document were quoted only once. Reference 1 provided the most quotes as this document reviewed the functions of vitamin C in more detail than Reference 2 or 3. Quotes about functions were categorised according to the body organ, component or physiological process to which they were linked, with the view to formulating a health statement in accordance with the proposed phraseology.

3. Comparison with information currently available to consumers

An audit of functional claims for vitamin C currently on the market was undertaken to provide a comparison of functions reported in the source documents with actual claims about vitamin C made on foods and dietary supplements. Also, information for consumers about common nutrients published on the UK Food Standards Agency website was also reported.

Recommendation:

Although this information provides and interesting comparison, an audit of this nature should be undertaken for all health claims on the market, not just nutrient function claims. Therefore an audit of nutrient function claims should be removed from Stage 2, 6a (i) of the process.

4. Developing health claims for 'well-established nutrient-functions'

A summary of the well-established functions for vitamin C were presented in 'Table 1: Summary of well-established nutrient functions for Vitamin C'. A small section of this table is presented below:

	Essential for	Required for	Helps in	Normal synthesis	Normal development	Normal growth	Normal function	Normal process
1. Collagen	✔			✔				
4. Gums	✔						✔	
9. Iron absorption			✔					✔
10. Antioxidant properties			✔					✔

Model claims were then formulated in accordance with the draft phraseology for nutrient-function claims set out in Stage 2, 5, above. For example:

1. *"Vitamin C is essential for the normal synthesis of collagen"*
4. *"Vitamin C is essential for the normal function of gums"*

9. "Vitamin C helps the body to absorb iron from food"
10."Vitamin C (a powerful antioxidant) helps to protect cells from the damage
* caused by free radicals"*

The JHCI Expert Committee's comments in relation to the overall process have been presented in this paper, rather than the specific details about the functions of vitamin C, which will be reported on separately.

Recommendations:

(i)   **The remit of the project is to identify well-established health 'statements' rather than 'claims', given that the project will not involve the assessment of health claims on food products. Once the list of well-established nutrient function statements has been published, companies applying these statements to food products, as health claims, should comply with the JHCI Code of Practice for Health Claims for Food (JHCI, 2000)**

(ii)   **The proposed phraseology, presented in section 5 above, should be replaced with:**

   'Nutrient (x) is necessary for/contributes to the normal development/function/process of (y)'

   Whereby:

   - ***'Necessary for'*** **was selected to prevent confusion over the meaning of 'essential', given that it has a specific definition with regards to particular dietary components.**
   - ***'Contributes to'*** **was selected to replace both 'required for' and 'helps in' given that the distinction is small between these two terms.**
   - ***'Normal development'*** **was retained to replace both 'normal synthesis' and 'normal growth' given that 'development' encompasses development, growth, regeneration and maintenance of tissues and structures.**
   - **The working definition of 'normal' will need to be confirmed under Stage 3 of the process when each nutrient is assessed.**

(iii) **The table of summarised results for each nutrient should be modified to reflect the amended phraseology, as follows:**

	Necessary for	Contributes to	Normal development	Normal function	Normal process
1. Collagen	✔		✔		
4. Gums	✔			✔	
9. Iron absorption		✔			✔
10. Antioxidant properties		✔			✔

    (iii)   **The scientific accuracy of statements must be maintained if technical jargon is simplified to aid consumer understanding.**

    (iv)   **British/European terminology should replace American terms whenever possible.**

5. Dosage and applicability of statements to food products

The vitamin C pilot considered whether JHCI should specify a minimum and maximum dosage for products to carry nutrient-function statements. The statements are about normal nutrient functions, not enhanced nutrient functions, therefore the level of the nutrient required to support the function will usually reflect quantities obtainable from natural food sources and a healthy balanced diet.

Recommendations:

    (i)   **Products must comply with the legally defined minimum nutrient requirements, as set out in the Food Labelling Regulations (refer section 5.1.1 JHCI Code).**

    (ii)   **The remit of this project does not include quantification of the nutrient function statements, or to recommend a nutrient content range prerequisite in order for products to carry the statements. The Expert Group on Vitamins and Minerals is currently establishing Safe Upper Limits for all nutrients in the Annex 1 to the Food Supplements Directive (2002/46/EC), which the JHCI will make reference to once the EVM has competed its work.**

    (iii)   **Labelling should not suggest to consumers that pharmacological doses of the nutrient are required to support normal functions, or that consuming high doses will deliver an enhanced function. Products that contain the nutrient in amounts significantly higher than the UK Reference Nutrient Intake for adults should not suggest to consumers that the beneficial effect cannot be obtained from a healthy diet and natural food sources (refer sections 6.2.6 & 6.2.7 JHCI Code of Practice).**

(iv) **All products carrying the health statements should be made in accordance with the general principles in the JHCI Code of Practice for Health Claims for Food (2000).**

6. Enhanced nutrient functions

The pilot considered whether it would be useful to include information about the nutrient's relationship to enhanced functions and reduced risk of disease.

Recommendation:

The process has been designed to assess any type of claim, in terms of whether or not it is defined as 'well-established', however, only 'normal' functions or processes in the body will be considered during the development of a list of well-established nutrient function statements.

SUMMARY OF RECOMMENDATIONS

- The project will relate to health 'statements' rather than health 'claims'
- A tailored mix of source documents for each nutrient will be reviewed to determine whether statements are well-established
- An audit of nutrient-function claims currently on the market will not be undertaken within this project
- The proposed phraseology has been amended significantly
- The JHCI will not develop maximum upper limits for products to carry the statements or quantify the nutrient function statements (although JHCI is likely to recommend that the latter is carried out at some future date by an authoritative body)
- Enhanced functions and disease risk reduction statements will not be considered during the development of the list of well-established nutrient function statements.

THE DRAFT FINAL PROCESS

Following the pilot study, the recommendations reported in this paper have been incorporated in to the draft final process, presented in paper JHCI/19/01,which will be used to proceed with assessing the nutrients in the Annex 1 to the Food Supplements Directive (2002/46/EC). As other nutrients are assessed, it may become apparent that further refinements to the process are required before it is finalised and submitted to the Food Standards Agency at the completion of the project in late June 2003.

ANNEX 3

Nomenclature of nutrients

1. Nutrients listed in Annex 1 of the Food Supplements Directive (2002/46/EC)

Vitamin A (beta-carotene)   Calcium
Vitamin D   Magnesium
Vitamin E   Iron
Vitamin K   Copper
Thiamin (B_1)   Iodine
Riboflavin (B_2)   Zinc
Niacin   Manganese
Pantothenic Acid   Sodium
Vitamin B_6 Potassium
Folate (folic acid, folacin)   Selenium
Vitamin B_{12} Chromium
Biotin   Molybdenum
Vitamin C   Fluoride
                               Chloride
                               Phosphorus

2. Nomenclature as listed in 'Directions for Contributors', British Journal of Nutrition':

<u>Nomenclature of Vitamins:</u> Most of the names for vitamins and relate compounds that are accepted by the Editors are those recommended by the IUNS Committee on Nomenclature (see *Nutrition Abstracts and Reviews* A (1978) **48**, 831-835).

Acceptable Name	*Other names**
Vitamin A	
Retinol	Vitamin A_1
Retinaldehyde, retinal	Retinene
Retinoic acid (all-*trans* or 13-*cis*)	Vitamin A_1 acid
3-Dehydroretinol	Vitamin A_2
Vitamin D	
Ergocalciferol, ercalciol	Vitamin D_2 califerol
Cholecalciferol, calciol	Vitamin D_3
Vitamin E	
α-, β- and γ-tocopherols plus tocotrienols	
Vitamin K	
Phylloquinone	Vitamin K_1
Menaquinone-n (MK-n)+	Vitamin K^2
Menadione	Vitamin K_3
	Menaquinone
	Menaphthone

Acceptable Name	Other names*
Vitamin B$_1$	
Thiamin	Aneurin(e), thiamine
Vitamin B$_2$	
Riboflavin	Vitamin G, riboflavine
	Lactoflavin
Niacin	
Nicotinamide	Vitamin PP
Nicotinic acid	
Folic Acid	
Pteroyl(mon)glutamic acid	Folacin, vitamin B$_c$ or M
Vitamin B$_6$	
Pyridoxine	Pyridoxol
Pyridoxal	
Pyridoxamine	
Vitamin B$_{12}$	
Cyanocobalamin	
Hydroxocobalamin	Vitamin B$_{12a}$ or B$_{12b}$
Aquocobalamin	
Methylcobalamin	
Adenosylcobalamin	
Inositol	
Myoinositol	Meso-inositol
Choline	
Pantothenic acid	
Biotin	Vitamin H
Vitamin C	
Absorbic acid	
Dehydroascorbic acid	

*Including some names which are still in use elsewhere, but are not used by the British Journal of Nutrition.

+ Details of the nomenclature for these and other naturally occurring quinones should follow the Tentative Rules of the IUPAC-IUB Commission on Biochemical Nomenclature (see European Journal of Biochemistry (1975) 53, 15-18).

Generic descriptors

The terms **vitamin A, vitamin C** and **vitamin D** may still be used where appropriate, for example in phrases such as 'vitamin A deficiency', 'vitamin D activity'.

Vitamin E. The term **vitamin E** should be used as the descriptor for all tocol and tocotrienol derivatives exhibiting qualitatively the biological activity of *a*-tocopherol. The term **tocopherols** should be used as the generic descriptor for all methyl tocols. Thus, the term **tocopherol** is not synonymous with the term **vitamin E.**

Vitamin K. The term **vitamin K** should be used as the generic descriptor for 2-methyl-1,4-naphthoquinone (menaphthone) and all derivatives exhibiting qualitatively the biological activity of phylloquinone (phytylmenaquinone).

Niacin. The term **niacin** should be used as the generic descriptor for pyridine 3-carboxylic acid and derivatives exhibiting qualitatively the biological activity of nicotinamide.

Vitamin B_6. The term **vitamin B_6** should be used as the generic descriptor for all 2-methylpyridine derivatives exhibiting qualitatively the biological activity of pyridoxine.

Folate. Due to the wide range of carbon-substituted, unsubstituted, oxidized, reduced and mono- or polyglutamyl side-chain derivatives of pteroylmonoglutamic acid, which exist in nature, it is not possible to provide a complete list. Authors are encouraged to use either the generic name or the correct scientific name(s) of the derivative(s), as appropriate for each circumstance.

Vitamin B_{12}. The term **vitamin B_{12}** should be used as the generic descriptor for all corrinoids exhibiting qualitatively the biological activity of cyanocobalamin. The term **corrinoids** should be used as the generic descriptor for all compounds containing the corrin nucleus and thus chemically related to cyanocobalamin. The term **corrinoid** is not synonymous with the term **vitamin B_{12}**.

Vitamin C. The terms **ascorbic acid** and **dehydroascorbic acid** will normally be taken as referring to the naturally occurring $_L$-forms. If the subject matter includes other optical isomers, authors are encouraged to include the $_L$- or $_D$-prefixes, as appropriate. The same is true for all those vitamins, which can exist in both natural and alternative isomeric forms.

ANNEX 4

Quotes from source documents

Contents:

Annex 5 (page 262) provides a detailed reference list for the source documents.

ANNEX 4.1

Vitamin A

Source documents for reviewing vitamin A

Reference 1.1:
Institute of Medicine Dietary Reference Intakes for vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium and zinc. Washington D.C. National Academy Press, 2001.

Reference 1.2:
Institute of Medicine Dietary Reference Intakes for Vitamin C, Vitamin E, selenium and carotenoids. Washington D.C. National Academy Press, 2000.

Reference 2.0:
Encyclopedia of Human Nutrition 2E. Editor-in-chief, Michele J. Sadler, editors, J.J. Strain, Benjamin Caballero. San Diego : Academic Press, c1999.

Reference 3.1:
Opinion of the Scientific Committee on Food (SCF) on the Tolerable Upper Intake Level of Preformed Vitamin A (retinol and retinyl esters). September 2002. (http://europa.eu.int/comm/food/fs/sc/scf/out145_en.pdf).

Reference 3.2:
Opinion of the Scientific Committee on Food (SCF) on the Tolerable Upper Intake Level of Beta Carotene. October 2000. (http://www.europa.eu.int/comm/food/fs/sc/scf/out80b_en.pdf).

Reference 4.1:
Revised Review of Vitamin A. Expert Group on Vitamins and Minerals. April 2002. (http://www.food.gov.uk/multimedia/pdfs/reviewvita.pdf).

Reference 5.0:
Handbook of Nutrition and Food. Edited by Carolyn D Berdanier...[et al.]. Boca Raton, Fla.: CRC Press, 2002.

1) Eyes and vision

Code | **Proposed statement**
VA1a: | *Vitamin A is necessary for the normal function of the eye*
VA1b: | *Vitamin A is necessary for normal vision*

Reference 1.1:
'Vitamin A is important for normal vision, ….' (pg 82)

'The 11-*cis*-retinaldehyde (retinal) form of vitamin A is required by the eye for the transduction of light into neural signals necessary for vision (Saari, 1994). The retinoic acid form is required to maintain normal differentiation of the cornea and conjunctival membranes, thus preventing xerophthalmia (Sommer and West, 1996), as well as for the photoreceptor rod and cone cells of the retina. Rods contain the visual pigment rhodopsin (opsin protein bound to 11-*cis*-retinal). The absorption of light catalyzes the photoisomerization of rhodopsin's 11-*cis*-retinal to all-*trans*-retinal in thousands of rods, which triggers the signaling to neuronal cells associated with the brain's visual cortex. After photoisomerization, all-*trans*-retinal is released, and for vision to continue, 11-*cis*-retinal must be regenerated. Regeneration of 11-*cis*-retinal requires the reduction of all-*trans*-retinol, thereby providing a local storage pool of retinyl esters. When needed, retinyl esters are hydrolyzed and isomerized to form 11-*cis*-retinal, which is oxidized to 11-*cis*-retinal and transported back to the photoreceptor cells for recombination with opsin to begin another photo cycle.' (pg 84, 85)

'The most specific clinical effect of inadequate vitamin A intake is xerophthalmia. …. The World Health Organisation (WHO, 1982) classified various stages of xerophthalmia to include night blindness (impaired dark adaptation due to slowed regeneration of rhodopsin), conjunctival xerosis, Bitot's spots, corneal xerosis, corneal ulceration, and scarring, all related to vitamin A deficiency. Night blindness is the first ocular symptom to be observed with vitamin A deficiency (Dowling and Gibbons, 1961), and it responds rapidly to treatment with vitamin A (Sommer, 1982).' (pg 95)

'The ability of the retina to adapt to dim light depends upon an adequate supply of vitamin A, because 11-*cis*-retinal is an integral part of the rhodopsin molecule of the rods. Without adequate levels of vitamin A in the retina, the function of the rods in dim light situations becomes compromised, resulting in abnormal dark adaptation (night blindness). Before clinically apparent night blindness occurs, abnormal rod function may be detected by dark adaptation testing. In addition to vitamin A deficiency, zinc deficiency and severe protein deficiency also may affect dark adaptation responses (Bankson et al., 1989; Morrision et al., 1978).' (pg 97)

'Before the clinical onset of xerophthalmia, mild vitamin A deficiency leads to early keratinizing metaplasia and losses of mucin-secreting goblet cells on the bulbar surface of the conjunctiva of the eye.' (pg 105)

Reference 2.0:
'Vitamin A' is the collective term for compounds that show the biological properties of retinol, including maintenance of epithelial tissue and visual function. This

classification includes retinol, retinyl esters and retinal (vitamin A aldehyde); retinoic acid is included even though it does not sustain visual function. These are isoprenoid compounds, having in common an 11-carbon polyene chain attached to a trimethyl-substituted cyclohexenyl ring. The term 'retinoids' refers to all compounds, natural or synthetic, that show some biological activity typical of vitamin A, such as promoting differentiation of cells in culture; not all retinoids can support all the functions of vitamin A, e.g. some are unable to contribute to vision.' (pg 1708)

'The major roles of vitamin A are in vision, differentiation of epithelial tissues, and in the immune system. Metabolism of vitamin A in the retina of the eye is unique, in keeping with the unusual role of vitamin A in that tissue. Vitamin A is stored in the retinal pigment epithelium as retinyl esters. All-*trans*-retinyl esters are simultaneously hydrolysed and isomerized to 11-*cis*-retinol, a compound unique to the eye. 11-*cis*-Retinol is then oxidized to 11-*cis*-retinal. 11-*cis*-Retinal is transferred from the pigment epithelium to the rod cells by interstitial retinoid binding protein (IRBP), a distinct binding protein (140,000 Da). In the rod cells, 11-*cis*-retinal binds …. to the protein opsin to form the visual pigment, rhodopsin. When a photon of light is absorbed by a rhodopsin complex, the 11-*cis*-retinal is isomerized to all-*trans*-retinal and released from the protein complex; the resulting conformation change of the protein initiates a cascade of reactions, resulting in a neural signal to the brain.' (pg 1711)

'In contrast to the high turnover rates of vitamin A in other tissues, vitamin A in the eye is highly conserved, with little leakage back to the liver. Prolonged vitamin A deficiency, however, leads to reduced sensitivity to light, usually first noted as impaired vision at night (night blindness). These effects of vitamin A deficiency are generally reversible by subsequent vitamin A supplementation.' (pg 1711)

'In a very different role, the cornea of the eye depends on vitamin A for proper cell differentiation and for secretion of protective glycoproteins. In vitamin A deficiency, these tissues are susceptible to attack by opportunistic bacteria; such attack may not be reversible and, especially on the corneal surface of the eye, may result in permanent scarring and permanent vision loss. These effects of vitamin A deficiency, unlike those in the retina, may not be reversible by subsequent vitamin A supplementation. Such vitamin A-dependent corneal degeneration (given the general name 'xerophthalmia') accounts for an estimated 500,000 new cases of blindness in preschool children in the world each year.' (pg 1711)

'It has been argued, without conclusive proof as yet, that retinoic acid is the active form of vitamin A required for cellular differentiation. Retinoic acid is an endogenous metabolite of vitamin A. Animals maintained on retinoic acid as sole source of vitamin A seem to grow normally and maintain good health, but become blind (because retinoic acid cannot be converted to retinal); some but not all species also show loss of testicular function. ' (pg 1711)

Reference 3.1:
'Retinal, the initial oxidised metabolite of retinol, is the chromophore of rhodopsin, a visual pigment of the cone cells of the pigmented epithelium of the retina. The photo-induced isomerisation of 11-*cis*-retinal into all-*trans*-retinal is the initial event of the

phototransduction cascade, which ends by the production of a signal to the ocular nerves.' (pg 4)

Reference 4.1:
'Vitamin A is essential to the processes of vision… Lack of vitamin A results in keratinisation of mucus secreting ciliated epithelium and other epithelial changes. The tissues most affected by this include the … cornea ...' (pg 17)

'Vitamin A is required for vision in the dark and for colour perception. The active form of vitamin A in this function is retinal. In the retinal pigment epithelial cells, all-t*rans*-retinol undergoes enzymatic isomerisation to 11-*cis*-retinal by retinol (alcohol) dehydrogenase. The 11-*cis*-retinal forms a Schiff's base with a specific lysyl residue in the membrane bound protein, opsin. The resultant rhodopsin, when exposed to light, isomerises to form a transoid intermediate. Thereafter follow several more protein conformational changes. The intermediate meta-rhodopsin II, interacts with a G protein, transducin, and activates phospodiesterase resulting in the hydrolysis of GTP to GMP via the formation of cGMP. cGMP maintains the opening of sodium channels in the rod outer segment. As the level of cGMP falls, sodium entry decreases and the rod cell- membrane hyperpolarises. Changes in membrane potential re transmitted to and integrated by the brain. Return to the basal state occurs by the reconversion of meta-rhodopsin II to opsin and all-*trans*-retinal. All-*trans*-retinal is then reduced to the corresponding isomer of retinol, and consequently isomerised back to 11-*cis*-retinol.' (pg 18)

'During this cycle, not all vitamin A is conserved and must be replaced from the circulation. A similar sequence of events is involved in the process of colour sensing in cone cells (Basu and Dickerson, 1996 and references therein; Olson, 1996 and references therein).' (pg 18)

Reference 5.0:
'Helps maintain normal vision in dim light – prevents night blindness and xerophthalmia.' (pg 78)

2) Skin and mucous membranes

Code   **Proposed statement**
VA2:   *Vitamin A is necessary for the normal structure and function of the skin and mucous membranes (such as in the lung, intestines, nose, eyes and female reproductive tract).*

Reference 1.1:
'Vitamin A is required for the integrity of epithelial cells throughout the body (Gudas et al., 1994). Retinoic acid, through the activation of retinoic acid (RAR) and retinoid X (RXR) receptors in the nucleus, regulates the expression of various genes that encode for structural proteins (e.g., skin keratins), enzymes (e.g., alcohol dehydrogenase), extracellular matrix proteins (e.g., laminin), and retinol binding proteins and receptors.' (pg 85)

'Because of the role of vitamin A in maintaining the structural integrity of epithelial cells, follicular hyperkeratosis has been observed with inadequate vitamin A intake (Chase et al., 1971; Sauberlich et al., 1974). Men who were made vitamin A deficient under controlled conditions were then supplemented with either retinol or β-carotene, which caused the hyperkeratosis to gradually clear (Sauberlich et al., 1974).' (pg 96)

Reference 2.0:
'The action of retinoids in differentiation is manifest in various systems, including maintenance of epithelial tissue (e.g. the lung, intestines and skin, and the cornea of the eye). In the absence of adequate vitamin A, cells of these tissues do not differentiate normally, but change structure (becoming stratified and cornified) and lose the ability to secrete glycoproteins. The common mechanism underlying these roles of retinoids in diverse tissues seem to involve the binding of retinoic acid (and perhaps retinol) to specific proteins associated with nuclear deoxyribonucleic acid (DNA). These nuclear retinoic acid receptor proteins (RARs), which are distinct from the cytoplasmic 'cellular retinoic acid binding proteins' (CRABPs), can then bind to specific regions of DNA, either promoting or inhibiting transcription of specific genes.' (pg 1711)

Reference 3.1:
'Retinoic acids, both all-*trans*-retinoic acid (TRA) and its 9-*cis* isomer (9CRA) act as regulators of genomic expression……' (pg 4)

'Retinoic acids are able to bind to specific nuclear receptors known as RAR and RXR receptors; RARs can bind either TRA or 9CRA, while RXRs bind only 9CRA. Upon ligand binding these nuclear receptors bind to specific response elements on DNA, and thus regulate gene expression.' (pg 4)

Reference 4.1:
'Vitamin A is essential to the processes of … cellular differentiation… Lack of vitamin A results in keratinisation of mucus secreting ciliated epithelium and other epithelial changes. The tissues most affected by this include the trachea, skin, salivary gland, cornea and testes.' (pg 17)

'Retinoic acid is now recognised as an important signalling molecule that, as a ligand to its nuclear receptors, alters gene expression at the level of transcription. Two sets of retinoic acid nuclear receptors have been identified, known as RAR and RXR, each having α, β and γ subgroups. The RARs and RXRs belong to a family of hormonal regulatory proteins, which include those for steroids, thyroid hormones and vitamin D. Each receptor consists of six domains: an amino-terminal activation domain, a DNA-binding domain, a hinge region, a ligand-binding domain, and a carboxy-terminal involved in heterodimerisation. The RAR receptors bind either all *trans*- or 9-*cis*-retinoic acid, whereas the RXR receptors bind only 9-*cis*-retinoic acid. RXR receptors form dimers with themselves as well as with RAR, the vitamin D receptor, the T3 receptor, PXR, PPAR, βCAR etc. Binding of these dimers to specific response elements usually results in increased gene transcription, although inhibitory interactions do occur (Gerster, 1997 and references therein; Evans, 1996; Basu and Dickerson, 1996 and references therein; Olson, 1996 and references therein).' (pg 18)

Reference 5.0:

'Deficiency symptoms: Rough, dry, scaly skin – a condition known as follicular hyperkeratosis (it looks like "gooseflesh"); increased sinus, sore throat, and abscesses in ears, mouth, or salivery glands; increased diarrhoea ...' (pg 78)

'Vitamin A is needed for the growth and repair of cells that line both the small and large intestines.' (pg 1334)

3) Embryonic Development

Code   **Proposed statement**
VA3:   *Vitamin A contributes to normal embryonic development*

Reference 1.1:
'Vitamin A is important for normal…, embryonic development, growth, …' (pg 82)

'Retinoic acid plays an important role in embryonic development. Retinoic acid, as well as RAR, RXR, cellular retinol-binding protein (CRBP), and cellular retinoic acid-binding proteins (CRABP-I and CRABP-II), is present in temporally specific patterns in the embryonic regions known to be involved in the development of structures posterior to the hindbrain (e.g., the vertebrae and spinal cord) (Morriss-Kay and Sokolova, 1996). Retinoic acid is also involved in the development of the limbs, heart, eyes, and ears (Dickman and Smith, 1996; Hofmann and Eichele, 1994; McCaffery and Drager. 1995).' (pg 85)

'An association of vitamin A deficiency and impaired ebryonic development is well documented in animals (Morriss-Kay and Sokolova, 1996; Wilson et al., 1953). In laboratory animals, fetal resorption is common in severe vitamin A deficiency, while fetuses that survive have characteristic malformations of the eye, lungs, urogenital tract, and cardiovascular system. Similar abnormalities are observed in rat embryos lacking nuclear retinoid receptors (Wendling et al., 1999). Morphological abnormalities associated with vitamin A deficiency are not commonly found in humans; however, functional defects of the lungs have been observed (Chytil, 1996).' (pg 95,96)

Reference 3.1:
'Of particular importance in the setting of an upper level is the role of retinoic acids during morphogenesis and embryonic development. It has long been recognised that abnormal fetal development is associated with either insufficient or excessive intakes of vitamin A and related compounds.' (pg 4)

'Moreover, RARs and RXRs show specific spatio-temporal patterns of expression in all developing systems during embryonic development, which suggests that retinoic acid signalling is involved in most, if not all, morphogenetic and patterning processes (Morriss-Kay and Sokolova, 1996).' (pg 4)

Reference 4.1:
'Retinol and retinoic acid are essential for morphogenesis in embryonic development and may be involved in control of *Hox* gene expression, vital for correct sequential development (Marshall *et al.*, 1996).' (pg 18)

4) Cell differentiation

Code   **Proposed statement**
VA4:   *Vitamin A is necessary for normal cell differentiation (such as in the immune system).*

Reference 1.1:
'Vitamin A is important for normal…and immune function.' (pg 82)

'Retinoids are necessary for the maintenance of immune function, which depends on cell differentiation and proliferation in response to immune stimuli. Retinoic acid is important in maintaining an adequate level of circulating natural killer cells that have antiviral and anti-tumor activity (Zhao and Ross, 1995). Retinoic acid has been shown to increase phagocytic activity in murine macrophages (Katz et al., 1987) and to increase the production of interleukin 1 and other cytokines, which serve as important mediators of inflammation and stimulators of T and B lymphocyte production (Trechsel et al., 1985). Furthermore, the growth, differentiation, and activation of B lymphocytes requires retinol (Blomhoff et al., 1992).' (pg 85, 86)

'Vitamin A deficiency has been associated with a reduction in lymphocyte numbers, natural killer cells, and antigen-specific immunoglobulin responses (Cantorna et al., 1995; Nauss and Newberne, 1985). A decrease in leukocytes and lymphoid organ weights, impaired T cell function, and decreased resistance to immunogenic tumors have been observed with inadequate vitamin A intake (Dawson and Ross, 1999, Wiedermann et al., 1993). A generalized dysfunction of humoral and cell-mediated immunity is common in experimental animals and is likely to exist in humans.' (pg 96)

'Epidemiological evidence suggests that host resistance to infection is impaired at lesser stages of vitamin A deficiency, prior to clinical onset of night blindness (Arroyave et al., 1979; Arthur et al., 1992; Barreto et al., 1994; Bloem et al., 1990; Ghana VAST Study Team, 1993; Loyd-Puryear et al., Salazar-Lindo et al., 1993)' (pg 98)

'There is sound evidence for a role of vitamin A in the maintenance of both humoral antibody responses and cell-mediated immunity. In experimental animals, both nonspecific immunity (Butera and Krakowka, 1986; Cohen and Elin, 1974) and antigen-specific responses, including delayed-type hypersensitivity (Smith et al., 1987), blastogenesis (Butera and Krakowka, 1986; Friedman and Sklan, 1989), and antibody production (Carman et al., 1989, 1992; Pasatiempo et al., 1990; Ross, 1996; Stephensen et al., 1993), have been shown to be altered by a deficiency of vitamin A or enhanced by vitamin A supplementation. The number and cytotoxic activity of natural killer cells (Dawson et al., 1999; Zhao et al., 1994) is reduced in vitamin A deficiency, although responsiveness to activation is maintained.' (pg 105)

'Several human studies have linked impairment in immunity to low plasma or serum vitamin A concentrations (Coutsoudis et al., 1992; Semba et al., 1992, 1996). However, there are no human studies using controlled diets that have evaluated immune function tests as a means to assess the adequacy of different levels of dietary vitamin A.' (pg 105, 106)
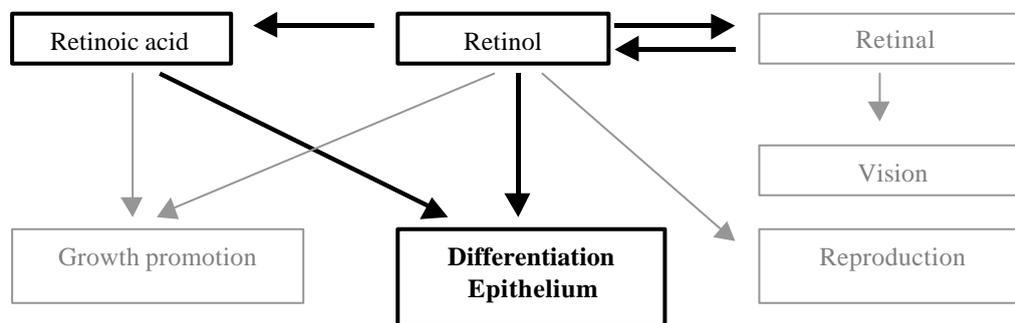
Reference 2.0:
'The major roles of vitamin A are in vision, differentiation of epithelial tissues, and in the immune system.' (pg 1711)

'Although animal studies have long shown a necessity for vitamin A in immune function, the molecular action of retinoids is still unknown. It also seems that some carotenoids function as such in immune function, and perhaps additionally as precursors of vitamin A.' (pg 1712)

Reference 4.1:
'…Requirements for vitamin A have also been implicated in the immune response, taste, hearing, and maintenance of appetite. Failure of cell division and differentiation can affect stem cells, and for example, result in impaired haematopoiesis.' (pg 17)

Figure 4: The functions of retinoic acid, retinal and retinal (adapted from Miller et al., 1998):

Most of the above processes are directly or indirectly dependent upon cellular differentiation and control of gene expression, with the majority of effects of vitamin A explained by the existence of complex signal transduction pathways, retinoid receptors, binding-proteins with bioactive vitamin A metabolites serving as physiological ligands. There is also a hypothesis that vitamin A is involved as a cofactor in the biosynthesis of cell surface glycoproteins that act as antigenic determinants, viral receptors, and markers of cellular identity (Gerster, 1997 and references therein; Basu and Dickerson, 1996 and references therein; Olson, 1996 and references therein).' (pg 17)

Reference 5.0:
'Vitamin A has a wide variety of functions, including specific roles in …immune status…'(pg 1315)

'Important for resisting infectious diseases…'(Table 65.1, pg 1334)

5) Growth

Code   **Proposed statement**
VA5:   *Vitamin A is necessary for normal growth*

Reference 1.1:
'Vitamin A is important for normal … gene expression, … growth, and ...' (pg 82)

Reference 4.1:
'Vitamin A is essential to the processes of …growth and cellular differentiation … Failure of cell division and differentiation can affect stem cells, and for example, result in impaired haematopoiesis.' (pg 17)

6) Reproduction

Code   **Proposed statement**
VA6:   *Vitamin A is necessary for normal reproduction*

Reference 1.1:
'Vitamin A is important for normal …, reproduction, ...' (pg 82)

Reference 2.0:
'It has been argued, without conclusive proof as yet, that retinoic acid is the active form of vitamin A required for cellular differentiation. Retinoic acid is an endogenous metabolite of vitamin A. Animals maintained on retinoic acid as sole source of vitamin A seem to grow normally and maintain good health, … some but not all species also show loss of testicular function. ' (pg 1711)
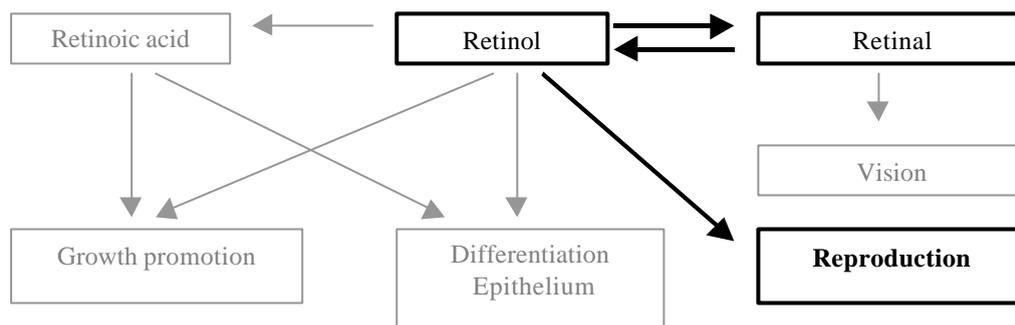
Reference 3.1:
'Of particular importance in the setting of an upper level is the role of retinoic acids during morphogenesis and embryonic development. It has long been recognised that abnormal fetal development is associated with either insufficient or excessive intakes of vitamin A and related compounds….Moreover, RARs and RXRs show specific spatio-temporal patterns of expression in all developing systems during embryonic development, which suggests that retinoic acid signalling is involved in most, if not all, morphogenetic and patterning processes (Morriss-Kay and Sokolova, 1996).' (pg 4)

Reference 4.1:
'Vitamin A is essential to the processes of … reproduction… and cellular differentiation …. Lack of vitamin A results in keratinisation of mucus secreting ciliated epithelium and other epithelial changes. The tissues most affected by this include the … testes.' (pg 17)

Figure 4: The functions of retinoic acid, retinal and retinal (adapted from Miller et al., 1998):

Retinoic acid — Retinol ⇄ Retinal

Vision

Growth promotion — Differentiation Epithelium — **Reproduction**

Most of the above processes are directly or indirectly dependent upon cellular differentiation and control of gene expression, with the majority of effects of vitamin A explained by the existence of complex signal transduction pathways, retinoid receptors, binding-proteins with bioactive vitamin A metabolites serving as physiological ligands. There is also a hypothesis that vitamin A is involved as a cofactor in the biosynthesis of cell surface glycoproteins that act as antigenic determinants, viral receptors, and markers of cellular identity (Gerster, 1997 and references therein; Basu and Dickerson, 1996 and references therein; Olson, 1996 and references therein).' (pg 17)

Reference 5.0:
'Vitamin A has a wide variety of functions, including specific roles in …growth and reproduction, …'(pg 1315)

7) Beta Carotene

Code   **Proposed statement**
VA7:   *Beta carotene can be converted to vitamin A. Vitamin A is necessary for / contributes to...*

Reference 1.1:
'There are a number of sources of dietary vitamin A. Preformed vitamin A is abundant in some animal-derived foods, whereas pro-vitamin A carotenoids are abundant in darkly colored fruits and vegetables, as well as oily fruits and red palm oil' (pg 82)

'For dietary provitamin A carotenoids-β-carotene, α-carotene, and β-cryptoxanthin-RAEs have been set at 12, 24, and 24 μg, respectively.' (pg 82)

'Although a large body of observational epidemiological evidence suggest that higher blood concentrations of β-carotenes and other carotenoids obtained from foods are associated with a lower risk of several chronic diseases, there is currently not sufficient evidence to support a recommendation that requires a certain percentage of dietary vitamin A to come from provitamin A carotenoids in meeting the vitamin A requirement.' (pg 83)

Reference 1.2:

'While there is evidence that β-carotene is an antioxidant in vitro, its importance to health is not known. The one clear function of certain carotenoids that is firmly linked to a health outcome is the provitamin A activity of some dietary carotenoids (α-carotene, β-carotene, and β-cryptoxanthin) and their role in the prevention of vitamin A deficiency.' (pg 325)

'Three of these carotenoids, namely α-carotene, β-carotene, and β-cryptoxanthin, can be converted into retinol and are thus referred to as provitamin A carotenoids.' (pg 326)

'In humans, the only known function of carotenoids is vitamin A activity (provitamin A carotenoids only). Carotenoids also are thought to have a variety of different actions, including possible antioxidant activity, immunoenhancement, inhibition of mutagenesis and transformation, inhibition of premalignant lesions, quenching of nonphotochemical fluorescence, and activity as a pigment in primate macula (Olson, 1999). Carotenoids have also been associated with various health effects: decreased risk of macular degeneration and cataracts, decreased risk of some cancers, and decreased risk of some cardiovascular events (Olson, 1999). However, as described above, the only known function of carotenoids in humans is to act as a source of vitamin A in the diet.' (pg 326)

'The effect of increasing β-carotene intake on several markers of antioxidant activity has been investigated in a series of studies involving humans. These studies have examined antioxidant marker activity in apparently healthy men and women as well as in subjects who were physiologically challenged (i.e., smokers and patients with coronary disease or cystic fibrosis). Studies of the effect of β-carotene intake on measures of antioxidant activity are summarized in Table … The dietary source of β-carotene ranged from modification of diets with normally consumed foods to giving supplements that provided as much as 120 mg/day of a highly bioavailable preparation. In general, subjects in most studies consumed β-carotene in amounts that would be difficult to achieve from foods alone and, as a result, relate to the pharmacological range of intakes. The findings reported in Table… indicate that β-carotene supplementation did not alter, or inconsistently alter, markers of anti-oxidant activity, which were somewhat dependent on β-carotene intake. In studies in which subjects were fed less than 25 mg/day of β-carotene, either from foods or as a supplement, changes in the markers for antioxidant activity were minimal. Exceptions noted were decreased deoxyribonucleic acid strand breaks observed when 22 mg/day of β-carotene was administered as carrot juice (Pool-Zobel et al., 1997) and lowered copper-induced oxidation of low-density lipoprotein when 12 or 24 mg/day of β-carotene was given along with vitamins C and E (Mosca et al., 1997)… feeding β-carotene in amounts greater than 25 mg/day generally resulted in inconsistent responses of the biological markers monitored.' (pg 331, 332)

'Appropriate communication among cells is essential for the coordination of biochemical functions in complex, multicellular organisms. One theory suggest that failure of signaling is one cause of cell overgrowth and eventually cancer. Two research groups have demonstrated that carotenoids stimulate gap junction

communication between cells in vitro (Sies and Stahl, 1997; Zhang et al., 1991).' (pg 333)

'It is not known whether the parent carotenoids or their metabolites are the active factors (Hanusch et al., 1995), nor is it known whether carotenoids influence this communication process in vivo. More study is needed to ascertain whether carotenoids play a direct role in cell-cell communication and, if so, what health outcomes are influenced by this action.' (pg 338)

'It is important to remember, however, that studies conducted with provitamin A carotenoids may yield results that are attributable to the conversion of carotenoids to vitamin A or other retinoids, not to the effects of the intact carotenoid.' (pg 338)

'Santos et al, (1996) showed that long-term β-carotene supplementation enhanced natural killer cell activity in men 65 to 86 years of age, but not in men 51 to 64 years of age; enhancement by β-carotene in this age group was confirmed in a subsequent study (Santos et al., 1998). Hughes et al, (1997) evaluated mechanisms by which β-carotene might enable immune cells to act more efficiently. Subjects were supplemented for 26 days with either 15 mg of β-carotene or a placebo. Subjects receiving the β-carotene treatment had increases in expression of adhesion molecules by monocytes, in ex vivo secretion of tumor necrosis factor-α, and in the percentage of monocytes expressing major histocompatibility complex II, a cell surface molecule responsible for presenting antigen to T-helper cells.' (pg 338)

'Other immunological effects that carotenoids are reported to increase are lymphocyte response to mitogens (Kramer and Burri, 1997) and total white blood cells and helper T cells in human immunodeficiency virus-related humans (Coodley et al., 1993). Whether these and the other effects noted are specific to carotenoids and are important in overall immunity is not confirmed.' (pg 338)

Reference 3.2:
'Some dietary carotenoids, such as β-carotene, serve as an important source of vitamin A, which is the major know function of carotenoids in humans…Carotenoids containing at least one unsubstituted β-ionone ring and a poliene chain are potential precursors of vitamin A. The preformed vitamin A is only present in animal products (e.g. liver, eggs, milk products), thus, in countries where the intake of animal products is low, carotenoids have to meet (i.e. by 80% or more in Asia and Africa) the vitamin A requirements.' (pg 2)

'The best-characterised natural functions of carotenoids are to serve as light-absorbing pigments during photosynthesis and protection of cells against photosensitization. Carotenoids provide considerable coloration and identification for many species, from vegetables to animals. In addition, carotenoids serve several other functions, such as radical quenching, antioxidant and anticarcinogenic activities in different animal sites and are regulators of cell function.' (pg 2)

'Carotenoids can act as antioxidants and free radical/reactive species scavengers (Tsuchiya *et al*., 1993; Everett *et al*., 1996; IARC, 1998; Omenn, 1998)…The role *in vivo* and in humans is less clear (IARC, 1998; Palozza, 1998; Lambert, 1999). The switch from antioxidant to pro-oxidant behaviour can be, for example, a function of

oxygen concentration (Edge and Truscott, 1997; Palozza, 1998). The pro-oxidant activity of β-carotene has been demonstrated at a high partial pressure of oxygen; because this is highest in the outermost cells of the lung, these cells might be particularly subject to the pro-oxidant effect of β-carotene (cited in Paolini *et al*., 1999).' (pg 4)

'Part of the effects of β-carotene can be mediated by the formation of retinoic acid (RA) that has a key function as a regulator of gene expression, morphogenesis, and growth in vertebrate embryos…RARβ plays an important role in lung development…' (pg 4)

ANNEX 4.2

Vitamin D

Source documents for reviewing vitamin D

Reference 1.4:
Institute of Medicine Dietary Reference Intakes for calcium, phosphorus, magnesium, vitamin D, and fluoride. Washington D.C. National Academy Press, 1997.

Reference 2.0:
Encyclopedia of Human Nutrition 2E. Editor-in-chief, Michele J. Sadler, editors, J.J. Strain, Benjamin Caballero. San Diego : Academic Press, c1999.

Reference 3.3:
Opinion of the Scientific Committee on Food (SCF) on the Tolerable Upper Intake Level of vitamin D. December 2002. (http://www.europa.eu.int/comm/food/fs/sc/scf/out157_en.pdf).

Reference 4.2:
Review of Vitamin D – Revised Version. Expert Group on Vitamins and Minerals. August 2002. (http://www.food.gov.uk/multimedia/pdfs/evm-00-11r.pdf).

Reference 6.1:
Nutrition and Bone Health: with particular reference to calcium and vitamin D. Report of the Subgroup on Bone Health, Working Group on the Nutritional Status of the Population of the Committee on Medical Aspects of Food and Nutrition Policy. Report on Health and Social Subjects No. 49. DH: The Stationery Office, 1998.

1) Calcium & phosphorus absorption and utilisation

Code **Proposed statement**
VD1: *Vitamin D is necessary for the normal absorption and utilisation of calcium & phosphorus*

Reference 1.4:
'Vitamin D's major biologic function in humans is to maintain serum calcium and phosphorus concentrations within the normal range by enhancing the efficiency of the small intestine to absorb these minerals from the diet (DeLuca, 1988; Reichel et al., 1989). 1,25 enhances the efficiency of intestinal calcium absorption along the entire small intestine, but primarily in the duodenum and jejunum. 1,25 (OH) $_2$D$_3$ also enhances dietary phosphorus absorption along the entire small intestine (Chen et al., 1974), but its major influence is in the jejunum and ileum. When dietary calcium intake is inadequate to satisfy the body's calcium requirement, 1,25 (OH) $_2$D, along with parathyroid hormone (PTH), mobilizes monocytic stem cells in the bone marrow to become mature osteoclasts (Holick, 1995; Merke et al., 1986). The osteoclasts, in

turn, are stimulated by a variety of cytokines and other factors to increase the mobilization of calcium stores from the bone. Thus, vitamin D maintains the blood calcium and phosphorus at supersaturating concentrations that are deposited in the bone as calcium hydroxyapatite.' (pg 253)

'Although the kidney supplies the body with $1,25 (OH)_2D$ to regulate calcium and bone metabolism, it is recognized that activated macrophages come lymphoma cells, and cultured skin and bone cells also make $1,25 (OH)_2D$ (Adams et al., 1990; Holick, 1995; Pillai et al., 1987). Although the physiologic importance of locally produced $1,25 (OH)_2D$ is not well understood, the excessive unregulated production of $1,25 (OH)_2D$ by activated macrophages and lymphoma cells is responsible for the hypercalciuria associated with chronic granulomatous disorders and the hypercalcemia seen with lymphoma (Adams, 1989; Davies et al., 1994).' (pg 254, 255)

Reference 2.0:
'In the intestine, $1,25(OH)_2D$ enhances the absorption of dietary calcium and phosphorus across the microvilli of the small intestinal absorptive cells. It also interacts with monocytic stem cells in the bone marrow to initiate their transformation into mature osteoclasts. Thus, $1,25(OH)_2D_3$ regulates serum calcium levels by enhancing the efficiency of intestinal calcium absorption and stimulating resorption of calcium from the bone. It remains controversial as to whether $1,25(OH)_2D$ has any direct action on the renal handling of either calcium or phosphorus.' (pg 363)

Reference 3.3:
'The principal physiological function of vitamin D in all vertebrates including humans is to maintain serum calcium and phosphorus concentrations in a range that support cellular processes, neuromuscular function, and bone ossification. Vitamin D accomplishes this goal by enhancing the efficiency of the small intestine to absorb dietary calcium and phosphorous, and by mobilising calcium and phosphorus from the bone (Holick, 1999; Holick et al., 1998).' (pg 2)

'The main mechanism of action of vitamin D is the interaction of $1,25(OH)_2D$ with the nuclear vitamin D receptor (Brown et al., 1999). VDR belongs to the super family of steroid nuclear receptors. Following ligand binding, VDR heterodimerises with retinoid X receptor (RXR) and acts as a ligand-activated transcription factor by binding to genomic vitamin D responsive elements (VDRE) in vitamin D-regulated genes. These include more than 50 other genes important for mineral homeostasis, vitamin D metabolism, energy metabolism, cell differentiation and proliferation, extracellular matrix proteins, oncogenes, growth factors, signal transduction proteins and peptide hormones. Genes can be both up-regulated or down-regulated, but the exact mechanism is unclear. Among genes down-regulated are PTH, osteocalcin, protein-kinase A inhibitors and interleukin-2 genes.' (pg 6)

'The most critical role of $1,25(OH)_2D$ I mineral homeostasis is to enhance the efficiency of the small intestine to absorb dietary calcium. This was clearly demonstrated in the VDR null mouse (Yoshizawa et al., 1997).' (pg 7)

'$1,25(OH)_2D$ also promotes the intestinal absorption of phosphate. However a significant phosphate absorption also occurs in $1,25(OH)_2D$-deficient states (Brown et al., 1999).' (pg 7)

'$1,25(OH)_2D$ enhances the mobilisation of calcium and phosphorus stores from bone at times of calcium deprivation.' (pg 7)

'$1,25(OH)_2D$ regulate calcium homeostasis in close co-operation with PTH, which is the principal hormone regulating extracellular ionised calcium from minute to minute. PTH stimulates $1,25(OH)_2D$ synthesis and $1,25(OH)_2D$ suppresses the synthesis and secretion of PTH and controls parathyroid growth through negative gene regulation. Studies in the VDR null mouse suggest that VDR is essential, but works in co-operation with calcium and phosphate (Brown et al., 1999).' (pg 7)

'$1,25(OH)_2D$ increases renal calcium reabsorption and calbinding expression, and it accelerates PTH dependent calcium transport in the distal tubule, which has the highest level of VDR. The enhancing effect of $1,25(OH)_2D$ on renal phosphate absorption might be an indirect action via PTH suppression (Brown et al., 1999).' (pg 7)

'Synthesis and cellular receptors for $1,25(OH)_2D$ have been found not only in the intestine, kidney and bone but also in many other tissues, suggesting that $1,25(OH)_2D$ is fundamental to the regulation of gene expression in many cell types in addition to its probable role in intracellular calcium regulation (Brown et al., 1999; Sehnder et al., 2002a and b).' (pg 7)

Reference 4.2:
'The active form of vitamin D regulates the intestinal absorption of calcium from the diet.' (pg 6)

'Transport is facilitated by two $1,25-(OH)_2D$ dependent calcium binding proteins, calbindin D9k (CaBP-D_{9k}, the most abundant) and calbindin D28k (CaBP-D_{28k}).' (pg 6)

'Thus, it is recognised that the major response of the intestine to vitamin D is an increase in calbindin synthesis, although the function of calbindin is not yet clear. There is some evidence to suggest that it is related to the intestinal transport of calcium.' (pg 6)

'Magnesium absorption through the gut involves both active and passive mechanisms but is much less tightly regulated by $1,25-(OH)_2D$ than calcium absorption. Transport of inorganic phosphate across the luminal brush border is dependent on the sodium-phosphate co-transporter; this is the step at which $1,25-(OH)_2D$ regulated phosphate absorption occurs, but the mechanisms are poorly understood (Sahota and Hosking, 1999).' (pg 6, 7)

'Calcium resorption at the distal nephron is regulated by $1,25-(OH)_2D_2$ and PTH. The mechanisms of the active calcium transcellular transport are similar to those in the enterocyte with $1,25-(OH)_2D_2$ stimulating the expression of calcium binding protein.

1,25-$(OH)_2D_2$ may also stimulate the synthesis of the plasma membrane pump, as well as regulating its activity (Sahota and Hosking, 1999).' (pg 7)

'…Reports exist that suggest an effect of 1,25-$(OH)_2$ vitamin D on stimulation of the renal absorption of calcium (DeLuca and Schnoes, 1976). 1,25-$(OH)_2D_3$ is able to localise in the nuclei of cells in the distal renal tubules, where it binds to the vitamin D receptor (VDR) (DeLuca and Zierold, 1998). Binding to the VDR is known to be essential to the prevention of rickets and the regulation of calcium and phosphorus.' (pg 7)

Reference 6.1:
'The active hormonal form, 1,25$(OH)_2$vitamin D, controls plasma calcium concentrations by modulating calcium absorption in the small intestine, phosphate resorption in the renal tubules and through calcium release from bone. A specific nuclear receptor for 1,25$(OH)_2$vitamin D (vitamin D receptor) occurs in tissues involved in calcium homeostasis, such as intestine, kidney and bone. (pg 40)

2) Cell division - skin, immune system

Code   **Proposed statement**
VD2a: *Vitamin D contributes to the normal cell division*
VD2b: *Vitamin D contributes to the normal structure of skin*
VD2c: *Vitamin D contributes to the normal function of the immune system*

Reference 1.4:
'A multitude of other tissues and cells in the body can recognize 1,25 $(OH)_2D$ (Stumpf et al., 1979). Although the exact physiologic function of 1,25 $(OH)_2D$ in the brain, heart, pancreas, mononuclear cells, activated lymphocytes, and skin remains unknown, its major biologic function has been identified as a potent antiproliferative and prodifferentation hormone (Abe et al., 1981); Colston et al., 1981; Eisman et al., Smith et al., 1987). There is little evidence that vitamin D deficiency leads to major disorders in these organ and cellular systems.' (pg 253)

Reference 2.0:
'There are a variety of other tissues – including the brain, gonads, pancreas, stomach, activated T an dB lymphocytes, monocytes and skin – that have nuclear VDR. Although the exact physiologic function of the ineraction 1,25$(OH)_2D$ with these VDRs is not well understood, it is known that *in vivo* and *in vitro* 1,25$(OH)_2D_3$ can inhibit proliferation and induce terminal differentiation of various normal and tumour cells including normal human keratinocytes. This is the reason why activated vitamin D compounds are now routinely used for the treatment of the hyperproliferative skin disorder, psoriasis.' (pg 363)

Reference 3.3:
'In the skin, 1,25$(OH)_2D$ plays an important role by inhibiting proliferation and stimulating differentiation of keratinocytes and vitamin D analogues are used in the treatment of psoriasis. In the immune system, 1,25$(OH)_2D$ modulates synthesis of interleukins and cytokines. Besides stimulating monocytes and macrophages, 1,25$(OH)_2D$ functions as an immunosuppressive agent by decreasing the rate of

proliferation and the activity of both T- and B cells and inducing suppressor T cells (Brown et al., 1999).' (pg 8)

'In addition, VDR is expressed in many other tissues, such as muscle and nervous tissue, liver, intestine, reproductive organs, pancreas, pituitary, thyroid gland and lung, where $1,25(OH)_2D$ apparently has important functions in regulation of cell proliferation and differentiation (Brown et al., 1999; Holick, 1999).' (pg 8)

Reference 3.3:
'The last couple of decades it has become increasingly apparent that vitamin D also has other important functions in tissues not primarily related to mineral metabolism (Brown et al., 1999; Holick, 1999). One example is the haematopoietic system, in which vitamin D affects cell differentiation and proliferation including such effects also in cancer cells...' (pg 2)

Reference 4.2:
'$1,25\text{-}(OH)_2D_2$ the hormonal form of vitamin D regulates calcium and phosphate metabolism by its action on three target tissues, small intestine, bone and kidney. In addition to these major sites of vitamin D action, many other body tissues and cells have receptors for and responses to $1,25\text{-}(OH)_2D$ vitamin D. These include pancreas, pituitary gland, lymphocytes and monocytes. The precise physiological function of vitamin D in these tissues is uncertain but an anti-proliferative and pro-differentiation action is apparent (FNB, 1997). $1,25\text{-}(OH)_2D_3$ interacts with a specific receptor protein in its target tissues, the receptor complex is taken up into the nucleus and recycled (DeLuca and Ziorold, 1998). The Vitamin D Receptor (VDR) binds to direct repeat response elements called DR-3 in the promoter regions of target genes to stimulate or suppress transcription. The VDR will only bind to the response elements if the retinoid X receptor is also present.' (pg 6)

3) Bone

Code   **Proposed statement**
VD3:   *Vitamin D is necessary for the normal structure of bone*

Reference 1.4:
'After vitamin D was recognized as being critically important for the prevention of rickets, the United States, Canada, and many other countries instituted a policy of fortifying some foods with vitamin D (Steenbock and Black, 1924).' (pg 257)

'Vitamin D deficiency is characterized by inadequate mineralization or demineralization of the skeleton. In children, vitamin D deficiency results in inadequate mineralization of the skeleton causing rickets, which is characterized by widening at the end of the long bones, rachitic rosary, deformations in the skeleton including frontal bossing, and outward or inward deformities of the lower limbs causing bowed legs and knocked knees, respectively (Goldring et al., 1995). In adults, vitamin D deficiency leads to a mineralization defect in the skeleton causing osteomalacia. In addition, the secondary hyperparathyroidism associated with vitamin D deficiency enhances mobilization of calcium from the skeleton, resulting in porotic bone (Favus and Christakos, 1996)' (pg 258)

'Vitamin D deficiency causes a decrease in ionized calcium in blood, which in turn leads to an increase in the production and secretion of PTH (Fraser, 1980; Holick, 1995).' (pg 258)

'The elevated PTH leads to an increase in the destruction of the skeletal tissue in order to release calcium into the blood.' (pg 258)

'It is well recognized that vitamin D deficiency causes abnormalities in calcium and bone metabolism.' (pg 258)

'Little information is available about the level of 25(OH)D that is essential for maintaining normal calcium metabolism and peak bone mass in older children and in young and middle-aged adults. For the elderly, there is mounting scientific evidence to support their increased requirement for dietary vitamin D in order to maintain normal calcium metabolism and maximize bone health (Dawson-Hughes et al., 1991; Krall et al., 1989; Lips et al., 1988).' (pg 259)

'The ultimate effect of vitamin D on human health is maintenance of a healthy skeleton. Thus, in reviewing the literature for determining vitamin D status, one of the indicators that has proven to be valuable is an evaluation of skeletal health. In neonates and children, bone development and the prevention of rickets, either in combination with serum 25(OH)D and PTH concentrations, or by itself, are good indicators of vitamin D status (Gultekin et al., 1987; Koo et al., 1995; Kruse et al., 1984; Markested et al., 1986; Meulmeester et al., 1990). For adults, bone mineral content (BMC), bone mineral density (BMD), and fracture risk, in combination with serum 25(OH)D and PTH concentrations, have proven to be the most valuable indicators of vitamin D status (Brazier et al., 1995; Dawson-Hughes et al., 1991, 1995; Lamberg-Allardt et al., 1989, 1993; Sorva et al., 1991; Webb et al., 1990).' (pg 260, 261)

Reference 2.0:
'Once $1,25(OH)_2D$ interacts with the VDR, the complex forms a heterodimer with retinoic acid X receptor (RXR). This new complex site on specific segments of vitamin D responsive genes known as vitamin D responsive elements (VDREs) to either increase or decrease transcriptional activity of the vitamin D sensitive genes such as osteocalcin, calcium binding protein (calbindin), PTH and osteonectin.' (pg 363)

'The onset of vitamin D deficiency decreases the efficiency of intestinal calcium absorption. There is a decline in blood ionized calcium which causes the parathyroid glands to produce and secrete more parathyroid hormone. This hormone tries to conserve calcium by enhancing tubular reabsorption of calcium in the kidney. However, in the face of developing hypocalcaemia which could disturb neuromuscular function and a wide variety of metabolic and cellular processes, the body calls upon $1,25(OH)_2D$ and PTH to mobilize stem cells to become functional osteoclasts, which in turn mobilize calcium from the skeleton. In addition, PTH causes a loss of phosphorus into the urine causing hypophosphataemia. Thus, in early vitamin D deficiency the serum calcium is normal; it is the low serum phosphorus that causes the extracellular Ca XPO_4 to be too low for normal mineralization of bone

matrix. This causes a disruption in the orderly sequence of events in the differentiation of hypertrophied chondrocytes in the epiphyseal plates, resulting in their disorganization and causing a widening of the epiphyseal plates (end of long hones), demineralization of the skeleton, and bony deformities.' (pg 364)

'Once the epiphyseal plates are closed later in adolescence, vitamin D deficiency can no longer cause bone deformities. Instead, there is an inability to mineralize newly deposited bone matrix leading to wide osteoid seams within the trabecular and cortical bone, causing the bone disease commonly known as osteomalacia.' (pg 364)

Reference 3.3:

'$1,25(OH)_2D$ is essential for development and maintenance of a mineralised skeleton. Deficiency results in rickets during growth and osteomalacia in adults. $1,25(OH)_2D$ induces bone formation by regulation of matrix proteins important for bone formation, such as osteocalcin, osteopontine, alkaline phosphatase, matrix-gla- protein and collagen, as well as mineral apposition. The bone forming osteoblasts express VDR and it appears that $1,25(OH)_2D$ inhibits osteoblast proliferation through VDR-dependent signal pathway, and promotes their differentiation (Kveiborg et al., 1999). Vitamin D does not appear to be absolutely essential for the ossification process, but enhances this through increasing serum levels of calcium and phosphate. It has been suggested that not only $1,25(OH)_2D$ is involved in bone mineralisation, but also $24,25(OH)_2D$ may be required (Brown et al., 1999).' (pg 7)

Reference 4.2:

'The action of vitamin D on bone is not well defined. Fraser (1981) proposes a role for the 1,25-dihydroxy derivative in bone resorption, possibly in the proliferation of macrophages (Sahot and Hosking, 1999). Bone formation begins with the differentiation of mesenchymal stromal cells to form mature osteoblasts. This is regulated by a number of facts including PTH and $1,25-(OH)_2D$. It has been proposed that another metabolite, $24,25-(OH)_2D_3$, plays a significant role in normal bone formation (Ornoy et al., 1978). $1,25-(OH)_2D$ and PTH are also involved in the regulation of the migration of the vesicles containing calcium phosphate from the cell processes of the osteoblast to the zone of mineralisation (Sahota and Hosking, 1999). As this process proceeds some of the osteoblasts are incorporated into bone as osteocytes, while others remain as lining cells covering the trabecular surfaces. However, it appears that there is no direct effect of vitamin D or any of its metabolites on bone mineralisation.' (pg 7)

'When considering the effects of $1,25-(OH)_2$ vitamin D on bone it should be noted that bone contains several cell types, which may respond in different ways to $1,25-(OH)_2D$. Osteoblasts have receptors for $1,25-(OH)_2D_3$ which appears to increase mineralisation and osteoblast differentiation. In this way, bone formation and hence growth are promoted (Braidman, 1990).' (pg 7, 8)

'To maintain a constant plasma calcium concentration, PTH works in conjunction with vitamin D on the osteoblasts in an unknown mechanism to mobilise calcium, and hence phosphate, from bone (DeLuca and Zeorold, 1998).' (pg 8)

Reference 6.1:

1,25(OH)$_2$vitamin D also appears to promote calcium deposition in growing ends of bones but the mechanisms are not fully understood but may be mediated via an effect on osteocalcin concentrations. 1,25(OH)$_2$vitamin D has several other functions not specifically related to calcium; deficiency causes impaired function of nerves and muscles, as well as behavioural changes such as depression.' (pg 40)

'Recent data relating plasma levels of PTH to those of 25(OH)vitamin D have led to the suggestion that elevation of PTH might define the level of 25(OH)vitamin D needed for bone health, beyond the avoidance of clinical deficiency.' (pg 40)

ANNEX 4.3

Vitamin E

Source documents for reviewing vitamin E

Reference 1.2:
Institute of Medicine Dietary Reference Intakes for vitamin C, vitamin E, selenium and carotenoids.
Washington D.C. National Academy Press, 2000.

Reference 2.0:
Encyclopedia of Human Nutrition 2E. Editor-in-chief, Michele J. Sadler, editors, J.J. Strain, Benjamin
Caballero. San Diego : Academic Press, c1999.

Reference 3.4:
Opinion of the Scientific Committee on Food (SCF) on the Tolerable Upper Intake Level of Vitamin E.
April 2003. (http://www.europa.eu.int/comm/food/fs/sc/scf/out195_en.pdf)

Reference 4.3:
Review of Vitamin E – Revised Version. Expert Group on Vitamins and Minerals. July 2002.
(http://www.food.gov.uk/multimedia/pdfs/evm-00-13r.pdf).

1) Antioxidant activity in cell membranes

Code   **Proposed statement**
VE1:   *Vitamin E is necessary for protecting cell membranes from some types of*
              damage caused by free radicals (such as the oxidation of polyunsaturated
              fatty acids).

Reference 1.2:
'Vitamin E is a chain-breaking antioxidant that prevents the propagation of free-
radical reactions (Burton and Ingold, 1986; Burton et al., 1983; Ingold et al., 1987;
Kamal-Eldin and Appelqvist, 1996; Packer, 1994; Tappel, 1962). The vitamin is a
peroxyl radical scavenger and especially protects polyunsaturated fatty acids (PUFAs)
within membrane phospholipids and in plasma lipoproteins Burton et al., 1983).
Peroxyl radicals (abbreviated ROO•) react with vitamin E (abbreviated Vit E_OH)
1,000 times more rapidly than they do with PUFA (abbreviated RH) (Packer, 1994).'
(pg 195)

'….α-Tocopherol can be oxidized to the tocopheroxyl radical-one electron oxidation
product-which can be reduced back to the unoxidized form by reducing agents such as
vitamin C.' (pg 199)

'In general, lipid peroxidation markers are elevated during vitamin E depletion and
their levels can be normalized upon vitamin E repletion. However, these markers are
not necessarily specific to vitamin E, since changes in intake of other antioxidants can

also change the levels of these markers. At present, there is no evidence that lowering lipid peroxidation marker levels is associated with health benefits.' (pg 203)

'When vitamin E intercepts a radical, a tocopheroxyl radical is formed (Burton and Ingold, 1981). This radical can be reduced by ascorbic acid or other reducing agents (Doba et al., 1985; Niki et al., 1982), thereby oxidizing the latter and returning vitamin E to its reduced state.' (pg 224)

Reference 2.0:
'Vitamin E activity is derived from tocopherols and tocotrienols, which are lipid-soluble compounds originally synthesized by plants. There are four forms (α-, β-, γ- and δ-) of each, all of which have antioxidant properties but differing biological activities. α-Tocopherol is the most effective lipid-soluble antioxidant present in membranes and lipoproteins, and its function in these locations is to protect the unsaturated bonds of phospholipids from damage caused by free radicals.' (pg 1878)

'Tocopherols function as lipid-soluble antioxidants which protect membranes and lipoproteins against damage caused by lipid oxidation. However, because the wide array of deficiency symptoms observed in different species is difficult to explain by way of a simple antioxidant hypothesis, this has led to suggestions that tocopherols may also have more specific roles, such as in nucleic acid and mitochondrial metabolism and in the maintenance of membrane integrity. At this time, however, conclusive evidence for non-antioxidant functions is lacking.' (pg 1880)

'Lipid oxidation is a process which may occur during normal aerobic cellular metabolism and during the metabolism of drugs. In the process, polyunsaturated fatty acids (PUFA), such as those in cellular and subcellular membranes give up loosely bound hydrogen atoms from an allylic CH_2 group to highly reactive free radicals and are converted to fatty acid radicals. The fatty acid radical usually rearranges to a conjugated diene and takes up molecular oxygen, producing a peroxyl radical and this compound attacks a second PUFA, resulting in the formation of a hydroperoxide and a second fatty acid radical. A chain reaction may then ensue. The hydroperoxides which are continuously produced as a part of the process may split in the presence of iron or copper to peroxyl or alkoxyl radicals, which serve to accelerate the chain reaction, or they may break down to aldehydes, ketones, alkanes and other products. Some of these compounds may bind to and disrupt cellular macromolecules such as DNS and proteins, while others have chemotactic properties which may induce inflammatory reactions.' (pg 1880)

'Lipid oxidation may be prevented or retarded in several ways….In the event that a chain reaction does occur, chain-breaking antioxidants can quench the reaction.' (pg 1880)

'α-Tocopherol accounts for most of the lipid-soluble chain-breaking antioxidant activity in mammalian tissues and plasma. It donates its phenolic H atom to peroxyl radicals, and in the process becomes an α-tocopheroxyl radical. However, this radical is relatively stable because the unpaired electron on the oxygen atom is delocalized throughout the aromatic ring structure. Under normal conditions it does not react with membrane PUFA, and so propagation of the chain reaction is inhibited.' (pg 1880)

'Membranes usually contain less than one 'α-tocopherol molecule per 1000-2000 phospholipids, and yet 'α-tocopherol is extremely effective in protecting membrane phospholipids against oxidative damage. The hydrophobicity of the isoprenoid side chain helps to fix 'α-tocopherol in the most fluid part of the membrane, close to the PUFA at risk of oxidative damage. In addition, 'α-tocopherol scavenges peroxyl radicals about 10,000 times faster than they can react with PUFA. The length of the side chain also plays a critical role in the overall effectiveness of 'α-tocopherol because it positions the phenolic OH group at the membrane surface, allowing any 'α-tocopheroxyl radicals formed to be converted back to 'α-tocopherol.' (pg 1880,1881)

'There is also a view that the criterion upon which the RDA for vitamin E ought to be based should be the amount needed for optimal protection of cells and tissues against oxidative damage.' (pg 1882)

Reference 3.4:
'The basic mode of action of tocopherols in human tissue is to prevent the oxidation of polyunsaturated fatty acids (PUFA) by trapping free radicals and donating hydrogen. It is effective in protecting the integrity of lipid and phospholipid membranes and thus the requirement for vitamin E and the recommended intake is determined to a large extent by the intake of PUFAs. It has been shown that increasing PUFA content of a diet low in á-tocopherol equivalents has adverse effects on tocopherol status (Horwitt, 1974; SCF, 1993).' (pg 5)

'Chronic marginal deficiency can be generally characterised by an enhanced susceptibility to lipid peroxidation and corresponding lipofuschinosis. In rats this fist results in weakening of the basement membranes of the muscle capillaries and a breakdown of endothelial cells.' (pg 6)

Reference 4.3:
'Information currently available indicates that all its nutritional effects are consistent with its role as a biological antioxidant. In this regard, vitamin E is thought to have basic functional importance in the maintenance of membrane integrity in virtually all cells of the body. The potent antioxidant properties of vitamin E were first demonstrated by Olcott and Matthill in 1931. It was later proposed that the major function of the vitamin was the protection of PUFAs from oxidation *in vivo* to hydroperoxides. Other oxidation reactions prevented are the conversion of free or protein-bound sulphydryls to disulphides. However, it was not until more recent years that the precise function of vitamin E was elucidated and its central role in protection against free-radical induce cellular damage was recognised (Chow 1985, Basu and Dickerson 1996).

'Potentially damaging free radicals are produced in cells under normal conditions either by homolytic cleavage of a covalent bond, or by a univalent oxidation or reduction. The PUFA's of biological membranes are particularly susceptible to attack by free radicals due to their 1,4-pentadiene systems, from which a hydrogen atom is readily removed. The lipoperoxyl free radicals thus formed can attack adjacent PUFA residues and thereby initiate a chain of free radical reactions, with widespread harmful consequences to membrane structure. Vitamin E breaks the chain of free radical formation by reacting with the free radicals and converting them to a non-harmful form. This action, termed free radical 'scavenging', involves the donation of a

hydrogen atom to a fatty acyl free radical (or superoxide radical) to prevent the attack of that species on other PUFAs (Lucy 1972, Chow 1985). As noted previously, in the course of this process, α-tocopherol is converted to an α-tocopherol radical which is more stable than fatty acid or peroxyl radicals and does not react with membrane PUFA. The α-tocopherol radical can then react with another radical to form a non radical product or can be re-converted to α-tocopherol (Bramley et al 2000).' (pg 5, 6)

'The clinical manifestations of vitamin E deficiency vary considerably between species. In general, however, the targets are the neuromuscular, vascular and reproductive systems. The various signs of vitamin E deficiency are believed to be manifestations of membrane dysfunction, the result of the oxidative degradation of polyunsaturated membrane phospholipids and/or the disruption of other critical cellular processes (Horwitt 1960). In a wide range of animal species, vitamin E deficiency causes an increase in the tendency for erythrocytes to lyse in a solution of hydrogen peroxide. Of the effects of vitamin E deficiency reported in experimental animals, this is the only feature of deficiency which occurs definitely in man and first suggested the possible role of vitamin E in maintenance of membrane stability.' (pg 6)

'There is clear evidence that the requirement for vitamin E increases with the amount of dietary PUFAs.' (pg 6)

2) Cell proliferation and differentiation

Code   **Proposed statement**
VE2:   *Vitamin E helps cells to grow and multiply*

Reference 1.2:
'….α-Tocopherol inhibits protein kinase C activity, which is involved in cell proliferation and differentiation, in smooth muscle cells (Boscoboinik et al., 1991; Chatelain et al., 1993; Clement et al., 1997; Stauble et al., 1994; Tasinato et al., 1995),…' (pg 195, 196)

Reference 4.3:
'Some non-antioxidant functions have been attributed to α but not β-tocopherol (Azzi and Stocker 2000). These include regulation of protein kinase C, modification of cell growth and proliferation, modification of gene transcription, protein phosphatase activation and modifications to gene expression. The authors state that the best evidence for a non-oxidant role is related to the recognition and transfer of a α-tocopherol.' (pg 6)

3) Immune system

Code   **Proposed statement**
VE3:   *Vitamin E contributes to the normal function of the immune system*

Reference 1.2:

'….α-Tocopherol inhibits protein kinase C activity, which is involved in …human platelets (Freedman et al., 1996), and monocytes (Cachia et al., 1998; Deveraj et al., 1996).' (pg 195, 196)

Reference 2.0:
'Vitamin E deficiency impairs immune responses, while supplementation with higher than recommended dietary levels enhances humoral and cell-mediated immunity and increases the efficiency of phagocytes.' (pg 1883)

'The mechanism of the immunostimulatory effect of vitamin E appears to be mainly related to its antioxidant function, although other antioxidants do not produce similar actions. Vitamin E could function either by decreasing concentrations of reactive oxygen species (e.g. hydrogen perioxide), thereby preventing oxidative damage to the stimulated immune and phagocytic cells, or by modulating production of arachidonic acid metabolites, such as prostaglandins.' (pg 1883)

'The release of reactive oxygen species is a characteristic feature of inflammation. These compounds (including superoxide anion, hydroxyl radical, hydrogen peroxide and singlet oxygen) may originate via the arachidonic acid cascade or during the respiratory burst which occurs during phagocytosis.' (pg 1883)

4) Vasodilation/circulation

Code   **Proposed statement**
VE4:   *Vitamin E contributes to the normal function of arteries*

Reference 1.2:
'Vitamin E enrichment of endothelial cells downregulates the expression of intercellular cell adhesion molecule (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), thereby decreasing the adhesion of blood cell components to the endothelium (Cominacini et al., 1997). Vitamin E also upregulates the expression of cytosolic phospholipase A_2 (Chan et al., 1998a; Tran et al., 1996) and cyclooxygenase-1 (Chan et al., 1998b). The enhanced expression of these two rate-limiting enzymes in the arachidonic acid cascade explains the observation that vitamin E, in a dose-dependent fashion, enhanced the release of prostacyclin, a potent vasodilator and inhibitor of platelet aggregation in humans (Szczeklik et al., 1985; Tran and Chan, 1990).' (pg 196)

'Vitamin E does inhibit LDL oxidation whether induced by cells in culture (Steinbrecher et al., 1984) or by copper ion in vitro (Dieber-Rotheneder et al., 1991; Jialal et al., Reaven et al., 1993). In addition, vitamin E could affect atherogenesis at a number of steps, based on the following in vitro observations:

- Vitamin E inhibits smooth muscle cell proliferation through the inhibition of protein kinase C (Azzi et al., 1995; Boscoboinik et al., 1991; Chatelain et al, 1993).
- Vitamin E inhibits platelet adhesion, aggregation, and platelet release reactions (Freedman et al., 1996; Higashi and Kikuchi, 1974; Ishizuka et al., 1998; Steiner and Anastasi, 1976).

- Vitamin E inhibits plasma generation of thrombin, a potent endogenous hormone that binds to platelet receptors and induces aggregation (Rota et al., 1998).
- Vitamin E decreases monocyte adhesion to the endothelium by downregulating expression of adhesion molecules (Devaraj et al., 1996; Faruqi et al., 1994; Islam et al., 1998; Martin et al., 1997; Molenaar et al., 1989) and decreasing monocyte superoxide production (Cachia et al., 1998; Islam et al., 1998)
- In human endothelial cells, vitamin E potentiates synthesis of prostyacyclin, a potent vasodilator and inhibitor of platelet aggregation (Chan and Leith, 1981; Szczeklik et al., 1985; Thorin et al., 1994; Tran and Chan, 1990)
- Vitamin E mediates upregulation of the expression of cytosolic phospholipase A_2 and cyclo-oxygenase (Chan et al., 1998a,b; Tran et al., 1996)
- Vitamin E enrichment of endothelial cells in culture inhibits the expression of intracellular cell adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1) induced by exposure to oxLDL (Cominacini et al., 1997).' (Pg 211, 212)

'Among the effects of vitamin E intakes from supplements are inhibition of LDL oxidation both in vitro and in vivo; inhibition of smooth muscle cell proliferation through inhibition of protein kinase C; inhibition of platelet adhesion, aggregation, and platelet release reactions; and inhibition of plasma generation of thrombin. In addition, supplemental intakes of vitamin E decrease monocyte adhesion to endothelium, decrease monocyte superoxide production, potentiate the synthesis of prostacyclin, upregulate the expression of phospholipase A_2 and cyclo-oxygenase, and inhibit the expression of ICAM-1 and VCAM-1 induced by exposure to oxLDL.' (pg 223)

Reference 2.0:
'Vitamin E modulates many of the important events in atherogenesis, including the oxidative modification of LDL. In the absence of vitamin E, LDL is easily oxidized and is taken up by macrophages in the arterial intima, leading to the accumulation of lipid-laden foam cells and later, fatty streaks. Its presence in the intima may also result in the release of cytotoxic compounds which damage the endothelial layer, leading to platelet aggregation, release of growth factors, and migration and proliferation of smooth muscle cells.' (pg 1882,1883)

Reference 4.3:
'The clinical manifestations of vitamin E deficiency vary considerably between species. In general, however, the targets are the neuromuscular, vascular and reproductive systems.' (pg 6)

ANNEX 4.4

Vitamin K

Source documents for reviewing vitamin K

Reference 1.1:
Institute of Medicine Dietary Reference Intakes for vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium and zinc. Washington D.C. National Academy Press, 2001.

Reference 2.0:
Encyclopedia of Human Nutrition 2E. Editor-in-chief, Michele J. Sadler, editors, J.J. Strain, Benjamin Caballero. San Diego : Academic Press, c1999.

Reference 3.5:
Opinion of the Scientific Committee on Food (SCF) on the Tolerable Upper Intake Level of Vitamin K. April 2003.
(http://www.europa.eu.int/comm/food/fs/sc/scf/out196_en.pdf).

Reference 4.4:
Revised Review of Vitamin K. Expert Group on Vitamins and Minerals. April 2002.
(http://www.food.gov.uk/multimedia/pdfs/evm-01-09p.pdf).

Reference 6.1:
Nutrition and Bone Health: with particular reference to calcium and vitamin D. Report of the Subgroup on Bone Health, Working Group on the Nutritional Status of the Population of the Committee on Medical Aspects of Food and Nutrition Policy. Report on Health and Social Subjects No. 49. DH: The Stationery Office, 1998.

1) Coagulation

Code   **Proposed statement**
VK1:   Vitamin K is necessary for normal coagulation (blood clotting).

Reference 1.1:
'Vitamin K functions as a coenzyme during the synthesis of the biologically active form of a number of proteins involved in blood coagulation and …' (pg 162)

'Vitamin K plays an essential role in the posttranslational conversion of specific glutamyl residues in a limited number of proteins to γ-carboxyglutamyl (Gla) residues (Suttie, 1993). These proteins include plasma prothrombin (coagulation factor II) and the plasma procoagulants, factors VII, IX, and X. Because under-γ-carboxylated forms of these proteins lack biological activity, the classical sign of a vitamin K deficiency has been a vitamin K-responsive increase in prothrombin time and, in severe cases, a hemorrhagic event.' (pg 163)

'A clinically significant vitamin K deficiency has usually been defined as a vitamin K-responsive hypoprothrombinemia and is associated with an increase in prothrombin time (PT) and, in severe cases, bleeding….There are numerous case reports of bleeding episodes in antibiotic-treated patients, and these have often been ascribed to an acquired vitamin K deficiency resulting from a suppression of menaquinone-synthesizing organisms. However, these reports are complicated by the possibility of general malnutrition in this patient population and by the antiplatelet of many of the same drugs (Suttie, 1995).' (pg 164)

'Although there is some interference in the hepatic synthesis of the vitamin K-dependent clotting factors that can be measure by sensitive assays, standard clinical measure of procoagulant potential are not changed.' (pg 165)

'In humans, an insufficiency of vitamin K leads to the secretion into plasma of biologically inactive, under-γ-carboxylated forms of the vitamin K-dependent clotting factors.' (pg 167)

'Concentrations of vitamin K in cord blood are usually less than 0.1 nmol/L or undetectable (Mandelbrot et al., 1988; Widdershoven et al., 1988), and elevated concentrations of undercarboxylated prothrombin (PIVKA-II) have been reported (Greer, 1995). Poor vitamin K status added to the fact that the concentrations of most plasma clotting factors are low at the time of birth increases the risk of bleeding during the first weeks of life, a condition known as hemorrhagic disease of the newborn (HDNB). Because HDNB can be effectively prevented by administration of vitamin K, infants born in the United States and Canada routinely receive 0.5 to 1 mg of phylloquinone intramuscularly or 2.0 mg orally within 6 hours of birth.' (pg 176)

Reference 2.0:
'Vitamin K serves as a cofactor of an enzyme that posttranslationally γ-carboxylates specific glutamate residues in a few proteins. These γ-carboxylglutamate (Gla) residues confer to proteins the ability of binding calcium with high affinity and specificity. The carboxylase itself is a Gla protein. Several of the blood coagulation factors (factors II, VII, IX and X) and coagulation inhibitors (proteins C and S) contain Gla without which they cannot be activated.' (pg 1928)

'Traditionally, blood-clotting assays have been used to monitor vitamin K activity…Blood-clotting assays are based on the fact that the coagulation factors II, VII, IX and X are vitamin-K dependent proteins which are incompletely carboxylated during vitamin K deficiency and thus have reduced activity.' (pg 1930)

'The earliest and most devastating effect of vitamin K deficiency concerns infants during their first weeks of life…This naturally low vitamin K status during intrauterine life is associated with low blood coagulability…For this reason most health services now either recommend or require prophylactic vitamin K administration for all newborns…' (pg 1931)

'Normal or near normal blood coagulation is usually maintained in older children and adults even in the absence of dietary vitamin K, presumably because the small amounts of bacterial menaquinones from the lower intestine are sufficient for this function….However, if vitamin K production by the normal intestinal flora is reduced

at the same time, as during antibiotic treatment or as a consequence of diarrhoea, significant and even dangerous bleeding may occur within days. In the absence of liver disease normal blood coagulation is restored within one or two days by the administration of vitamin K.' (pg 1931,1932)

Reference 3.5:
'The prime physiological relevance of phylloquinone is the synthesis of coagulation proteins (Ferland, 1998; Olson, 1999 and 2000).' (pg 4)

Reference 4.4:
'Vitamin K was first identified in 1935 by Dam, who identified it as the fat-soluble factor necessary for the coagulation of blood. The primary function of vitamin K is to catalyse the synthesis of prothrombin by the liver. In the absence of vitamin K, hypoprothrombinaemia occurs in which blood clotting time may be greatly prolonged. Blood coagulation is a highly complex process, the mechanism of which is not fully understood. It involves cells such as thrombocytes, platelets and erythrocytes, numerous protein factors and Ca^{2+}. Essentially, a cascade of protein factors catalyses the reaction prothrombin to thrombin, the latter protein then converting soluble fibrinogen into insoluble fibrin which forms the basis of the blood clot. Vitamin K is known to be involved in the hepatic synthesis of at least four of the protein factors, which include prothrombin (factor II), proconvertin (factor VII), thromboplastin (factor IX) and the Stuart-Prower factor (factor X) (Committee on Nutrition 1961, Basu and Dickerson 1996).' (pg 9, 10)

'Vitamin K is thought to be necessary for formation of Ca^{2+} binding sites on prothrombin (Gallop *et al* 1980, Olson 1984). These are essential for prothrombin to be bound to phospholipids, for activation to thrombin. In the presence of dicoumarol, a very potent antagonist of vitamin K the prothrombin produced *in vivo* has a very low Ca^{2+} binding capacity. The Ca^{2+} binding sites of prothrombin are formed by the introduction of a second carboxyl group into the glutamyl side-chains, located in the amino-terminal region of the protein. Once carboxylated, the glutamates are referred to as γ-carboxyglutamic acid (GLA). When the action of vitamin K is blocked by dicoumarol, calcium ions cannot bind to prothrombin because the protein lacks added carboxyl groups. The formation of vitamin K epoxide is an obligatory step in the action of vitamin K in the biosynthesis of prothrombin.' (pg 10)

'Like prothrombin, factors VII, IX, and X have been found to have a series of glutamic acid residues and vitamin K is also needed for the carboxylation of these residues (Gallop *et al* 1980, Olson 1984). The vitamin K-dependent carboxylation is carried out by a liver microsomal enzyme, through a molecular mechanism that is not fully understood. It is believed to require reduced vitamin K (or its epoxide) and CO_2. The process appears to be coupled with the simultaneous epoxidation of vitamin K hydroquinone, the active form of the vitamin.' (pg 10)

2) Bone

Code   **Proposed statement**
VK2:   *Vitamin K contributes to the normal structure of bone*

Reference 1.1:
'Vitamin K functions as a coenzyme during the synthesis of the biologically active form of a number of proteins involved in … and bone metabolism.' (pg 162)

'Two structurally related vitamin K-dependent proteins (Price, 1988), osteocalcin found in bone and matrix Gla protein originally found in bone but now known to be more widely distributed, have received recent attention as proteins with possible roles in the prevention of chronic disease (Ferland, 1998).' (pg 163)

'Small amounts of the bone protein, osteocalcin, circulate in plasma, and like PIVKA-II, under-γ-carboxylated osteocalcin (ucOC) has been considered an indicator of suboptimal vitamin K status….Only recently has direct assessment of ucOC been possible with the development of a monoclonal antibody specific for the undercarboxylated form of osteocalcin (Vergnaud et al., 1997).' (pg 168,169)

'…a number of reports have correlated decreased bone mineral density (BMD) or increased fracture rate with a five to eight-fold increase in ucOC. Concurrently, it has been observed that vitamin K intakes similar to those reported for the general population did not ensure complete carboxylation of osteocalcin (Bach et al., 1996; Sokoll and Sadowski, 1996) and that ucOC could be decreased by increasing vitamin K intake (Binkley et al., 1999); Booth et al., 1999b; Douglas et al., 1995; Knapen et al., 1989, 1993). These reports have led to the suggestion that vitamin K requirements for bone function are probably much higher than those needed to maintain normal hemostasis and that the recommendation for vitamin K should be much higher than current recommendations (Weber, 1997).' (pg 169)

'Although there is little doubt that vitamin K intake affects the degree of osteocalcin λ-carboxylation …. the physiological significance of diet-induced changes prevent the use of ucOC for estimating an average requirement for vitamin K.' (pg 170)

'More recently, lower circulating phylloquinone and menaquinone concentrations have been observed in subjects with reduced BMD (Kanai et al., 1997; Tamatani et al., 1998) though other studies have not confirmed this finding (Rosen et al., 1993)….The role of vitamin K in bone metabolism has also been investigated by studying the vitamin K bone protein osteocalcin and its undercarboxylated from ucOC. The extent to which osteocalcin is undercarboxylated has been assessed with respect to age, bone status, and risk of hip fracture (Binkley and Suttie, 1995; Vermeer et al., 1996). Although ucOC was reported to increase with age in some studies (Knapen et al., 1998; Liu and Peacock, 1998; Plantalech et al., 1991), other reports have not confirmed this finding (Sokoll and Sadowski, 1996). Negative correlations have also been reported between ucOC and BMD, but the strength of the associations has varied depending on the population studied (Knapen et al., 1998; Liu and Peacock, 1998; Vergnaud et al., 1997). Although the observed relationship between ucOC and BMD is of interest, it requires further investigation as significant inverse relationships have also been observed between BMD and total osteocalcin (Liu and Peacock, 1998; Ravn et al., 1996) and between BMD and the active (carboxylated) form of osteocalcin (Knapen et al., 1998).' (pg 170, 171)

'Whether vitamin K intake is a significant etiological component of osteoporosis is difficult to establish on the basis of the studies performed thus far.' (pg 172)

Reference 2.0:
'Three additional Gla proteins have been completely characterized, but have functions not related to haemostasis; bone Gla protein (BGP, osteocalcin), matrix Gla protein (MGP) and …The function of BGP, which is produced almost exclusively in osteoblasts and odontoblasts, is still obscure; it is most likely related to the control of mineralizing activities by these cells.' (pg 1928)

'At least three vitamin K-dependent proteins (BGP, MGP and protein S) are produced in bone, suggesting that vitamin K may be important for bone health…the improvement of vitamin K status has been shown to minimize loss of bone minerals. While it remains to be seen whether supplemental vitamin K in later age actually reduces bone fracture risk, long-term vitamin K status appears to be important for bone health.' (pg 1932)

'Little is known about the mechanism(s) through which vitamin K status influences bone. Bone is constantly remodelled by osteoclastic breakdown and subsequent osteoblastic rebuilding. BGP has been suggested as a mediator that links osteoclastic and osteoblastic activities; undercarboxylated BGP has been found to be ineffective for this function. As a consequence, the osteoclastic breakdown cycle may continue longer during suboptimal than during optimal vitamin K status. Another mechanism may be an inhibiting effect of vitamin K on interleukin 6 (IL-6) production. IL-6 relays the action on osteoblasts of various mediators such as parathyroid hormone (PTY) to osteoclasts (which do not have PTY receptors themselves). Vitamin K might thus dampen the catabolic effect of such hormones and limit bone mineral loss. Finally, there may be a more or less direct effect of vitamin K on PTH levels. It has recently been observed that secondary hyperparathyroidism due to renal failure is much less prevalent among patients with optimal vitamin K status compare to those with poorer vitamin K status.' (pg 1932)'

Reference 3.5:
'…vitamin K is also essential for the synthesis of a number of proteins…the bone Gla-protein, osteocalcin, which is exclusively synthesised by osteoblasts and odontoblasts, and which is a negative regulator of bone formation.' (pg 4)

'Vitamin K is required for the ã-carboxylation of glutamate in 2 proteins induced by the vitamin D hormone in bone. Osteocalcin is a 49-residue protein with 3 carboxyglutamic acid residues, is water soluble, adheres to the bone mineral hydroxyapatite and is secreted by osteoblasts.' (pg 4)

'The level of osteocalcin carboxylation has been proposed as an indicator of the nutritional state of the bone with respect to vitamin K. Circulating levels of undercarboxylated osteocalcin may be a sensitive marker of vitamin K inadequacy. These levels of undercarboxylate osteocalcin have been reported to be increased both in postmenopausal women and in individuals who sustain hip fracture (Binkley and Suttie, 1995; Vermeer et al., 1995; Szulc et al., 1993 and 1994; Knapen et al., 1998; Luukinen et al,. 2000).' (pg 4)

Reference 4.4:

'Proteins containing GLA have been identified in bone (Price 1988). There appear to be at least two GLA-containing proteins in bone, called bone GLA protein (BGP) or osteocalcin, and matrix GLA protein (MGP). The functions of these proteins have not been clearly defined, but there is an accumulation of evidence suggesting that they may participate in the modulation of bone mineralisation.' (pg 10)

'Osteocalcin is one of the most abundant non-collagenous proteins in the extra-cellular matrix of the bone. Its precise function is uncertain but it appears to be a marker of osteoblast activity (Shearer, 1995). Osteocalcin contains three GLA residues spaced at the same interval as calcium ions in the hydroxyapatite lattice. The appearance of osteocalcin in bones has been shown, using embryonic chick bones, to coincide with the beginning of mineralisation. Injection of vitamin K antagonists into eggs containing developing embryos, has been shown to result in a reduction of the GLA content of osteocalcin by 20-50% (Hauschka *et al* 1978).' (pg 10)

'Undercarboxylated (partially functional) osteocalcin may be associated with low bone mineral density and risk of hip fracture (Shearer, 1995, DH 1998) in older women….Schaafsma et al (2000) studied the effect of daily vitamin D_3 and phylloquinone supplements in postmenopausal women with normal and low bone mineral density….At baseline, women with normal BMD had significantly higher percentage carboxylated osteocalcin (%carbOC) and across the whole group, %carbOC was positively correlated with BMDs of the lumbar spine and femoral neck. After 6 and 12 months women with normal BMD who had received phylloquinone (alone or with vitamin D) had significantly higher %carbOC compared to the placebo group and to baseline. In women with low BMD %carbOC rose significantly from baseline values in both groups but the phylloquinone-vitamin D group were not significantly different to the vitamin D group.' (pg 11)

'The function of MGP is unclear, but is has been related to the action of the active metabolite of vitamin D ($1,25-(OH)_2D_3$) and therefore the mobilisation and deposition of bone calcium (Price and Baukol 1980).' (pg 11)

'It has been suggested that kidney GLA protein (KGB) is involved in the reabsorption of Ca^{2+} by the kidney tubules, a function related to vitamin D action. It is thought that KGB may solubilise calcium salts in urine. Sakamoto *et al* (1999) reported that urinary calcium excretion was lower in subjects considered to have high dietary vitamin K intakes.' (pg 11)

Reference 6.1:
'Vitamin K status might influence bone health as several vitamin K-dependent proteins, including osteocalcin and matrix gla-protein, are involved in bone mineralisation. Low dietary intake of vitamin K is associated with an elevated proportion of under-carboxylated (partially functional) osteocalcin and this has been associated with low BMD and increased risk of hip fracture in older women.' (pg 53)

3) Arteries

Code   **Proposed statement**
VK3:   *Vitamin K contributes to the normal function of arteries*

Reference 1.1:
'A role for vitamin K in atherosclerosis was hypothesized when proteins containing Gla residues were isolated from hardened atherosclerotic plaque (Gijsbers et al., 1990; Levy et al, 1979). These were later identified as osteocalcin and matrix Gla proteins (Ferland, 1998).' (pg 173)

Reference 2.0:
'Three additional Gla proteins have been completely characterized, but have functions not related to haemostasis; bone Gla protein (BGP, osteocalcin), matrix Gla protein (MGP) and …MGP has been shown to modulate the nucleation of calcium crystals in a variety of tissues and is essential for the prevention of arterial wall calcification.' (pg 1928)

Reference 3.5:
'…vitamin K is also essential for the synthesis of a number of proteins…matrix Gla-protein (MGP) which is synthesised in most soft tissues, but predominantly in cartilage (by chondrocytes) and in vessel wall (by vascular smooth muscle cells) and which is a potent inhibitor of soft tissue calcification'. (pg 4)

'Matrix carboxyglutamic acid (Gla) protein contains 79 amino acid residues of which 5 are Gla residues. It is hydrophobic, insoluble in plasma, and is associated with the matrix of cartilage and bone as well as with the tunica media of the arterial vessel wall (Olson, 2000).' (pg 4)

4) Embryonic development

Code   **Proposed statement**
VK4:   *Vitamin K contributes to normal embryonic development*

Reference 2.0:
'Three additional Gla proteins have been completely characterized, but have functions not related to haemostasis; …and growth-arrest specific protein (gas6)…Gas6 is the specific ligand of the tyrosine-kinase receptor axl which participates in growth and differentiation modulating cell signalling.' (pg 1928)

Reference 3.5:
'…vitamin K is also essential for the synthesis of a number of proteins…growth arrest-specific gene 6 protein (Gas6), which is a ligand for tyrosine kinases and has strong apoptopic activity in cultured cells.' (pg 4)

Reference 4.4:
'Growth arrest-specific protein (Gas 6) is vitamin K dependent and may be a ligand for tyrosine kinases. In addition, sequence analysis suggests a possible role for vitamin K in cell signalling. It has been suggested (Israels, *et al*, 1997) that the level

of vitamin K in the newborn is tightly regulated because of the involvement of vitamin K dependent proteins in tyrosine kinases signalling and thus in growth regulation in the developing foetus. Tight control of vitamin K levels would be necessary to ensure normal embryonic development.' (pg 12)

Summary Table
Reference 2.0, (pg 1929):
Human vitamin K-dependent proteins:

Name	No. of Gla residues	Function
Factor II	10	Blood coagulation cascade
Factor VII	10	Blood coagulation cascade
Factor IX	12	Blood coagulation cascade
Factor X	11	Blood coagulation cascade
Protein C	9	Inhibitor of coagulation
Protein S Receptor	11	Activator of protein C, ligand of dte (growth modulation)
Protein Z	13	Enhancement of blood coagulation
gas6	5	Ligand of axl receptor (growth modulation)
Osteocalcin (bone Gla protein, BGP)	2-3	Regulation of bone mineralization (mechanism unknown)
Matrix Gla protein (MGP)	5	Modulator of calcium crystal nucleation
Vitamin K dependent carboxylase	?	Carboxylation of Gla proteins
Galactocerebroside sulfotransferase	?	Sulfatide biosynthesis in brain
n-Sulfatidase	?	Sphingolipid catabolism

ANNEX 4.5

Thiamin (B1)

Source documents for reviewing thiamin (B1)

Reference 1.3:
Institute of Medicine Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B_6, folate, vitamin B_{12}, pantothenic acid, biotin and choline. Washington D.C. National Academy Press 2000.

Reference 2.0:
Encyclopedia of Human Nutrition 2E. Editor-in-chief, Michele J. Sadler, editors, J.J. Strain, Benjamin Caballero. San Diego : Academic Press, c1999.

Reference 3.6:
Opinion of the Scientific Committee on Food (SCF) on the Tolerable Upper Intake Level of Vitamin B_1. July 2001.
(http://www.europa.eu.int/comm/food/fs/sc/scf/out93_en.pdf).

Reference 4.5:
Revised Review of Thiamin. Expert Group on Vitamins and Minerals. August 2002.
(http://www.food.gov.uk/multimedia/pdfs/evm0014p.pdf).

Reference 5.0:
Handbook of Nutrition and Food. Edited by Carolyn D Berdanier...[et al.]. Boca Raton, Fla.: CRC Press, 2002.

1) Carbohydrate metabolism

Code   **Proposed statement**
Th1:   *Thiamin is necessary for the normal metabolism of carbohydrates*

Reference 1.3:
'Thiamin functions as a coenzyme in the metabolism of carbohydrates and branched-chain amino acids.' (pg 58)

'Chemically, thiamin consists of substituted pyrimidine and thiazole rings linked by a methylene bridge. It exists mainly in various inter-convertible phosphorylated forms, chiefly thiamin pyrophosphate (TPP). TPP, the coenzymatic form of thiamin, is involved in two main types of metabolic reactions: decarboxylation of α-ketoacids (e.g., pyruvate, αketoglutarate, and branched-chain keto acids) and transketoloation (e.g., among hexose and pentose phosphates).' (pg 58, 59)

Reference 2.0:
'The active form of thiamin in the body is TDP which acts as a coenzyme/cofactor in the oxidative phosphorylation of α-ketoacids and in the transketolase reactions. These

two reaction pathways are involved in glucose metabolism so that thiamin is mainly required for energy metabolism.' (pg 1859)

'The decarboxylation and oxidation of pyruvate gives acetyl-S-Coenzyme A, which then enters the tricarboxylic acid (Krebs) cycle where further oxidation yields carbon dioxide and water. This oxidative decarboxylation is accomplished by a multienzyme pyruvate dehydrogenase complex (PDHC), comprising three enzymes, a TDP-dependent pyruvate decarboxylase, a lipoic acid-bound dihydrolipoyl transacetylase and a dihydrolipoyl dehydrogenase (an FAD-dependent enzyme) which reoxidizes the reduced lipoic acid. An analogous series of reactions involving the α-ketoglutarate dehydrogenase complex (αKGDHC) catalyses the conversion of α-ketoglutarate to succinyl-S-CoA in the tricarboxylic acid (TCA) cycle. The decarboxylation of the three branched-chain α-ketoacids derived from the deamination of leucine, isoleucine and valine, namely α-ketoisocaproic acid, α-keto-β-methylvaleric acid and α-ketoisovaleric acid, is achieved by a multienzyme complex similar to those described above.' (pg 1859)

'Here the transketolase TDP reacts with the appropriate ketosugars to break the carbon-to-carbon bond between C2 and C3 to form a TDP-glycoaldehyde intermediate which is transferred to a suitable acceptor aldehyde in the pentose or hexose monophosphate shunt (HMPS) pathway for the oxidation of glucose. Glycolysis is the main pathway for the oxidation of glucose and this second metabolic pathway for glucose is important, not so much for energy production (as is the TCA cycle), as for the production of pentoses for RNA and DNA synthesis and NADPH for the biosynthesis of fatty acids and other products, while also supplying intermediate sugars for glycolysis.' (pg 1859-1861)

'The UK dietary reference value (DRV) for thiamin is expressed per energy intake because of its essential role in energy metabolism....The thiamin requirement is related to metabolic rate and is greatest when carbohydrate is the energy source.' (pg 1862)

'In maple syrup urine disease (MSUD), also called branched-chain ketoaciduria, where the branched-chain α-ketoacids derived from the three amino acids are not decarboxylated but are excreted in the urine, patients are not thiamin-deficient but many illustrate a dependency and a response to large doses of thiamin or TDP.' (pg 1863)

Reference 3.6:
'Vitamin B_1 mainly acts in α-ketoacid decarboxylation (e.g. pyruvate, α-ketoglutarate and branched-chain α-ketoacid acids), in transketolation (e.g. among hexose and pentose phosphates) ...' (pg 2)

'Animal experiments have shown that the rate of vitamin B_1 utilisation depends on the amount of carbohydrate metabolised. Because the principal metabolic role is in energy-yielding metabolism the requirement is related to energy intake.' (pg 3)

Reference 4.5:

'The major coenzymatic form of thiamin is thiamin pryrophosphate (TPP0), which requires ATP, Mg $^{2+}$ and thiaminpyrophospokinase for its synthesis. TPP functions as coenzyme in the following enzymic reactions:

(i)   Non-oxidative decarboxylation of α-ketoacids 0 catalysed by pyruvate decarboxylase (mainly plants and yeast, first step in alcoholic fermentation) $RCOCOOH \rightarrow RCHO + CO_2$.

(ii)   Oxidative decarboxylation – catalysed by the pyruvate, α-ketoglutarate (and other α-keto acids) and branched-chain amino acid (leucine, isoleucine and valine) dehydrogenase multienzyme complex systems. All these enzymes are intramitochondrial and produce acetyl-coenzyme A (CoA), succinyl CoA and the appropriate derivatives of branched chain amino acids, respectively, which are important in carbohydrate and lipid metabolism.

(iii)   Transketolation – catalysed by cytosolic transketolase. This is an important reaction in the pentose phosphate pathway, and allows the reversible conversion of three-, four-. five-, six- and seven- carbon sugars by the transfer of two- or three-carbon moieties. This pathway provides the major source of pentose sugars for the synthesis of nucleic acids and NADPH for fatty acid synthesis. (Rindi, 1996 and references therein).' (pg 6)

2) Neurological and cardiac systems

Code   Proposed statement
Th2:   Thiamin is necessary for normal neurological and cardiac function.

Reference 1.3:
'The clinical signs of deficiency include … mental changes such as apathy, decrease in short-term memory, confusion, and irritability; muscle weakness; and cardiovascular effects such as an enlarged heart (Horwitt et al., 1948; Inouye and Katsura, 1965; Platt, 1967; Williams et al., 1942; Wilson, 1983).' (pg 59)

'A controlled-diet, dose-response experiment was conducted with nine girls aged 16 to 18 years to examine the thiamin requirement (Hart and Reynolds, 1957)….The authors noted that the subjects became irritable and uncooperative and lost the ability to concentrate when fed the low-thiamin diet – symptoms also noted by others in the early stage of thiamin deficiency.' (pg 67, 68)

Reference 2.0:
'Thiamin, as TTP, may have a part to play in nerve cell transmissions.' (pg 1859)

'Polyneuritis, which is so often a feature of thiamin deficiency, is evidence of a specific function in neural tissues, and the existence of TPP in brain and other neural tissues suggests a direct role for thiamin in neural excitation. It has been repeatedly demonstrated that the stimulation of nerves or treatment with certain neuroactive drugs results in a decrease in the level of TDP and particularly TTP in the nerve, concomitant with an increase in free TMP in the surrounding fluid. It has been postulated that TTP plays an essential role in nerve transmission involving a gating

mechanism for Na+ and K+ transport via (Na+-K+) - ATPase. This is supported by the fact that nerves contain a constant and significant level of TTP (10%) and also that patients with Leigh disease (Subacute necrotizing encephalomyelopathy) have a deficiency of TTP (but normal TDP), and this is accompanies by severe neurological involvement. The presence of an inhibitor of TTP synthesis from TDP is though to be the contributing factor here.' (pg 1861)

'Thiamin has also been shown to bear a relationship to the levels and functions of various neurotransmitters namely the serotenergic, adrenergic and cholinergic systems. Whether, or to what extent, changes in these systems are responsible for the neurologic symptoms of thiamin deficiency remains to be established.' (pg 1861)

'Beriberi is the traditional thiamin deficiency disease and … is characterized by involvement of the nervous and cardiac systems, with one or other system being predominantly affected: the cardiac system in wet beriberi and the nervous system in dry beriberi. In addition to beriberi, the decreased activity of cerebral thiamin-dependent congenital lactic acidosis, intermittent ataxia of childhood, Leigh disease and the Wernicke-Korsakoff syndrome. Recent evidence also suggests that thiamin neurochemistry is disrupted in Alzheimer's disease. The role of thiamin deficiency in Wernicke-Korsakoff syndrome is well established. This syndrome and Alzheimer's disease are both associated with marked loss of cholinergic neurons in the nucleus basalis and with memory loss, suggesting that alterations of thiamin-dependent enzymes and/or disrupted neurotransmissions could also be implicated in the pathophysiology of Alzheimer's disease.' (pg 1861)

Reference 3.6:
'Vitamin B$_1$ mainly acts in … and possibly in nerve conduction.' (pg 2)

Reference 4.5:
'Thiamin is a pharmacologic antagonist of acetylcholine, which may explain the nervous lesions caused by thiamin deficiency (Baugartner, 1991 and references therein).' (pg 6)

'A non-coenzymatic function for TPP has been proposed in nervous tissue. TPP is concentrated in neuronal cells and other excitable tissues such as skeletal muscle …' (pg 6)

'Clinical deficiency in humans, and various animals, results in the disease known as beriberi, the major manifestations of which mainly affect the cardiovascular (wet beriberi) and nervous systems (dry beriberi). Cardiovascular manifestations of beriberi include cardiac hypertrophy and dilatation, particularly of the right ventricle, tachycardia, respiratory stress and oedema of the legs. Neurological manifestations typically affect the lower extremities and include exaggerated tendon reflexes, polyneuritis and sometimes paralysis. In later stages of the disease, the upper extremities are also affected, resulting in muscle weakness and pain and convulsions. "Burning feet" syndrome may also be a manifestation of thiamin deficiency, appearing early on in the course of polyneuropathy. In more severe cases, both cardiovascular and neurological symptoms may be present and the disease can be fatal.' (pg 7)

Reference 5.0:
'Deficiency symptoms: …abnormalities of the electrocardiogram. Severe thiamin deficiency of long duration culminates in beriberi, the symptoms of which are…disturbances of heart function…' (pg 84)

'More recent evidence suggests that thiamin has a role beyond that of a coenzyme in regulating transmission of impulses in peripheral nerves. …The symptoms, as they progress, are often followed by… and tachycardia. When thiamin deficiency is advanced, the patient usually exhibits prominent cardiovascular and neurological features. Cardiac findings include an enlarged heart, tachycardia, edema, and ST-segment and T-wave changes. There is high output failure due, at least in part, to the peripheral vasodilation. The clinical syndrome has a number of similarities to apathetic hyperthyroidism, with which it often confused.' (pg 1321)

ANNEX 4.6

Riboflavin (B$_2$)

Source documents for reviewing riboflavin (B$_2$)

Reference 1.3:
Institute of Medicine Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B$_6$, folate, vitamin B$_{12}$, pantothenic acid, biotin and choline. Washington D.C. National Academy Press 2000.

Reference 2.0:
Encyclopedia of Human Nutrition 2E. Editor-in-chief, Michele J. Sadler, editors, J.J. Strain, Benjamin Caballero. San Diego : Academic Press, c1999.

Reference 3.7:
Opinion of the Scientific Committee on Food (SCF) on the Tolerable Upper Intake Level of Vitamin B$_2$. November 2000. (http://www.europa.eu.int/comm/food/fs/sc/scf/out80i_en.pdf).

Reference 4.6:
Revised Review of Riboflavin. Expert Group on Vitamins and Minerals. August 2002. (http://www.food.gov.uk/multimedia/pdfs/reviewriboflavin).

Reference 7.0:
Introduction to Human Nutrition. Edited on behalf of The Nutrition Society by Michael J. Gibney, Hester H. Vorster and Frans J. Kok. Blackwell Science, September 2002.

1) Release of energy from food

Code **Proposed statement**
Ri1:   *Riboflavin contributes to the normal release of energy from food.*

Reference 1.3:
'The primary form of the vitamin is an integral component of the coenzymes, flavin mononucleotide (FMN) and flavin-adenine dinucleotide (FAD) (McCormick, 1994; McCormick and Greene, 1994; Merrill et al., 1981). It is in these bound coenzyme forms that riboflavin functions as a catalyst for redox reactions in numerous metabolic pathways and in energy production (McCormick and Greene, 1994).' (pg 87, 88)

'The redox reactions in which flavocoenzymes participate include flavoprotein-catalyzed dehydrogenations that are both pyridine nucleotide (niacin) dependent and independent, reactions with sulfur-containing compounds, hydroxylations, oxidative decarboxylations (involving thiamin as its pyrophosphate), dioxygenations, and reduction of oxygen to hydrogen peroxide (McCormick and Greene, 1994). There are obligatory roles of flavocoenzymes in the formation of some vitamins and their coenzymes. For example, the biosynthesis of two niacin-containing coenzymes from tryptophan occurs via FAD-dependent kynurenine hydroxylase, an FMN-dependent oxidase catalyzes the conversion of the 5'-phosphates of vitamin B$_6$ to coenzymic

pyridoxal 5'-phoshpate, and an FAD-dependent dehydrogenase reduces 5,10-methylene-tetrahydrofolate to the 5'-methyl product that interfaces with the B_{12}-dependent formation of methionine from homocysteine and thus with sulfur amino acid metabolism.' (pg 88)

'Riboflavin interrelates with other B vitamins, notably niacin, which requires FAD for its formation from tryptophan, and vitamin B_6, which requires FN for conversion to the coenzyme pyridoxal 5'-phosphate (McCormick, 1989).' (pg 96)

Reference 2.0:
'The first example of serious metabolic disturbance, seen in moderate riboflavin deficiency, is the disturbance of fatty acid oxidation. The normal first stage in the spiral process of β-oxidation of fatty acids within the mitochondria is the removal of two hydrogen atoms from the two carbons located α and β to the activated carboxyl end of the chain. The fatty acyl coenzyme A substrate is acted upon by one of several fatty acyl-CoA dehydrogenase flavoprotein enzymes (e.g. long-chain acyl-CoA: (acceptor) 2,3- oxidoreductase EC 1.3.99.13), each of which is specific for a small range of acyl chains. The second stage in this process involves transfer of the electrons via another flavoenzyme, known as 'electron transferring flavoprotein dehydrogenase' (electron-transferring-flavoprotein; ubiquinone oxidoreductase EC 1.5.5.1), and thence to the cytochrome chain and to oxygen. These flavoenzymes, unlike the flavoenzymes that are linked to carbohydrate oxidation, are highly sensitive to dietary riboflavin depletion.' (pg 1726)

'Several studies have documented an apparent increase in riboflavin requirements accompanying an increase physical exercise in human subjects. This may reflect the fact that anabolic influences and the accretion of new lean body mass creates a demand for the vitamin, for mitochondrial accretion.' (pg 1727)

'Although even a severe riboflavin deficiency is less obviously life-threatening than some other types of malnutrition that are commonly encountered in the Third World, it can nevertheless be a major source of debility, through skin lesions and metabolic dysfunctions, and riboflavin nutrition thus deserves an important place in future public health programmes.' (pg 1729)

Reference 3.7:
'Riboflavin is a precursor of certain essential coenzymes such as flavin mononucleotide (FMN) and flavin-adenine dinucleotide (FAD). In these coenzyme forms riboflavin functions as a catalyst for redox reactions including flavoprotein-catalyzed dehydrogenations that are either pyridine nucleotide dependent or independent reactions with sulphur-containing compounds, hydroxylations, oxidative carboxylations, dioxygenations and the reduction of oxygen to hydrogen peroxide. Flavo-coenzymes are also involved in the biosynthesis of niacin-containing coenzymes from tryptophan via FAD-dependent kynurenine hydroxylase, the FMN dependent conversion of the 5'–phosphates of vitamin B_6 to pyridoxal 5'-phosphate and the FAD-dependent dehydrogenation of 5,10-methylene-tetrahydrofolate to the 5'–methyl product, with the vitamin B_{12} – dependent formation of methionine and sulphur amino metabolism.' (pg 2)

Reference 4.6:

'Clinically, riboflavin promotes normal growth, is required for the breakdown of fat, and assists in the synthesis of steroids and glycogen and formation of red blood cells. FAD plays roles in oxidation-reduction reactions as well, interacting with a group of enzymes known as flavoproteins.' (pg 6)

2) Transport and metabolism of iron

Code   **Proposed statement**
Ri2:   *Riboflavin contributes to the normal transport and metabolism of iron in the body.*

Reference 1.3:
'The signs of riboflavin deficiency (ariboflavinosis) in humans are … normochromic, normocytic anemia associated with pure erythrocyte cytoplasia of the bone marrow (Wilson, 1983). Riboflavin deficiency is most often accompanied by other nutrient deficiencies….' (pg 90)

Reference 2.0:
'An important interaction of riboflavin with iron economy has been suspected for many years, partly because iron-deficient animals failed to respond readily to iron supplements if they were also riboflavin-deficient, and also because the redox system involving riboflavin and its coenzymes has been shown to interact very readily with the redox system between ferric and ferrous iron.' (pg 1726)

'Some studies in experimental animals have shown that not only is there evidence for some impairment of absorption of iron in riboflavin-deficient animals, and of its distribution between discrete compartments within the body, but also – more surprisingly and strikingly – a major increase in rates of iron loss from the intestinal mucosa, resulting in impaired retention of the body iron stores. This enhanced rate of iron loss is accompanied by hyperproliferation of crypt cells and increased cellular transit along the villi, leading to an excessive proportion of immature villi, and probably also to a reduction in absorptive area. These studies begin to explain how a combination of iron deficiency and riboflavin deficiency, which is frequently encountered in human populations in many developing countries, may lead to a gradual deterioration of iron status, which is often accompanies by other intestinal lesions and by impaired gut function.' (pg 1726,1727)

Reference 3.7:
'Riboflavin …is thought also to be necessary for the absorption of iron, since it is common for iron deficiency to accompany a deficiency in riboflavin (Butler and Topham 1993).' (pg 6)

Reference 7.0:
'Riboflavin deficiency is sometimes associated with hypochromic anaemia as a result of impaired iron absorption…the mobilisation of iron bound to ferritin in mucosal cells for transfer to transferrin requires oxidation by a flavin-dependent enzyme.' (pg 149)

3) Mucous membranes

Code   **Proposed statement**
Ri3:   *Riboflavin contributes to the normal structure of mucous membranes (such as the surface of the tongue, the mouth, eyes and intestines).*

Reference 1.3:
'The signs of riboflavin deficiency (ariboflavinosis) in humans are sore throat; hyperemia and edema of the pharyngeal and oral mucous membranes; cheilosis; angular stomatitis; glossitis (magenta tongue); seborrheic dermatitis; and…' (pg 90)

'…lens opacities in humans have been associated with high glutathione reductase activity (with FAD) (Leske et al., 1995)…' (pg 94)

Reference 2.0:
'Some studies in experimental animals have shown that not only is there evidence for some impairment of absorption of iron in riboflavin-deficient animals, and of its distribution between discrete compartments within the body, but also – more surprisingly and strikingly – a major increase in rates of iron loss from the intestinal mucosa, resulting in impaired retention of the body iron stores. This enhanced rate of iron loss is accompanied by hyperproliferation of crypt cells and increased cellular transit along the villi, leading to an excessive proportion of immature villi, and probably also to a reduction in absorptive area. These studies begin to explain how a combination of iron deficiency and riboflavin deficiency, which is frequently encountered in human populations in many developing countries, may lead to a gradual deterioration of iron status, which is often accompanies by other intestinal lesions and by impaired gut function.' (pg 1726,1727)

Reference 4.6:
'Riboflavin helps to maintain the integrity of mucous membranes, skin, eyes and the nervous system…' (pg 6)

Reference 7.0:
Riboflavin deficiency is characterized by lesions of the margin of the lips (cheilosis) and corners of the mouth (angular stomatitis), a painful desquamation of the tongue, so that it is red, dry and atrophic (magenta tongue), and sebhorreic dermatitis, with filiform excrescences, affecting especially the nasolabial folds, eyelids and ears, with abnormalities of the skin around the vulva and anus and at the free border of prepuce. There may also be conjunctivitis with vascularization of the cornea and opacity of the lens. This last is the only lesion of aribflavinosis for which the biochemical basis is known: glutathione is important in maintaining the normal clarity of crystalline in the lens, and glutathione reductase is a flavoprotein that is particularly sensitive to riboflavin depletion.' (pg 148)

4) Fetal Growth

Code   **Proposed statement**
Ri4:   *Riboflavin contributes to normal fetal growth*

Reference 1.3:
'Maternal riboflavin intake (estimated from a crosscheck dietary history) was positively associated with foetal growth in a study of 372 pregnant women (Badart-Smook et al., 1997), but the data are insufficient to warrant use of foetal growth as an indicator for setting the riboflavin requirement for pregnant women. For pregnancy an additional riboflavin requirement of 0.3mg/day is estimated based on increased growth in maternal and fetal compartments and a small increase in energy utilization.' (pg 110, 111)

Reference 2.0:
'Riboflavin is secreted into milk, the concentration being species-specific and to a moderate extend dependent on maternal status and intake. Riboflavin is also required by the developing fetus, which implies a need for active transport from the maternal to the fetal circulation during pregnancy, the flavin concentration being greater on the fetal side. Studies from India have identified a specific riboflavin concentration carrier protein (RCP) present in bird (e.g. chicken) eggs, which is considered to be specific for riboflavin, and is essential for normal embryonic development….A homologous protein…has been shown to occur in…two species of monkeys and also in humans. There remains some controversy over the interpretation of these data and other, less specific, riboflavin binders in blood may also play an important role. These studies have provided an intriguing example of the role of specific vitamin-transporting mechanisms, designed to ensure that the vitamin needs of developing embryos has been provided by the demonstration that riboflavin analogues can cause teratogenic changes, even in the absence of any detectable damage to maternal tissues.' (pg 1724,1725)

Reference 4.6:
'Clinically, riboflavin promotes normal growth…' (pg 6)

5) Eyes

Code   **Proposed statement**
Ri5:   *Riboflavin contributes to the normal structure of eyes*

Reference 1.3:
'…lens opacities in humans have been associated with high glutathione reductase activity (with FAD) (Leske et al., 1995)…' (pg 94)

Reference 4.6:
'Riboflavin helps to maintain the integrity of mucous membranes, skin, eyes and the nervous system…' (pg 6)

Reference 7.0:
'Riboflavin deficiency is characterized by …There may also be conjunctivitis with vascularization of the cornea and opacity of the lens. This last is the only lesion of aribflavinosis for which the biochemical basis is known: glutathione is important in maintaining the normal clarity of crystalline in the lens, and glutathione reductase is a flavoprotein that is particularly sensitive to riboflavin depletion.' (pg 148)

6) Red blood cells

Code   **Proposed statement**
Ri6:   *Riboflavin contributes to the normal structure of red blood cells*

Reference 1.3:
'…assessing riboflavin status involves the determination of erythrocyte glutathione reductase (EGR) activity…The EGR value is an enzymatic and hence functional indicator that is conventionally determined with and without the addition on flavin-adenine dinucleotide (FAD) – the coenzyme required for the activity of EGR…' (pg 90 - 91)

'Erythrocyte flavin has been used as an indicator of the cellular concentration of the vitamin in its coenzyme forms because these coenzymes comprise over 90 percent of flavin (Burch et al., 1948).' (pg 91)

Reference 2.0:
'… Riboflavin is also required by the developing fetus, which implies a need for active transport from the maternal to the fetal circulation during pregnancy, the flavin concentration being greater on the fetal side. Studies from India have identified a specific riboflavin concentration carrier protein (RCP) present in bird (e.g. chicken) eggs, which is considered to be specific for riboflavin, and is essential for normal embryonic development….A homologous protein…has been shown to occur in…two species of monkeys and also in humans. There remains some controversy over the interpretation of these data and other, less specific, riboflavin binders in blood may also play an important role...' (pg 1724 - 1725)

Reference 4.6:
'Clinically, riboflavin … assists in the … formation of red blood cells.' (pg 6)

ANNEX 4.7

Niacin

Source documents for reviewing niacin

Reference 1.3:
Institute of Medicine Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B_6, folate, vitamin B_{12}, pantothenic acid, biotin and choline. Washington D.C. National Academy Press 2000.

Reference 2.0:
Encyclopedia of Human Nutrition 2E. Editor-in-chief, Michele J. Sadler, editors, J.J. Strain, Benjamin Caballero. San Diego : Academic Press, c1999.

Reference 3.8:
Opinion of the Scientific Committee on Food (SCF) on the Tolerable Upper Intake Levels of Nicotinic Acid and Nicotinamide (Niacin) April 2002.
(http://www.europa.eu.int/comm/food/fs/sc/scf/out80j_en.pdf).

Reference 4.7:
Review of Niacin – Revised Version. Expert Group on Vitamins and Minerals. August 2002.
(http://www.food.gov.uk/multimedia/pdfs/evm-01-11r.pdf).

Reference 6.3:
Dietary Reference Values for Food Energy and Nutrients for the United Kingdom. Report of the Panel on Dietary Reference Values. Report on Health and Social Subjects No. 41. DH: The Stationery Office, 1991.

Reference 7.0:
Introduction to Human Nutrition. Edited on behalf of The Nutrition Society by Michael J. Gibney, Hester H. Vorster and Frans J. Kok. Blackwell Science, September 2002.

1) Release of energy from food

Code   **Proposed statement**
Ni1:   *Niacin is necessary for the normal release of energy from food.*

Reference 1.3:
'In the form of the coenzymes NAD and NADP, niacin functions in many biological redox reactions. NAD functions in intracellular respiration and as a codehydrogenase with enzymes involved in the oxidation of fuel molecules such as glyceraldehyde 3-phoshpate, lactate, alcohol, 3-hydroxybutyrate, pyruvate, and α-ketoglutarate…Three classes of enzymes cleave the β-*N*-glycosylic bond of NAD to free nicotinamide and catalyze the transfer of ADP-ribose in non-redox reactions (Lautier et al., 1993). Two of the three classes catalyze ADP-ribose transfer to proteins; mono-ADP-ribosyltransferases and poly-ADP-ribose polymerase (PARP). The third class

promotes the formation of cyclic ADP-ribose, which mobilizes calcium from intracellular stores in many types of cells (Kim et al., 1994).' (pg 124)

Reference 2.0:
'Some of the most important and characteristic functions of NAD manifest in the principal cellular catabolic pathways, responsible for liberation of energy during the oxidation of energy-producing fuels.' (pg 1293)

Reference 3.8:
'Niacin is the term used to describe two related compounds, nicotinic acid and nicotinamide, both of which have biological activity. Niacin is not strictly speaking a vitamin because it is formed from the metabolism of tryptophan, and is not *per se* essential to the body, providing that there is an adequate supply of the essential amino acid tryptophan (Horwitt *et al.*, 1981). Niacin is the precursor for two cofactors, NAD (nicotinamide adenine dinucleotide) and NADP (nicotinamide adenine dinucleotide phosphate), which are essential for the functioning of a wide range of enzymes involved in redox reactions.' (pg 2)

'The co-enzymes NAD and NADPH are involved in a large number of redox reactions essential for the normal functioning of mammalian cells.' (pg 2)

Reference 4.7:
'Niacin is the functional component of two important coenzymes, NAD and NADP (nicotinamide adenine dinucleotide and its phosphorylated relative), which activate over 20 dehydrogenase enzymes essential to electron transport and other cellular respiratory reactions. Most dehydrogenases are specific to either NAD or NADP, however, a small number of dehydrogenases use both nicotinamide coenzymes (Levy *et al* 1983).' (pg 6)

'In spite of their great structural similarity, NAD and NADP have quite different metabolic roles. NAD functions as an electron carrier for intracellular respiration as well as a co-dehydrogenase with enzymes involved in the oxidation of fuel molecules, such as glyceraldehyde 3-phosphate, lactate, pyruvate and α-ketoglutarate dehydrogenases.' (pg 6)

Reference 7.0:
'The best defined role of niacin is in the metabolism of metabolic fuels, and the functional nicotinamide part of the coenzymes NAD and NADP, which play a major role in oxidation and reduction reactions.' (pg 151)

'In general, NAD^+ is involved as an electron acceptor in energy-yielding metabolism, being oxidized by the mitochondrial electron transport chain, while the major coenzyme for reductive synthetic reactions is NADPH. An exception to this general rule is the pentose phosphate pathway of glucose metabolism, which results in the reduction of $NADP^+$ to NADPH, and is the principal metabolic source of reductant for fatty acid synthesis.' (pg 151)

2) DNA replication and growth

Code   **Proposed statement**
Ni2a: *Niacin is necessary for the normal repair and replication of DNA*
Ni2b: *Niacin contributes to normal growth in the developing fetus*

Reference 1.3:
'The enzyme PARP is found in the nuclei of eukaryotic cells and catalyzes the transfer of many ADP-ribose units from NAD to an acceptor protein and also to the enzyme itself. These nuclear poly ADP-ribose proteins seem to function in DNA replication and repair and in cell differentiation. DNA damage greatly enhances the activity of PARP (Stierum et al., 1994); PARP activity is strongly correlated with cellular apoptosis (Stierum et al., 1994).' (pg 124)

'A possible functional measure for niacin status could be polyadenosine diphosphate (ADP) ribosylation, because ADP ribosylation may contribute to gene stability (poly-ADP-ribose polymerase in the nucleus) and may function in deoxyribonucleic acid (DNA) replication and repair (Stierum et al., 1994).' (pg 127)

'To derive the EAR for pregnant women, it is estimated that the need for niacin increases by 3mg/day of NEs to cover increased energy utilization and growth in maternal and fetal compartments, especially during the second and third trimesters.' (pg 136)

Reference 2.0:
'NAD is essential for the synthesis and repair of DNA. NAD has, in addition, a role in supplying ADP ribose moieties to lysine, arginine and asparagine residues in proteins such as histones, DNA lyase II and DNA-dependent RNA polymerase, and to polypeptides such as the bacterial diphtheria and cholera toxins. In the nucleus, poly (ADP ribose) synthetase is activated by binding to DNA breakage points and is involved in DNA repair. It is also concerned with condensation and expansion chromatin during the cell cycle and in DNA replication. Niacin status affects the level of ADP ribolysation of proteins. A high level of poly (ADP ribose) synthetase activity, which is found in some tumours, can result in low levels of NAD.' (pg 1293)

'The two pyridine nucleotide coenzymes, … and known nowadays as 'NAD' and 'NADP' (nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate), are involved in hundreds of enzyme-catalysed redox reactions *in vivo*. Although a minority of these diverse reactions can use either of the two niacin-derived cofactors, most are highly specific for one or the other.' (pg 1292)

Reference 3.8:
'In addition, NAD is the source for ADP-ribose, which is used in repairing DNA breakage caused by mutagens and other toxins.' (pg 2)

Reference 4.7:
'The niacin co-factor NAD is also required for important non-redox reactions. It is the substrate for three classes of enzymes that cleave the β-N-glycosylic bond of NAD to free nicotinamide and catalyse the transfer of ADP-ribose to proteins (Jacob and Swendseid, 1996).' (pg 6)

Reference 7.0:
'In addition to its coenzyme role, NAD has a function as the course of ADP-ribose for the ADP-ribosylation of a variety proteins and poly(ADP-ribosylation) and hence activation of nucleoproteins involved in the DNA repair mechanism.' (pg 151)

'In the nucleus, poly(ADP-ribose)polymerase is activated by binding to breakage points in DNA. The enzyme is involved in activation of the DNA repair mechanism in response to strand breakage caused by radical attack or UV radiation. In cells that have suffered considerable DNA damage, the activation of poly(ADP-ribose) polymerase may deplete intracellular NAD to such an extent that ATP formation is impaired, leading to cell death.' (pg 151, 152)

3) Fatty acid and steroid synthesis

Code   **Proposed statement**
Ni3:   *Niacin contributes to the normal structure of some steroids, which are required to make hormones*

Reference 1.3:
'NADP functions in reductive biosyntheses such as in fatty acid and steroid syntheses and, like NAD, as a codehydrogenase – as in the oxidation of glucose 6-phosphate to ribose 5-phosphate in the pentose phosphate pathway.' (pg 124)

Reference 2.0:
'NADP, however, functions mainly in the reductive reactions of lipid biosynthesis, and the reduced form of this coenzyme is generated via the pentose phosphate cycle.' (pg 1293)

Reference 4.7:
'NADP functions as a hydrogen donor in reductive biosyntheses, such as in fatty acid and steroid syntheses, and like NAD as a codehydrogenase, such as in the oxidation of glucose-6-phosphate to ribose 5-phosphate in the pentose phosphate pathway (Jacob and Swendseid 1996).' (pg 6)

4) Skin and mucous membranes

Code   **Proposed statement**
Ni4:   *Niacin is necessary for the normal structure and function of skin and mucous membranes (such as in the intestines).*

Reference 1.3:
'Pellagra is the classic manifestation of a severe niacin deficiency. It is characterised by a pigmented rash that develops symmetrically in areas exposed to sunlight; changes in the digestive tract that are associated with vomiting, constipation or diarrhea, and a bright red tongue; ...' (pg 125-126)

Reference 2.0:

'The most characteristic clinical signs of severe niacin deficiency in humans are dermatosis (hyperpigmentation, hyperkeratosis, desquamation – especially where exposed to the sun), anorexia, achlorhydria, diarrhoea, angular stomatitis, cheilosis, magenta tongue, anaemia, …The picture in other species is not radically different; however deficient dogs and cats typically exhibit 'black tongue' (pustules in the mouth, excessive salivation) and bloody diarrhoea; … fowl exhibit inflammation of the upper gastrointestinal tract, dermatitis, diarrhoea and damage to the feathers.' (pg 1295)

Reference 3.8:
'The condition that is characteristic of a deficiency of both tryptophan and preformed niacin is pellagra…and is characterised by spinal pains, "magenta tongue", digestive disturbances and subsequently erythema with drying and expurgation of the skin….' (pg 2)

Reference 4.7:
'The most common symptoms of niacin deficiency are divided into three categories: changes in the skin; mucosa of the mouth, stomach and intestinal tract; and... The changes in the skin are amongst the most characteristic in human beings. They are called 'pellagra', which means 'raw skin'. These symptoms are most pronounced in the parts of the skin which are exposed to sunlight. In severe deficiency, the human tongue and gastric mucosa become inflamed; the tongue becomes bright red and swells. ...' (pg 7)

Reference 6.3:
'Niacin deficiency results in pellagra, which is characterised by a severe sunburn-like skin lesion in areas of the body exposed to sunlight, and in ares such as the knees, ankles, wrists and elbows which are subjected to pressure. Diarrhoea is a characteristic, but not inevitable, symptom of pellagra...' (pg 99)

Reference 7.0:
'Pellagra is characterized by a photosensitive dermatitis, like severe sunburn, typically with a butterfly-like pattern of distribution over the face, affecting all parts of the skin that are exposed to sunlight. Similar skin lesions may also occur in areas not exposed to sunlight, but subject to pressure, such as the knees elbows, wrists and ankles. … and there may be diarrhoea. Untreated pellagra is fatal...' (pg 152)

5) Neurological system

Code   **Proposed statement**
Ni5:   *Niacin is necessary for normal neurological function.*

Reference 1.3:
'Pellagra is the classic manifestation of a severe niacin deficiency. It is characterised by … neurological symptoms including depression, apathy, headach, fatigue and loss of memory.' (pg 125-126)

Reference 2.0:

'The most characteristic clinical signs of severe niacin deficiency in humans are … neuropathy (headache, dizziness, tremor, neurosis, apathy). …The picture in other species is not radically different; … pigs show neurological lesions affecting the ganglion cells; rats exhibit damage to the peripheral nerves (cells and axons); …' (pg 1295)

Reference 3.8:
'The condition that is characteristic of a deficiency of both tryptophan and preformed niacin is pellagra… Various nervous manifestations, such as spasms, ataxic paraplegia and mental disturbances occur in severe cases.' (pg 2)

Reference 4.7:
'The most common symptoms of niacin deficiency are divided into three categories: changes in the skin; mucosa of the mouth, stomach and intestinal tract; and changes in the nervous sytem…. The neurological symptoms experienced can include fatigue, sleeplessness, depression, loss of memory and visual impairment (Gopalan and Rao).' (pg 7)

Reference 6.3:
'Niacin deficiency results in pellagra,… In advanced cases there may be dementia with intermittent periods of lucidity.' (pg 99)

Reference 7.0:
'Advanced pellagra is also accompanied by dementia (more correctly a depressive psychosis), … The depressive psychosis is similar to schizophrenia and the organic psychoses, but clinically distinguishable by the sudden lucid phases which alternate with the most florid psychiatric signs. It is probable that these mental symptoms can be explained by a relative deficit of the essential amino acid tryptophan, and hence reduced synthesis of the neurotransmitter 5-hydroxytryptamine (serotonin), and not to a deficiency of niacin per se.' (pg 152)

ANNEX 4.8

Pantothenic Acid

Source documents for reviewing pantothenic acid

Reference 1.3:
Institute of Medicine Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B₆, folate, vitamin B₁₂, pantothenic acid, biotin and choline. Washington D.C. National Academy Press 2000.

Reference 2.0:
Encyclopedia of Human Nutrition 2E. Editor-in-chief, Michele J. Sadler, editors, J.J. Strain, Benjamin Caballero. San Diego : Academic Press, c1999.

Reference 3.9:
Opinion of the Scientific Committee on Food (SCF) on the Tolerable Upper Intake Level of Pantothenic Acid. April 2002. (http://www.europa.eu.int/comm/food/fs/sc/scf/out80k_en.pdf).

Reference 4.8:
Review of Pantothenic Acid. Expert Group on Vitamins and Minerals. August 2002. (http://www.food.gov.uk/multimedia/pdfs/panto.pdf).

1) Fat metabolism

Code   **Proposed statement**
Pa1:   *Pantothenic acid is necessary for the normal metabolism of fat.*

Reference 1.3:
'Pantothenic acid is vital to the synthesis and maintenance of co-enzyme A (CoA), a cofactor and acyl group carrier for many enzymatic processes, and acyl carrier protein, a component of the fatty acid synthase complex (Tahiliani and Beinlich, 1991). As such, pantothenic acid is essential to almost all forms of life. Most tissues transport pantothenic acid into cells for the synthesis of CoA.' (pg 357)

'The synthesis of CoA from pantothenate is regulated primarily by pantothenate kinase, an enzyme that is inhibited by the pathway end products, CoA and acyl CoA. Thus CoA production does not reflect the amount of available pantothenate (Tahiliani and Beinlich, 1991).' (pg 358)

Reference 2.0:
'The primary role of pantothenic acid is in acyl group activation for lipid metabolism, involving thiol acylation of CoA or of ACP both of which contain 4-phosphopantotheine, the active group of which is β-mercaptoethylamine. Coenzyme A is essential for oxidation of fatty acids, of pyruvate and of α-oxoglutarate, for metabolism of sterols, and for acetylation of other molecules, so as to modulate their transport characteristics or functions.' (pg 1512)

'Beta-oxidation within the peroxisomes is also CoA-dependent, and is downregulated by pantothenate deficiency. The rate of CoA synthesis is under close metabolic control by energy-yielding substrates, such as glucose and free fatty acids, at the initial activation step, catalysed by pantothenate kinase (ATP: pantothenate 4-phosphotransferase, EC 2.7.1.33). This feedback control is thought to be a mechanism for conservation of cofactor requirements.' (pg 1512)

'In addition to the now well-established roles of CoA in the degradation and synthesis of fatty acids, of sterols and of other compounds synthesized from isoprenoid precursors, there are also a number of acetylation and long-chain fatty acylation processes which seem to require CoA as part of their essential biological catalytic sites… The acetylation of amino sugars and some other basic reactions of acetyl-CoA and succinyl-CoA in intermediary metabolism have been known since the 1980's. (pg 1512,1513)

Reference 3.9:
'Pantothenic acid plays a central role in intermediary metabolism as part of the coenzyme A (CoA) molecule and as part of the pantotheine functional group in the acyl-carrier protein (Acyl-CP). This vitamin serves therefore as a cofactor in acyl-group activation and transfer in fatty acid and carbohydrate metabolism, as well as in a wide range of (other) acylation reactions (see Fox, 1984 and Plesofsky-Vig, 1996 for reviews).' (pg 2)

Reference 4.8:
'Pantothenate, usually in the form of CoA-containing species (e.g. acetyl CoA, succinyl CoA) performs multiple roles within cellular metabolism and in the synthesis of many essential molecules… (reviewed by Plesofsky-Vig, 1999) …Within the tricarboxylic acid cycle, β-oxidation of fatty acids and oxidative degradation of amino acids.' (pg 8)

2) Molecule structure

Code   **Proposed statement**
Pa2:   *Pantothenic acid contributes to the normal structure of numerous essential molecules in the body*

Reference 1.3:
'… and in the synthesis of fatty acids and membrane phospholipids, amino acids, steroid hormones, vitamins A and D, porphyrin and corrin rings, and neurotransmitters. It is also required for the acetylation and acylation of proteins and the synthesis of α-tubulin (Plesofsky-Vig, 1996) (pg 358)

Reference 2.0:
'Acyl carrier protein, which is synthesized from apo-ACP and coenzyme A, is involved specifically in fatty acid synthesis. Its role is to activate acetyl, malonyl and intermediate-chain fatty acyl groups during their anabolism by the biotin-dependent fatty acid synthase complex (i.e. acyl-CoA; malonyl-CoA-acyl transferase (decarboxylating, oxoacyl and enoyl-reducing and thioester-hydrolysing), EC 2.3.1.85). (pg 1512)

'The organ with the highest concentration of pantothenate is liver, followed by adrenal cortex, because of the requirement of steroid hormone metabolism there.' (pg 1512)

'… the biochemical functions, and hence the basis for the dietary requirement of pantothenic acid, arise entirely from its occurrence as an essential component of CoA and of ACP, which cannot be synthesized *de novo* in mammals from simpler precursors.' (pg 1512)

'… the addition of acetyl or fatty acyl groups to certain proteins in order to modify and control their specific and essential properties is a more recent discovery. The first category of these modifications comprises the acetylation of the *N* terminal amino acid in certain proteins, which actually occurs in at least half of all the known proteins that are found in higher organisms. The specific amino acids that are recipients of these acetyl groups are most commonly methionine, alanine or serine. The purposes of this terminal acetylation process are not entirely clear and may be multiple, including modifications of function (e.g. of hormone function), of binding and site recognition, of tertiary peptide structure, and of eventual susceptibility to degradation. Another possible site of protein acetylation is the side chain of certain internal lysine residues, whose side chain ε-amino group may become acetylated in some proteins, notably the basic histone proteins of the cell nucleus, and the α-tubulin proteins of the cytoplasmic microtubules, which help to determine cell shape and motility.' (pg 1513)

'Proteins can also be modified by acylation with certain long-chain fatty acids, notably the 16-carbon saturated fatty acid, palmitic acid, and the 14-carbon saturated fatty acid, myristic acid. Although structurally very similar to each other, these two fatty acids seek entirely different protein locations for acylation and also have quite different functions. They have recently been explored with particular emphasis on viral and yeast proteins, although proteins in higher animals, in organs such as lung and brain, can also become acylated with palmitoyl moieties. Palmitoyl-CoA is also required for the transport of residues through the Golgi apparatus during protein secretion. It is believed that these protein acylations may enable and control specific protein interactions, especially in relation to cell membranes, and proteins which are palmitoylated are generally also found to be associated with the plasma membrane. Signal transduction (e.g. of the human β_2-adrenergic receptor) is one process which appears to be controlled by palmitoylation, and other palmitoylated proteins possess some structural importance, for example in the case of the protein-lipid complex of brain myelin. Clearly these subtle protein-modifications, all of which depend on CoA and hence on pantothenic acid, have a wide-ranging significance for many biological processes which is still being actively explored.' (pg 1513)

Reference 4.8:
'Pantothenate, usually in the form of CoA-containing species (e.g. acetyl CoA, succinyl CoA) performs multiple roles within cellular metabolism and in the synthesis of many essential molecules….(reviewed by Plesofsky-Vig, 1999):…Fatty acid and membrane phospholipid synthesis; Amino acid synthesis (leucine, arginine, methionine); Synthesis of isoprenoid derivatives, such as cholesterol, steroid hormones, dolichol, vitamin A, vitamin D, haem A; Synthesis of δ-amino-laevulinic acid, the precursor of porphyrin and corrin rings (vitamin B_{12}, haemoglobin,

cytochromes); Synthesis of neurotransmitters (eg acetylcholine); Acetylation, acylation, myristolation, palmitoylation and isoprenylation of proteins.' (pg 8)

ANNEX 4.9

Vitamin B$_6$

Source documents for reviewing vitamin B$_6$

Reference 1.3:
Institute of Medicine Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B$_6$, folate, vitamin B$_{12}$, pantothenic acid, biotin and choline. Washington D.C. National Academy Press 2000.

Reference 2.0:
Encyclopedia of Human Nutrition 2E. Editor-in-chief, Michele J. Sadler, editors, J.J. Strain, Benjamin Caballero. San Diego : Academic Press, c1999.

Reference 3.10:
Opinion of the Scientific Committee on Food (SCF) on the Tolerable Upper Intake Level of Vitamin B$_6$. October 2000. (http://www.europa.eu.int/comm/food/fs/sc/scf/out80c_en.pdf).

Reference 4.9:
Revised review of Vitamin B$_6$. Expert Group on Vitamins and Minerals. August 2002. (http://www.food.gov.uk/multimedia/pdfs/EVM/00/19/P.pdf).

Reference 5.0:
Handbook of Nutrition and Food. Edited by Carolyn D Berdanier...[et al.]. Boca Raton, Fla.: CRC Press, 2002.

1) Protein metabolism

Code   **Proposed statement**
VB$_6$1:   *Vitamin B$_6$ is necessary for the normal metabolism of protein*

Reference 1.3:
'Vitamin B$_6$ (B$_6$) comprises a group of six related compounds: pyridoxal (PL), pyridoxine (PN), pyridoxamine (PM), and their respective 5'-phosphates (PLP, PNP, and PMP).' (pg 150)

'PLP is a coenzyme for more than 100 enzymes involved in amino acid metabolism, including aminotransferases, decarboxylases, race-mases, and dehydratases. It is a coenzyme for δ-aminolevulinate synthase … and for cystathionine β-synthase and cystathioninase, enzymes involved in the transsulfuration pathway from homocysteine to cysteine. The carbonyl group of PLP binds to proteins as a Schiff's base with the ε-amine of lysine. For practically all PLP enzymes the initial step in catalysis involves formation of a Schiff's base between an incoming amino acid, via its α-amino group, and the carbonyl group of PLP. Much of the total PLP in the body is found in muscle bound to phosphorylase. PLP is a coenzyme in the phosphorylase reaction and is also directly involved in catalysis.' (pg 151)

'The major pathway of tryptophan catabolism proceeds via the PLP-dependent kynureninase reaction (Shane and Contractor, 1980). The xanthurenic acid pathway also involves PLP-dependent enzymes. However, under conditions of B_6 deficiency, this minor pathway is used to a greater extent, leading to the increased excretion of abnormal tryptophan metabolites.' (pg 157)

'Homocysteine catabolism proceeds via transsulfuration to cysteine and involves two PLP-dependent enzymes.' (pg 158)

'Because of PLP's role as a coenzyme for many enzymes involved in amino acid metabolism, it has been proposed that B_6 requirements are influenced by protein intake. Many studies have demonstrated that increased protein intake causes a relative decrease in B_6 status indicators (Baker et al., 1964; Hansen et al., 1996b; Linkswiler, 1978; Miller et al., 1985; Sauberlich, 1964).' (pg 161)

Reference 2.0:
'Vitamin B_6 has a central role in amino acid metabolism as it is the coenzyme for a variety of reactions, including transamination and decarboxylation.' (pg 1916)

'The metabolically active vitamer is pyridoxal phosphate. This is involved in many reactions of amino acid metabolism, where the carbonyl group is the reactive moiety; in glycogen phosphorylase, where it is the phosphate group which is important in catalysis; and in the release of hormone receptors from tight nuclear binding, where again it is the carbonyl group that is important. Glycogen phosphorylase catalyses the sequential phosphorolysis of glycogen to release glucose-1-phosphate; it is thus the key enzyme in the utilization of muscle and liver glycogen reserves.' (pg 1918)

'Pyridoxal phosphate-dependent enzymes catalyse a number of important reactions in amino acid metabolism, including transamination to yield oxo (keto) acids, decarboxylation to yield amines, and a variety of side chain elimination and rearrangement reactions.' (pg 1918)

'In the absence of the substrate, pyridoxal phosphate is bound to the enzyme by the formation of a Schiff base to the ε-amino group of a lysine residue. The first reaction between the substrate and the coenzyme is transfer of the aldimine linkage from this ε-amino group to the α-amino group of the substrate. The ring nitrogen of pyridoxal phosphate exerts a strong electron-withdrawing effect on the aldimine, and this leads to weakening of all three bonds about the α-carbon of the substrate; which bond is cleaved will depend on the orientation of the Schiff base relative to reactive groups of the catalytic site.' (pg 1918)

'Cleavage of the α-carbon-carboxyl bond of the Schiff base leads to decarboxylation of the amino acid, followed by release of the corresponding amine and reformation of the internal Schiff base to lysine. A number of the products of the decarboxylation of amino acids are important as neurotransmitters and hormones, and as the diamines and polyamines involved in the regulation of DNA metabolism; the decarboxylation of phosphatidylserine to phosphatidylethanolamine is important in phospholipid metabolism.' (pg 1918)

'Hydrolysis of the α-carbon-amino bond of the Schiff base results in the release of the 2-oxo-acid corresponding to the amino acid substrate, and leaves pyridoxamine phosphate at the catalytic site of the enzyme. In this case there is no reformation of the internal Schiff base to the reactive lysine residue. This is the half-reaction of transamination. The process is completed by reaction of pyridoxamine phosphate with a second oxo-acid substrate, followed by the reverse of the reaction sequence.' (pg 1920)

'Transamination is of central importance in amino acid metabolism, providing pathways for the catabolism of all amino acids other than lysine (which does not undergo transamination). Many of these reactions are linked to the amination of 2-oxoglutarate to glutamate or glyoxylate to glycine, which are substrates for oxidative deamination, reforming the oxo-acids. Equally, transamination reactions provide a pathway for the synthesis of those amino acids for which there is an alternative source of the oxo-acid (the nonessential amino acids).' (pg 1920)

Reference 3.10:
'Pyridoxal phosphate plays an essential role in the metabolism of many amino acids, and deficiency of this coenzyme can lead to many manifestations. Clinical signs include retarded growth, acrodynia, alopecia, skeletal changes and anaemia, while changes in neurotransmitters, such as dopamine, serotonin, norepinephrine (noradrenaline), tryptamine, tyramine, histamine, GABA and taurine, affect brain function and can lead to seizures and convulsions.' (pg 2)

'The active form of the vitamin is pyridoxal phosphate, which is a coenzyme that is recognised as being required for the function of more than 60 enzymes involved with transamination, deamination, decarboxylation or desulfuration reactions.' (pg 2)

'Tryptophan metabolism is dependent on vitamin B_6 status, because the enzyme kynureninase, requires pyridoxal phosphate. This enzyme is especially sensitive to vitamin B_6 depletion.' (pg 3)

'Vitamin B_6 is involved in the metabolism of sulphur-containing amino acids (methionine, taurine and cysteine (Sturman, 1986).' (pg 3)

Reference 4.9:
'As with the other B complex vitamins, pyridoxine is involved in the functioning of enzymes involved in the release of energy from food. The active, coenzyme forms of pyridoxine are pyridoxal 5' phosphate and pyridoxamine 5' phosphate. Pyridoxal phosphate is involved as a coenzyme in over 60 enzyme reactions (Basu and Dickerson, 1996) particularly in the metabolic transformation of amino acids, including decarboxylation, transamination, dehydration, desulphydration, cleavage, racemisation and synthesis.' (pg 11)

'Transaminases (aminotransferases) catalyse the transamination reaction whereby α-amino acids are converted to α-keto acids resulting in the formation of different α-amino acids. Most amino acids can undergo these reversbile reactions, and the reaction is reponsible for the formation of non-essential amino acids from keto acids. Examples of this are aspartate, which is formed from oxaloacetate, and alanine which is formed from pyruvate (Basu and Dickerson, 1996).' (pg 12)

'Pyridoxine is involved in several enzyme reactions in the metabolism of tryptophan. In individuals who are vitamin B_6 deficient, a number of metabolites of tryptophan, in particular xanthurenic acid, are excreted in urine in abnormally large quantities. This phenomenon is employed in the diagnostic tryptophan load test. The enzyme kynureinase is involved in the catabolism of tryptophan, cleaving the 3-hydroxyanthranilate ring, a pathway via which nicotinamide is formed in the body.' (pg 12)

'Vitamin B_6 is also a cofactor in the conversion of tryptophan to 5-hydroxytryptamine and of methionine to cysteine (Sturman, 1978) …' (pg 12)

2) Transport and metabolism of iron

Code   **Proposed statement**
VB62:   *Vitamin B6 is necessary for the normal transport and metabolism of iron in the body.*

Reference 1.3:
'…It is a coenzyme for δ-aminolevulinate synthase which catalyzes the first step in heme biosynthesis…' (pg 151)

'The classical clinical symptoms of B_6 deficiency are … microcytic anemia (Snyderman et al., 1953)…Microcytic anemia reflects decreased hemoglobin synthesis. The first enzyme and committed step in heme biosynthesis, aminolevulinate synthase, uses PLP as a coenzyme…' (pg 153)

Reference 2.0:
'A number of studies have shown that between 10 and 20% of the apparently healthy population have low plasma concentrations of pyridoxal phosphate or abnormal erythrocyte transaminase activation coefficient…' (pg 1922)

Reference 3.10:
'Pyridoxal phosphate plays an essential role in the metabolism of many amino acids, and deficiency of this coenzyme can lead to many manifestations. Clinical signs include … anaemia…' (pg 2)

Reference 4.9:
'… Pyridoxal and pyridoxal phosphate also bind to haemoglobin increasing the oxygen-binding capacity and preventing sickling in sickle-cell haemoglobin. Vitamin B_6 is also involved in the biosynthesis of haem and … (Bender, 1999).' (pg 12)

3) Hormones

Code   **Proposed statement**
VB_63:   *Vitamin B_6 is necessary for the normal function of some hormones*

Reference 1.3:
'The classical clinical symptoms of B_6 deficiency are …epileptiform convulsions (Bessey et al., 1957); Coursin, 1954) and depression and confusion (Hawkins and Barsky, 1948)…. Because PLP is also a coenzyme of decarboxylases that are involved in neurotransmitter synthesis, defects in some of these enzymes could explain the onset of convulsions in B_6 deficiency…However, it has not been definitely shown whether the convulsions are due to the reduced level of one of these neurotransmitters in particular. Guilarte (1993) proposed that the convulsions are caused by abnormal tryptophan metabolites that accumulate in the brain in B_6 deficiency.' (pg 153)

Reference 2.0:
'…It is also the coenzyme of glycogen phosphorylase, and has a role in the actions of steroid and other hormones which act by modulation of gene expression.' (pg 1916)

'The metabolically active vitamer is pyridoxal phosphate. This is involved in many reactions of amino acid metabolism, where the carbonyl group is the reactive moiety; in glycogen phosphorylase, where it is the phosphate group which is important in catalysis; and in the release of hormone receptors from tight nuclear binding, where again it is the carbonyl group that is important…' (pg 1918)

'Vitamin B_6 has a role in the action of those hormones which act by binding to a nuclear receptor protein and modulating gene expression. Such hormones include androgens, oestrogens, progesterone, glucocorticoids, calcitriol (the active metabolite of vitamin D), retinol and retinoic acid, and the thyroid hormones. Target tissue specificity of hormone action is ensured by the presence of receptor proteins which are responsible for both nuclear uptake and the interaction with control regions of DNA. Pyridoxal phosphate reacts with a lysine residue in the receptor protein and releases the hormone-receptor complex from tight nuclear binding. It thus acts to terminate hormone action and release receptor proteins for reutilization. In experimental animals, vitamin B_6 deficiency results in increased and prolonged nuclear uptake and retention of steroid hormones in target tissues, and there is enhanced sensitivity to low doses of hormones. In cells in culture, pyridoxal phosphate depletion results in enhanced induction of marker enzymes, while high intracellular concentrations of pyridoxal phosphate impair enzyme induction in response to the hormone.' (pg 1920)

Reference 3.10:
'Pyridoxal phosphate plays an essential role in the metabolism of many amino acids, and deficiency of this coenzyme can lead to many manifestations… changes in neurotransmitters, such as dopamine, serotonin, norepinephrine (noradrenaline), tryptamine, tyramine, histamine, GABA and taurine, affect brain function and can lead to seizures and convulsions.' (pg 2)

Reference 4.9:
'Decarboxylases catalyse the decarboxylation of amino acids to amines, these reactions include the formation of a number of neuroactive amines such as histamine from histidine, serotonin from tryptophan, γ-aminobutyric acid (GABA) from glutamic acid and adrenaline/noradrenaline and tyramine/dopamine from tyrosine (Bender, 1999, Basu and Dickerson, 1996).' (pg 12)

'Vitamin B_6 is also a cofactor in the conversion of tryptophan to 5-hydroxytryptamine and of methionine to cysteine (Sturman, 1978) and has been claimed to modify the action of steroid hormones *in vivo* by interacting with steroid-receptor complexes (Disorbo *et al.*, 1980). This interaction occurs via B_6 inhibiting the induction of hepatic tyrosine aminotransferase by glucocorticoids, probably by formation of a Schiff base link to the DNA-binding site of the complex (Basu and Dickerson, 1996) …Vitamin B_6 is also involved …in the decarboxylation of phosphatidylserine to phosphatidylethanolamine in phospholipid synthesis (Bender, 1999).' (pg 12)

4) Homocysteine metabolism

Code   **Proposed statement**
$VB_6$4:   *Vitamin B_6 contributes to the maintenance of normal blood homocysteine levels*

Reference 1.3:
'It is a coenzyme for … cystathionine β-synthase and cystathioninase, enzymes involved in the transsulfuration pathway from homocysteine to cysteine.' (pg 151)

'Inadequate intakes of B_6 have also been reported to impair platelet function and clotting mechanisms (Branstrom et al., 1990; Subarao and Kakkar, 1979), but these effects may also be due to the hyperhomocysteinemia noted in such patients (Brattstrom et al., 1990).' (pg 154)

'…fundamental tests for B_6 status, including the increase in homocysteine after a methionine load…' (pg 155 – 156)

'Homocysteine catabolism proceeds via transsulfuration to cysteine and involves two PLP-dependent enzymes. Homocysteine can also be remethylated to methionine via folate and vitamin B_{12}-dependent enzymes. Thus plasma concentrations of homocysteine are influenced by B_6 and folate, and to a lesser extent, B_{12} intakes (Selhub et al., 1993).' (pg 158)

'The increase in plasma homocysteine concentration after a methionine load or a meal is responsive to and primarily affected by B_6 status…Results from population-based studies using data adjusted for folate and B_{12} status and for age indicate that B_6 status as measured by PLP is inversely correlated with nonfasting plasma homocysteine concentration (Selhub et al., 1993).' (pg 159)

'For B_6 the data are compatible with the Framingham study (Selhub et al., 1993), in which the lowest deciles of B_6 intake were associated with higher circulating homocysteine. … At these high B_6 intakes, there is little effect of B_6 intake on homocysteine levels, which are mainly affected by changes in intake at much lower intakes.' (pg 159)

Reference 3.10:

'Vitamin B_6 is involved in the metabolism of sulphur-containing amino acids (methionine, taurine and cysteine (Sturman, 1986). The disease states of homocystinuria and cystathioninuria are due to inborn errors of metabolism involving the enzymes cystathionine â–synthase (EC 4.2.1.22) and gamma-cytathionase (EC 4.4.1.1). ' (pg 3)

Reference 4.9:
'Vitamin B_6 is also a cofactor in the conversion of tryptophan to 5-hydroxytryptamine and of methionine to cysteine (Sturman, 1978) …' (pg 12)

Reference 5.0:
'Vitamin B_6 has a significant role to play, along with folate and vitamin B_{12}, the reduction of elevated homocysteine levels associated with increased risk of cardiovascular disease – specifically, coronary artery disease and stroke.' (pg 1338)

ANNEX 4.10

Folate

Source documents for reviewing folate

Reference 1.3:
Institute of Medicine Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B$_6$, folate, vitamin B$_{12}$, pantothenic acid, biotin and choline. Washington D.C. National Academy Press 2000.

Reference 2.0:
Encyclopedia of Human Nutrition 2E. Editor-in-chief, Michele J. Sadler, editors, J.J. Strain, Benjamin Caballero. San Diego : Academic Press, c1999.

Reference 3.11:
Opinion of the Scientific Committee on Food (SCF) on the Tolerable Upper Intake Level of Folate. October 2000. (http://www.europa.eu.int/comm/food/fs/sc/scf/out80e_en.pdf).

Reference 4.10:
Review of Folic Acid. Expert Group on Vitamins and Minerals. August 2002. (http://www.food.gov.uk/multimedia/pdfs/evm0018p.pdf).

Reference 6.2:
Folic Acid and the Prevention of Disease Report of the Committee on Medical Aspects of Food and Nutrition Policy. Report on Health and Social Subjects No. 50. DH: The Stationery Office, 2000

1) Cell division

Code	Proposed statement
Fo1:	*Folate is necessary for normal cell division (such as in the gastro-intestinal tract).*

Reference 1.3:
'The folate coenzymes are involved in numerous reactions that involve (1) deoxyribonucleic acid (DNA) synthesis, which depends on a folate coenzyme for pyrimidine nucelotide biosynthesis (methylation of deoxyuridylic acid to thymidylic acid) and thus is required for normal cell division; (2) purine synthesis (formation of glycinamide ribonucleotide and 5-amino-4-imidazole carboxamide ribonucleotide); (3) generation of formate into the formate pool (and utilization of formate); …' (pg 197)

'Both folate and vitamin B$_{12}$ are required for the formation of 5,10-methylenetetrahydrofolate and involved in thymidylate synthesis by way of a vitamin B$_{12}$-containing enzyme. The formation of 5,10-methylene tetrahydrofolate depends on the regeneration of the parent compound (tetrahydrofolate) in the homocysteine-to-methionine conversion. This reaction involves the removal of a methyl group from methyl folate and the delivery of this group to homocystine for the synthesis of

methionine. Folate is involved as a substrate (5-methyl-tetrahydrofolate) and vitamin B_{12} as a coenzyme. The 5,10-methylenetetrahydrofolate delivers its methyl group to deoxyuridylate to convert it to thymidylate for incorporation into DNA. In either a folate or vitamin B_{12} deficiency, the megaloblastic changes occurring in the bone marrow and other replicating cells result from lack of adequate 5,10-methylene-tetrahydrofolate.' (pg 199)

'Folate requirements increase substantially during pregnancy because of the marked acceleration in single-carbon transfer reactions, including those required for nucleotide synthesis and thus cell division. During pregnancy, cells multiply in association with uterine enlargement, placental development, expansion of maternal erythrocyte number, and fetal growth (Cunningham et al., 1989). (pg 233)

Reference 2.0:
'Folate functions metabolically as an enzyme cofactor in the synthesis of nucleic acids and amino acids. Deficiency of the vitamin leads to impaired cell replication and other metabolic alterations particularly related to methionine synthesis.' (pg 803)

'Single carbon units are removed from folate by a number of reactions. The … single-carbon units from 10-formyl-THF are used for the biosynthesis of purines… one-carbon transfer from 5,10-methylene-THF to deoxyuridylate to form thymidylic acid, a precursor of DNA, is of crucial importance to the cell.' (pg 804)

'In summary, the biochemical function of folate coenzymes is the transfer and utilization of these one-carbon units in a variety of essential reactions including (1) *de novo* purine biosynthesis (formylation of glycinamide ribonucleotide (GAR) and 5-amino-4-imidazole carboxamide ribonucleotide (AICAR)); (2) pyrimidine nucleotide biosynthesis (methylation of deoxyuridylic acid to thymidylic acid); …; and (4) generation and utilization of formate.' (pg 806)

'An important determinant of folate uptake into cells is their mitotic activity, as would be expected given the dependence of DNA biosynthesis on folate coenzyme function. Folate accumulation is more rapid in actively dividing cells than in quiescent cells, a factor probably related to the induction and activity of folylpoly-γ-glutamate synthase. This enzyme catalyses the addition of glutamate by γ-peptide linkage to the initial glutamate moiety of the folate molecule. Although polyglutamate derivatization may be considered a storage strategem, this elongation is the most efficient coenzyme form for normal one-carbon metabolism.' (pg 807)

Reference 3.11:
'Folates play an important role in the transfer of C_1-groups (i.e. methyl-, methylene- and formyl-groups), maintaining the methylation balance, such as in the biosynthesis of DNA bases and ...' (pg 2)

'In tissues folates are retained as polyglutamates and the folate coenzymes can be interconverted in numerous (de-)methylation reactions, such as in DNA synthesis (formation of thymidilate from deoxyuridine)…' (pg 3)

Reference 4.10:

'Deficiency of folate results in a reduction in *de novo* DNA biosynthesis and, thus, impairment of cell replication, with the most obvious effects apparent in rapidly dividing cell-types, such as red blood cells and other cells generated by the bone marrow, enterocytes, and skin cells. This condition is recognised, haematologically, as a macrocytic anaemia, with characteristic red cell precursors (megaloblasts) present in bone marrow aspirates, and the clinical manifestation of megaloblastic anaemia.' (pg 15)

'Various THF-polyglutamates function metabolically as coenzymes and substrates in one-carbon metabolism. Transfer of a one-carbon unit from serine to THF *via* pyridoxal phosphate (PLP)-dependent serine hydroxymethyltransferase (SHMT), in the coupled serine to glycine conversion pathway, produces 5,10 methylene-THF. This reduced folate cofactor serves as the substrate to generate 5,10 methenyl-THF (5, 10 formyl-THF, anhydroleucovorin) (dehydrogenase reaction) and 10-formyl-THF (cyclohydrolase reaction), which are required for synthesis of the purine ring. 5,10 Methylene-THF also provides the methyl group for methylation of deoxyuridine monophosphate (dUMP, deoxyuridylic acid) for the *de novo* synthesis of deoxythymide monophosphate (dTMP, thymidylic acid) catalysed by thymidylate synthase. This reaction generates DHF, which is reduced to THF by the enzyme dihydrofolate reductase (DHFR). (pg 14)

'Folate coenzymes within the cell are involved in one-carbon transfer reactions, including those involved in phases of amino acid metabolism, purine and pyrimidine synthesis, and the formation of the primary methylating agent, S-adenosylmethionine (SAM).' (pg 15)

'Deficiency of folate results in a reduction in *de novo* DNA biosynthesis and, thus, impairment of cell replication, with the most obvious effects apparent in rapidly dividing cell-types, such as red blood cells and other cells generated by the bone marrow, enterocytes, and skin cells…' (pg 15)

'Various THF-polyglutamates function metabolically as coenzymes and substrates in one-carbon metabolism. Transfer of a one-carbon unit from serine to THF *via* pyridoxal phosphate (PLP)-dependent serine hydroxymethyltransferase (SHMT), in the coupled serine to glycine conversion pathway, produces 5,10 methylene-THF. This reduced folate cofactor serves as the substrate to generate 5,10 methenyl-THF (5, 10 formyl-THF, anhydroleucovorin) (dehydrogenase reaction) and 10-formyl-THF (cyclohydrolase reaction), which are required for synthesis of the purine ring. 5,10 Methylene-THF also provides the methyl group for methylation of deoxyuridine monophosphate (dUMP, deoxyuridylic acid) for the *de novo* synthesis of deoxythymide monophosphate (dTMP, thymidylic acid) catalysed by thymidylate synthase. This reaction generates DHF, which is reduced to THF by the enzyme dihydrofolate reductase (DHFR). (pg 14)

Reference 6.2:
'The one-carbon units that are attached to intracellular folate include formyl, methylene and methenyl methyl groups; they are derived from serine and also probably from formate. They are used in the biosynthesis of pyrimidines and purines, and thus for the synthesis of DNA in cell division...' (pg 18)

2) Developing neural tube

Code   **Proposed statement**
Fo2:   *Folate is necessary for the normal structure of the neural tube in developing embryos*

Reference 1.3:
'Folate requirements increase substantially during pregnancy because of the marked acceleration in single-carbon transfer reactions, including those required for nucleotide synthesis and thus cell division. During pregnancy, cells multiply in association with uterine enlargement, placental development, expansion of maternal erythrocyte number, and fetal growth (Cunningham et al., 1989).' (pg 233)

'…A defect in enzymes involved in homocysteine metabolism is suggested by altered folate, vitamin B_{12}, homocysteine, and methylmalonate values in mothers of infants with NTDs (Mills et al., 1995; Steegers-Theunissen et al., 1994); the prevention of some human NTDs by folate administration; and the prevention of NTDs in some rodent models by methionine (Essien, 1992; Vanaerts et al., 1994). These enzymes are 5,10-methylenetetrahydrofolate reductase (MTHFR), cystathionine β-synthase, and methionine synthase. Interestingly, families with homocystinuria caused by severe mutations in genes for each of these enzymes do not exhibit NTDs (Haworth et al., 1993; Kang et al., 1991b).' (pg 244, 245)

'The mechanism by which folate could reduce NTD risk is not known. Increasing folate intake and thus the concentrations of folate derivatives in tissues might overcome a metabolic deficiency in the production of proteins or in DNA synthesis at the time of neural tube closure (Mills et al., 1995). Another hypothesis is that folate does not prevent the occurrence of NTD but selectively increases the abortion rate of affected fetuses (Hook and Czeizel, 1997). Certainly, more research is needed to understand the effect of folate on embryonic and fetal development.' (pg 258)

'To summarize the data, a reduced risk of NTD has been observed for women who took a folate supplement of 360 to 800 μg/day in addition to a dietary folate intake of 200 to 300 μg/day. Folate intake is positively associated with erythrocyte folate concentration (Bower and Stanley, 1989; Brown et al., 1997; Cuskelly et al., 1996; Daly et al., 1997), and NTD risk is inversely associated with both folate intake (Bower and Stanley, 1989; Shaw et al., 1995c; Werler et al., 1993) and erythrocyte folate concentration (Daly et al., 1995).' (pg 258, 259)

'Although it is recognized that there are still uncertainties about the relationship among folate intake, erythrocyte folate, and NTD risk and the extent to which there are differences in the absorption of folate from food compared with supplements, the evidence is still judged sufficient to support a recommendation to reduce the risk of NTD.' (pg 259)

Reference 2.0:
'The debate on folate requirements of normal pregnancy has been overtaken by the finding that periconceptual consumption of folic acid has a significant protective effect against the occurrence and recurrence of neural tube defects (NTD). …' (pg 809)

'… It appears that folic acid exerts its protective effect by overcoming a partial block in folate metabolism rather than by correcting a nutritional deficiency. A functional variant of the gene for 5,10-methylene-THF reductase, the 'thermolabile variant' associated with NTD, may express its aberrancy through an inability to bind its flavin cofactor properly, an inability shown to be corrected experimentally (in the bacterial enzyme at least) by increasing the folate concentration. It is likely that other variants of this gene and/or variants of other genes associated with folate metabolism are involved not only in NTD but also in vascular diseases related to hyper-homocysteinaemia.' (pg 809)

Reference 3.11:
'… an intake >400µg/day is considered protective against neural tube defect (NTD).' (pg 3)

Reference 4.10:
'Health experts recommend peri-conceptual folic acid supplementation in women for the prevention of neural tube defects in developing foetuses. The Department of Health recommends that all women planning a pregnancy take 400µg of folic acid from when they cease contraception to the twelfth week of pregnancy to reduce the risk of them having a NTD-affected pregnancy.' (pg 5)

Reference 6.2:
'Low folate status, even when serum and red blood cell folate levels are in the conventional normal range, is associated with an increased risk of neural tube defects.' (pg 19)

3) Neurotransmitters

Code   **Proposed statement**
Fo3:   *Folate is necessary for the normal structure of some neurotransmitters*

Reference 1.3:
'The folate coenzymes are involved in numerous reactions that involve … amino acid interconversions, including the catabolism of histidine to glutamic acid, interconversion of serine and glycine, and conversion of homocysteine to methionine. Folate-mediated transfer of single-carbon units from serine provides a major source of substrate in single-carbon metabolism. The conversion of homocysteine to methionine serves as a major source of methionine for the synthesis of *S*-adenosyl-methionine, an important in vivo methylating agent (Wagner, 1996).' (pg 197)

'The mechanism by which folate modifies brain functions has been sought for more than two decades and is generally hypothesized to be related to its role in single-carbon metabolism (Alpert and Fava, 1997). In particular, methylene tetrahydrofolate is the methyl donor in methionine synthesis from homocysteine and is postulated to be important in maintaining adequate methionine pools for *S*-adenosylmethionine (SAM) biosynthesis (Bottiglieri et al., 1994). SAM is the cofactor in key methylation reactions in catecholamine synthesis and metabolism in brain (Turner, 1977); catecholamines are transmitters known to be important in maintaining affective state,

and exogenous SAM has been shown by some to elevate mood (Bell et al., 1998). Folate has also been linked to the maintenance of adequate brain levels of tetrahydropterin (Hamon et al., 1986), a key cofactor in the hydroxylation reactions leading the synthesis of transmitters such as serotonin and the catecholamines (Turner, 1977). Methylation reactions involving folate may be important in maintaining neuronal and glial membrane lipids (Hirata and Axelrod, 1980), which could have effects on more general brain functions as reflected in changes in mood, irritability, and sleep.' (pg 268, 269)

'Although available information may suggest that a link exists between folate deficiency and abnormal mental function, more than three decades of research have not produced a definitive connection.' (pg 269)

Reference 2.0:
'Folate functions metabolically as an enzyme cofactor in the synthesis of nucleic acids and amino acids. Deficiency of the vitamin leads to impaired cell replication and other metabolic alterations particularly related to methionine synthesis.' (pg 803)

'In summary, the biochemical function of folate coenzymes is the transfer and utilization of these one-carbon units in a variety of essential reactions including … (3) amino acid interconversions – the interconversion of serine to glycine, catabolism of histidine to glutamic acid, and conversion of homocysteine to methionine (which also requires vitamin B_{12}); …' (pg 806)

Reference 3.11:
'Folates play an important role in the transfer of C_1-groups (i.e. methyl-, methylene- and formyl-groups), maintaining the methylation balance, such as in …amino acid metabolism.' (pg 2)

'In tissues folates are retained as polyglutamates and the folate coenzymes can be interconverted in numerous (de-)methylation reactions, such as in …amino acid interconversions, such as the remethylation of homocysteine to methionine. In this latter methionine synthase (MS) reaction vitamin B_{12} is also involved as a cofactor.' (pg 3)

Reference 4.10:
'Folate coenzymes within the cell are involved in one-carbon transfer reactions, including those involved in phases of amino acid metabolism …and the formation of the primary methylating agent, S-adenosylmethionine (SAM).' (pg 15)

'Chronic, severe folate deficiencyhas also, rarely, been associated with neurological changes and depression (*cited in* Weir & Scott 1999).' (pg 15)

'The other pathway that requires 5,10 methylene-THF is the biosynthesis of 5-methyl-THF, catalysed by the enzyme methylene THF reductase (MTHFR), using NADPH as a cofactor. This pathway is:- 1] an absolute requirement for the *de novo* synthesis of 5-methyl-THF, the predominant form of intracellular folate, and 2] irreversible under normal physiological conditions. The N-5 methyl group of 5-methyl-THF can be used metabolically only for transfer to homocysteine, resulting in the regeneration of methionine. In addition to its role as an amino acid, methionine serves as a methyl

group donor *via* conversion to S-adenosyl methionine (SAM), an important biological methylating agent involved in many methyltransferase reactions. The conversion of homocysteine to methionine, *via* methyl transfer from 5-methyl-THF, is catalysed by the enzyme methionine synthase (homocysteine-methyl-transferase) and requires methyl-Cobalamin (vitamin B_{12}) as a cofactor.' (pg 14)

Reference 6.2:
'…As well as being essential for the action of the enzyme thymidylate synthase, and thus having a crucial role in DNA synthesis, 5,10-methylene-THF also indirectly supplies methyl groups for the "methylation cycle". 5-Methyl-THF is formed by reduction of 5,10-methylene-THF under the action of the enzyme 5,10-methylene-THF reductase. The methyl group is transferred from 5-methyl-THF to methionine, catalysed by the vitamin B_{12} –dependent enzyme methionine synthase. S-adenosylmethionine (SAM) is synthesised from methionine, and acts as a methyl donor in the methylation of a range of diverse compounds with different functions. The methyltransferase reactions give rise to S-adenosylhomocysteine (SAH), which is immediately metabolised to homocysteine.' (pg 18)

'The folate level in cerebrospinal fluid is three times higher than that in plasma, and nerve tissue concentrates folate at the expense of other organs. This may account for the apparent protection of neural tissue from folate deficiency...' (pg 19)

'The concentration of folate in the form of methylfolate in the cerebrospinal fluid is approximately three times higher than that in the serum. Through the homocysteine/methionine pathway and ultimately through S-adenosylmethionine, methylfolate provides the methyl group in innumerable methylation reactions in the nervous system, involving, for example, nucleoproteins, proteins, phospholipids, monoamines and neurotransmitters. Methylfolate may also influence monoamine metabolism and mood through the biopterin pathway.' (pg 51)

4) Blood formation

Code   **Proposed statement**
Fo4:   *Folate is necessary for normal blood formation.*

Reference 1.3:
'Both folate and vitamin B_{12} are required for the formation of 5,10-methylenetetrahydrofolate and involved in thymidylate synthesis by way of a vitamin B_{12}-containing enzyme. The formation of 5,10-methylene tetrahydrofolate depends on the regeneration of the parent compound (tetrahydrofolate) in the homocysteine-to-methionine conversion. This reaction involves the removal of a methyl group from methyl folate and the delivery of this group to homocystine for the synthesis of methionine. Folate is involved as a substrate (5-methyl-tetrahydrofolate) and vitamin B_{12} as a coenzyme. The 5,10-methylenetetrahydrofolate delivers its methyl group to deoxyuridylate to convert it to thymidylate for incorporation into DNA. In either a folate or vitamin B_{12} deficiency, the megaloblastic changes occurring in the bone marrow and other replicating cells result from lack of adequate 5,10-methylene-tetrahydrofolate.' (pg 199)

'Within weeks of the development of early morphological abnormalities in the marrow, subtle changes appear in the peripheral blood (Eichner et al., 1971) when hypersegmentation of the neutrophils becomes apparent.' (pg 200)

'… When folate supply to the bone marrow becomes rate limiting for erythropoiesis, macrocytic cells are produced. …' (pg 200)

'As folate depletion progresses further, the mean cell volume increases above normal. Neutrophil hypersegmentation (defined as more than 5 percent five-lobed or any six-lobed cells per 100 granulocytes) is typically present in the peripheral blood at this stage of macrocytosis and the neutrophil lobe average is elevated.' (pg 200)

'Macrocytic anemia then develops, as first evidenced by a depression of the erythrocyte count. Eventually, all three measures of anemia (hematocrit, hemoglobin concentration, and erythrocyte concentration) are depressed. At this point, macroovalocytes and macrocytes are usually detectable in the peripheral blood, and hypersegmentation is more impressive (Lindenbaum et al., 1988).'(pg 200)

'Because folate is taken up only by the developing erythrocyte in the bone marrow and not by the circulating mature erythrocyte during its 120-day lifespan, erythrocyte folate concentration is an indicator of long-term status.' (pg 201)

Reference 2.0:
'Folate deficiency alone, manifested clinically as megaloblastic anaemia, is the most common vitamin deficiency in developed countries.' (pg 803)

Reference 4.10:
'Deficiency of folate results in a reduction in *de novo* DNA biosynthesis and, thus, impairment of cell replication, with the most obvious effects apparent in rapidly dividing cell-types, such as red blood cells and other cells generated by the bone marrow, enterocytes, and skin cells. This condition is recognised, haematologically, as a macrocytic anaemia, with characteristic red cell precursors (megaloblasts) present in bone marrow aspirates, and the clinical manifestation of megaloblastic anaemia.' (pg 15)

Reference 6.2:
'… Deficiency of this vitamin particularly affects rapidly dividing tissues such as bone marrow and mucous membranes, leading to symptoms of anaemia and sore tongue. The diagnosis is based on macrocytic red blood cells and hypersegmented neutrophils in the peripheral blood, on typical morphological changes of megaloblastosis in bone marrow, on low concentrations of folate in serum and red blood cells, and on the exclusion of vitamin B_{12} deficiency.' (pg 53)

5) Homocysteine metabolism

Code   **Proposed statement**
Fo5:   *Folate contributes to the maintenance of normal blood homocysteine levels*

Reference 1.3:

'The conversion of homocysteine to methionine serves as a major source of methionine for the synthesis of S-adenosyl-methionine, an important in vivo methylating agent (Wagner, 1996).' (pg 197)

'The formation of 5,10-methylene tetrahydrofolate depends on the regeneration of the parent compound (tetrahydrofolate) in the homocysteine-to-methionine conversion. This reaction involves the removal of a methyl group from methyl folate and the delivery of this group to homocystine for the synthesis of methionine.' (pg 199)

'Inadequate folate intake first leads to a decrease in serum folate concentration, then to a … rise in homocysteine concentration, …' (pg 199)

'Plasma homocysteine concentration increase when inadequate quantities of folate are available to donate the methyl group that is required to convert homocysteine to methionine. Controlled metabolic and epidemiological studies provide evidence that plasma homocysteine rises with reductions in blood folate indices. … Many investigators have reported that plasma homocysteine is significantly elevate in individuals who have been diagnosed as folate deficient on the basis of established serum folate, plasma folate, or erythrocyte folate norms (Allen et al., 1993; Chadefaux et al., 1994; Curtis et al., 1994; Kang et al., 1987; Savage et al., 1994; Stabler et al., 1988; Ubbink et al., 1993).' (pg 201/202)

'Thus, in studies of different types, a similar inverse relationship between folate intake and plasma homocysteine values is seen for pre- and postmenopausal women, adult men, and the elderly.' (pg 202)

'Folate is required in the form of methyltetrahydrofolate as a substrate for methionine synthase. Therefore, the remethylation of homocysteine depends on adequate quantities of folate.' (pg 261)

'The inverse relationship between folate intake and homocysteine concentration is well established. However, there are conflicting data on the association among indicators of folate status or metabolism, homocysteine concentration, and risk of vascular disease. Whether increasing intake of folate could reduce the risk of vascular disease remains to be demonstrated. Folate may reduce the risk of cardiovascular disease through other mechanisms.' (pg 263)

Reference 2.0:

'A solitary transfer of one-carbon units takes place at the methanol level of oxidation. It involves the transfer of the methyl group from 5-methyl-THF to homocysteine to form methionine and THF. This reaction is catalysed by the enzyme methionine synthase and requires vitamin B_{12} as cofactor.' (pg 804)

'An important consequence of folate deficiency is the inability to remethylate homocysteine. Indeed, there is an inverse correlation between the levels of folate and homocysteine in the blood of humans. Many clinical studies, beginning with the observations of the children with homocysteinuria presenting with vascular abnormalities and thromboembolism, have demonstrated an association between

hyperhomocysteinaemia and increased risk of premature atherosclerosis in the coronary, carotid and peripheral vasculature. The prevalence of hyperhomocysteinaemia compared with normal controls. Even mild hyperhomocysteinaemia is recognized to be an independent risk factor for cardiovascular disease. Metabolically, homocysteine may be disposed of by the methionine synthase reaction (dependent on folate as well as vitamin B_{12}), the transsulfuration pathway (dependent on vitamin B_6) and by the choline degradation pathway. Marginal deficiencies of these three vitamins are associated with hyperhomocysteinaemia. Of the three, however, folic acid administration has been shown to be the most effective in lowering homocysteine, blood levels. Convincing evidence of the potential role of folate intake in the prevention of vascular disease, probably by lowering blood levels of homocysteine, has been demonstrated by a significant inverse relationship between serum folate levels and fatal coronary heart disease.' (pg 806)

Reference 3.11:
'…amino acid interconversions, such as the remethylation of homocysteine to methionine.' (pg 3)

Reference 4.10:
'The N-5 methyl group of 5-methyl-THF can be used metabolically only for transfer to homocysteine, resulting in the regeneration of methionine. In addition to its role as an amino acid, methionine serves as a methyl group donor *via* conversion to S-adenosyl methionine (SAM), an important biological methylating agent involved in many methyltransferase reactions. The conversion of homocysteine to methionine, *via* methyl transfer from 5-methyl-THF, is catalysed by the enzyme methionine synthase (homocysteine-methyl-transferase) and requires methyl-Cobalamin (vitamin B_{12}) as a cofactor.' (pg 14)

Reference 6.2:
'The 5-methyl-THF needed for methionine synthase is provided by 5,10-methylene-THF reductase. The activities of this enzyme and those of methionine synthase and cystathionine synthase keep the levels of homocysteine in cells and in plasma normally within a narrow range. These three enzymes depend, respectively, on folate and vitamin B_{12} and vitamin B_6. Thus reduced status of any of these nutrients can cause an elevation in plasma homocysteine. In practice, high plasma homocysteine levels are most likely to be related to low folate status, rather than low status of vitamins B_6 or B_{12}.' (pg 18)

ANNEX 4.11

Vitamin B$_{12}$

Source documents for reviewing vitamin B$_{12}$

Reference 1.3:
Institute of Medicine Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B$_6$, folate, vitamin B$_{12}$, pantothenic acid, biotin and choline. Washington D.C. National Academy Press 2000.

Reference 2.0:
Encyclopedia of Human Nutrition 2E. Editor-in-chief, Michele J. Sadler, editors, J.J. Strain, Benjamin Caballero. San Diego : Academic Press, c1999.

Reference 3.12:
Opinion of the Scientific Committee on Food (SCF) on the Tolerable Upper Intake Level of Vitamin B$_{12}$. October 2000. (http://www.europa.eu.int/comm/food/fs/sc/scf/out80d_en.pdf).

Reference 4.11:
Revised review of Vitamin B$_{12}$. Expert Group on Vitamins and Minerals. August 2002. (http://www.food.gov.uk/multimedia/pdfs/EVM/00/20/P).

Reference 5.0:
Handbook of Nutrition and Food. Edited by Carolyn D Berdanier...[et al.]. Boca Raton, Fla.: CRC Press, 2002.

1) Cell division and blood formation

Code   **Proposed statement**
VB$_{12}$1a: *Vitamin B12 is necessary for normal cell division (such as in the gastro-intestinal tract).*
VB$_{12}$1b: *Vitamin B12 contributes to normal blood formation.*

Reference 1.3:
'B$_{12}$ is a cofactor for two enzymes: methionine synthase and L-methylmalonyl-CoA mutase. Methionine synthase requires methylcobalamin as a cofactor for the methyl transfer from methyltetrahydrofolate to homocysteine to form methionine and tetrahydrofolate…An adequate supply of B$_{12}$ is essential for normal blood formation and…' (pg 307)

'As in folate deficiency, the underlying mechanism of anemia is an interference with normal deoxyribonucleic acid (DNA) synthesis. This results in megaloblastic change, which causes production of larger-than-normal erythrocytes (macrocytosis). This leads first to an increase in the erythrocyte distribution width index and ultimately to an elevated mean cell volume. Oval macrocytes and other abnormally shaped erythrocytes are present in the blood… By the time anemia has become established, there is usually also some degree of neutropenia and thrombocytopenia because the

megaloblastic process affects all rapidly dividing marrow elements. The hematological complications are completely reversed by treatment with $B_{12.}$ (pg 311)

Reference 2.0:
'Methionine synthase or N^5 – methyl tetrahydrofolate: homocysteine methyltransferase, which uses methyl-Cbl as a cofactor, forms an integral link between two essential metabolic processes of internal metabolism: the synthesis of the nucleic acids (RNA and DNA) via purines and pyrimidines, and the methylation reactions via *S*-adenosylmethionine.' (pg 397)

'Serine, which is synthesized from glucose passes its beta carbon moiety to tetrahydrofolate (THF) to produce N^5,N^{10} –methylene-THF and glycine in the cytoplasm of the cell. The product N^5,N^{10} –methylene-THF then stands at a metabolic crossroads. On the one hand, it can either (1) in conjunction with deoxyuridine monophosphate synthesize thymidylate which in turn produces a pyrimidine base of DNA, or (2) produce N^{10} –formyl-THF which inserts carbons 2 and 3 into the purine ring. On the other hand, it can be reduced to N^5 – methyl-THF which is required for the remethylation of homocysteine to methionine via methionine synthase, which is subsequently converted to *S*-adenosylmethionine (Ado-Met) via *S*-adenosylmethionine synthetase. Ado-Met, the universal methylator is essential for 35 methylation reactions in internal metabolism which have important synthetic and regulatory functions…Another essential function of methionine synthase is to act as a gatekeeper for the entry of folate into the cell.' (pg 397)

'Ado-Met (*S*-adenosylmethionine) function is also controlled by the level of its product, adenosylhomocysteine (Ado-Hcy), which is its main inhibitor. Thus, the ratio of the level of Ado-Met to that of Ado-Hcy has often been described as the 'methylation ratio'. When homocysteine levels rise, the back reaction of *S*-adenosylhomocysteine hydrolase is favoured and Ado-Hcy levels increase. This leads to inhibition of the methylation reactions and their regulation of internal metabolism.' (pg 397, 398)

'Inhibition of methionine synthase as a result of methyl-Cbl deficiency leads to reduced synthesis of THF and methionine, and to the accumulation of homocysteine and N^5 – methyl-THF. This leads to reduced availability of N^5, N^{10} – methylene-THF for thymidylate synthesis. Also … deficiency of methionine and Ado-Met leads to enhance conversion of N^5, N^{10} – methylene-THF to N^5 – methyl-THF which under physiological conditions is irreversible, thus further depleting the supply of N^5, N^{10} – methylene-THF and thymidylate for nucleic acid synthesis. This forms the basis of what has been termed the 'methyl-folate trap' hypothesis. The resulting nucleic acid deficiency induces a megaloblastic anaemia in the bone marrow which is identical to that induced by folate deficiency.' (pg 398, 399)

Reference 3.12:
'The key symptom in vitamin B_{12} deficiency is macrocytic megaloblastic anemia. These haematological abnormalities are indistinguishable from those seen in folate deficiency, because of the interrelated function of both vitamins (Herbert, 1986).' (pg 2)

Reference 4.11:

'In the form of methyl Cbl, vitamin B_{12} participates as a cofactor to the enzyme methionine synthase in the methylation of homocysteine (Hcy) which involves transfer of the methyl group from N^5-methyltetrahydrofolate (N^5-methyl-THF-glu$_{1-5}$). As such, vitamin B_{12} plays a pivotal role in one-carbon (methyl donor) metabolism, vital to many aspects of cellular metabolism, including the synthesis of the building block precursors to DNA and RNA.' (pg 12)

'The methionine formed may be converted to S-adenosylmethionine (SAM). SAM acts as the universal methyl donor in more than 100 methylation reactions within the cell, all of which are essential for internal metabolism. In particular, SAM is the major direct donor of methyl groups in the synthesis of polyamines (e.g. spermidine and putrescine *[important in cell and tissue growth]*).' (pg 13)

'The N^5-THF-glu$_1$ formed in the methionine synthase reaction is converted to the polyglutamated form N^5-THF-glu$_5$ by folyl-γ-glutamate synthetase which is the central folate acceptor molecule in the folate one-carbon cycle. In turn, N^5-THF-glu$_5$ receives the β-carbon moiety from serine, via serine hydroxymethyltransferase, to give glycine and N^5,N^{10} – methylene-THF-glu$_5$. N^5,N^{10} – methylene-THF-glu$_5$ either acts as a methyl donor in the conversion of deoxyuridylate monophosphate to thymidylate monophosphate (the precursor to the pyrimidine base thymidine) in a reaction catalysed by thymidine synthetase, is converted to N^{10} formyl-THF-glu$_5$, which provides carbons 2 and 8 in the synthesis of the purine bases, or is reduced to methyl-THF-glu$_5$, which can serve to re-methylate homocysteine to methionine.' (pg 13)

2) Neurological system

Code   **Proposed statement**
VB$_{12}$2:   *Vitamin B12 is necessary for the normal structure and function of the neurological system.*

Reference 1.3:
'An adequate supply of B_{12} is essential for normal …and neurological function.' (pg 307)

'Neurological complications are present in 75 to 90 per cent of individuals with clinically observable B_{12} deficiency and may, in about 35 per cent of cases, be the only clinical manifestation of B_{12} deficiency.' (pg 311)

'Neurological manifestations include sensory disturbances in the extremities (tingling and numbness), which are worse in the lower limbs. Vibratory and position sense are particularly affected. Motor disturbances, including abnormalities of gait, also occur. Cognitive changes may occur, ranging from loss of concentration to memory loss, disorientation, and frank dementia, with or without mood changes. In addition, visual disturbances, insomnia, impotency, and impaired bowel and bladder control may develop.' (pg 312)

'In some cases, neurological manifestations may be the earliest clinical sign of low B_{12} values (Beck, 1991; Karnaze and Carmel, 1990; Lindenbaum et al., 1998; Martin et al., 1992).' (pg 328)

Reference 2.0:
'The other effect of methionine synthase inhibition by Cbl deficiency is both to reduce the endogenous supply of methionine and Ado-Met and to increase the levels of homocysteine and Ado-Hcy. This causes a reduction of the Ado-Met/Ado-Hcy ration (the methylation ratio), which as explained above will inhibit the methylation reactions which in turn produces the neuropathy.' (pg 399)

Reference 3.12:
'Another key symptom of vitamin B_{12} deficiency are neurological complications, such as paraesthesia, leg weakness, memory loss, etc, due to progressive lesions in the lateral and posterior columns of the spinal cord (subacute combined degeneration of the spinal cord). Neurological symptoms occur in about 75-90% of all individuals with (untreated) vitamin B_{12} deficiency, and appear generally at a later stage.' (pg 2)

Reference 4.11:
'The methionine formed may be converted to S-adenosylmethionine (SAM). SAM acts as the universal methyl donor in more than 100 methylation reactions within the cell, all of which are essential for internal metabolism. In particular, SAM is the major direct donor of methyl groups in the synthesis of polyamines (e.g. spermidine and putrescine *[important in cell and tissue growth]*).' (pg 13)

3) Energy production

Code   **Proposed statement**
$VB_{12}3$:   Vitamin B_{12} contributes to normal energy production

Reference 1.3:
'B_{12} is a cofactor for two enzymes: methionine synthase and L-methylmalonyl-CoA mutase. … L-Methymalonyl-CoA mutase requires adenosylcobalamin to convert L-methymalonyl-CoA to succinyl-CoA in an isomerization reaction...' (pg 307)

Reference 2.0:
'Methylmalonyl-CoA mutase requires adenosyl-Cbl. Methylmalonyl semialdehyde is produced by a series of compounds which include amino acids (valine, isoleucine, methionine and threonine), along with cholesterol, thymine and odd-chain fatty acids. Methylmalonyl semialdehyde is then metabolized via propionyl-CoA to methylmalonyl-CoA. Methylmalonyl-CoA is normally converted to succinyl-CoA and propionic acid via *SR*-methylmalonyl-CoA racemase, and subsequently the adenosylcobalamin-dependent *R*-methylmalonyl-CoA mutase. However, when Cbl deficiency is present, (1) the mutase function is impaired and *S*-methylmalonyl-CoA is converted to methylmalonic acid (MMA) via *S*-methylmalonyl-CoA hydrolase, a vitamin-independent enzyme; and (2) propionyl-CoA levels will be elevated, and in association with oxaloacetic acid and citrate synthase will produce 2-methylcitric acids 1 and 2. Consequently, in Cbl deficiency states concentrations of

methylmalonyl-CoA, its hydrolytic product MMA, and 2-methylcitric acids 1 and 2 are raised.' (pg 397)

Reference 3.12:
Vitamin B_{12} plays a specific role in amino acid metabolism, i.e. in methylation reactions, together with folate, in the methionine synthase reaction, and in the rearrangement of methylmalonyl CoA into succinyl CoA (for review see Herbert, 1984; Ellenbogen & Cooper, 1991). (pg 3)

Reference 4.11:
'Methionine synthase also acts as gatekeeper for the entry of folate into the cell. Folate enters in the form of N^5 –methyl-THF-glu_1 and can only remain inside the cell following demethylation via methionine synthase. Consequently, the uptake of folate into the cell is also dependent on the methyl Cbl form of vitamin B_{12}.' (pg 13)

'As deoxyadenosylCbl (adoCbl), vitamin B_{12} has the role of obligate cofactor for the enzymatic conversion of L-methylmalonyl CoA to succinyl CoA by methylmalonyl CoA mutase.' (pg 13)

4) Homocysteine metabolism

Code   **Proposed statement**
$VB_{12}4$:   *Vitamin B_{12} contributes to the maintenance of normal blood homocysteine levels*

Reference 1.3:
'Vitamin B_{12} (cobalamin) functions as a coenzyne for a critical methyl transfer reaction that converts homocysteine to methionine.' (pg 306)

'B_{12} is a cofactor for two enzymes: methionine synthase and L-methylmalonyl-CoA mutase. Methionine synthase requires methylcobalamin as a cofactor for the methyl transfer from methyltetrahydrofolate to homocysteine to form methionine and tetrahydrofolate…' (pg 307)

'Serum total homocysteine concentration is commonly elevated in elderly persons whose folate status is normal but who have a clinical response to treatment with B_{12} (Stabler et al., 1996). Because a lack of folate, vitamin B_6, or both also results in an elevated serum and plasma homocysteine concentration, this indicator has poor specificity…' (pg 314)

'Lindenbaum and colleagues (1990) reported that metabolites that arise from B_{12} insufficiency are more sensitive indicators of B_{12} deficiency than is the serum B_{12} value. This was found in patients with pernicious anemia or previous gastrectomy who experienced early haematological relapse: serum methylmalonic acid (MMA), total homocysteine, or both were elevated in 95 percent of the instances of relapse whereas the serum B_{12} value wa low (less than 150 pmol/L [200pg/mL] in 69 percent….At present, the techniques developed to measure serum MMA and homocysteine (capillary gas chromatography and mass spectrometry) are costly and may be beyond the scope of routine laboratories.' (pg 316)

Reference 2.0:
'Serine, which is synthesized from glucose passes its beta carbon moiety to tetrahydrofolate (THF) to produce N^5,N^{10} –methylene-THF and glycine in the cytoplasm of the cell. The product N^5,N^{10} –methylene-THF then stands at a metabolic crossroads. … it can be reduced to N^5 – methyl-THF which is required for the remethylation of homocysteine to methionine via methionine synthase, which is subsequently converted to *S*-adenosylmethionine (Ado-Met) via *S*-adenosylmethionine synthetase.' (pg 397)

'The availability of methyl-Cbl and the substrates of the reaction controlled by methionine synthase – namely homocysteine and N^5 – methyl-THF – control its function. The availability of these substrates is in turn tightly controlled by the availability of dietary methionine and its products Ado-Met.' (pg 397)

'Plasma homocysteine is derived from intracellular homocysteine which occurs as a product of dietary methionine metabolism. The level is maintained by four enzymes, *S*-adenosylhomocysteine hydrolase (which increases it), and cystathionine synthase, methylene reductase and methionine synthase (which act to reduce it). The latter three enzymes have as cofactors the vitamins pyridoxine, folate and cobalamin respectively.' (pg 399)

Reference 3.12:
Vitamin B_{12} plays a specific role in amino acid metabolism, i.e. in methylation reactions, together with folate, in the methionine synthase reaction, and in the rearrangement of methylmalonyl CoA into succinyl CoA (for review see Herbert, 1984; Ellenbogen & Cooper, 1991). (pg 3)

Reference 4.11:
'In the form of methyl Cbl, vitamin B_{12} participates as a cofactor to the enzyme methionine synthase in the methylation of homocysteine (Hcy) which involves transfer of the methyl group from N^5-methyltetrahydrofolate (N^5-methyl-THF-glu$_{1-5}$).' (pg 12)

'N^5,N^{10} – methylene-THF-glu$_5$ either …or is reduced to methyl-THF-glu$_5$, which can serve to re-methylate homocysteine to methionine.' (pg 13)

Reference 5.0:
'Higher blood levels of vitamins B_6, B_{12}, and folic acid are associated with low levels of homocysteine, and supplementing with these vitamins helps to lower homocysteine levels.' (pg 1339)

ANNEX 4.12

Biotin

Source documents for reviewing biotin

Reference 1.3:
Institute of Medicine Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B₁₂, pantothenic acid, biotin and choline. Washington D.C. National Academy Press 2000.

Reference 2.0:
Encyclopedia of Human Nutrition 2E. Editor-in-chief, Michele J. Sadler, editors, J.J. Strain, Benjamin Caballero. San Diego : Academic Press, c1999.

Reference 3.13:
Opinion of the Scientific Committee on Food (SCF) on the Tolerable Upper Intake Level of Biotin. September 2001. (http://www.europa.eu.int/comm/food/fs/sc/scf/out106_en.pdf).

Reference 4.12:
Revised Review of Biotin. Expert Group on Vitamins and Minerals. April 2002. (http://www.food.gov.uk/multimedia/pdfs/biotin.pdf).

1) Energy metabolism

Code   Proposed statement
Bi1:   Biotin contributes to normal fat metabolism and energy production

Reference 1.3:
'The second biotin-dependent carboxylase, pyruvate carboxylase, catalyzes the carboxylation of pyruvate to form oxaloacetate, which serves as an intermediate in the tricarboxylic acid cycle. Oxaloacetate thus formed is converted to glucose in the liver, kidney, and other gluconeogenic tissues. A third biotin-dependent carboxylase, β-methylcrotonyl-CoA carboxylase, is required for the degradation of leucine, a branch-chained amino acid…A fourth biotin-dependent carboxylase, propionyl-CoA carboxylase, carboxylates propionyl-CoA to form D-methylmalonyl-CoA, which is racemized to the L-isomer, then undergoes isomerization to succinyl-CoA, and subsequently enters the tricarboxylic acid cycle.' (pg 375)

Reference 2.0:
'Pyruvate carboxylase (EC 6.4.1.1) catalyses the incorporation of bicarbonate into pyruvate to form oxaloacetate, an intermediate in the Krebs tricarboxylic acid cycle. Thus, pyruvate carboxylase (PC) catalyses an anaplerotic reaction. In gluconeogenic tissues (i.e. liver and kidney), the oxaloacetate can be converted to glucose. Deficiency of PC is probably the cause of the lactic acidaemia, central nervous system lactic acidosis and abnormalities in glucose regulation observed in biotin deficiency and biotinidase deficiency.' (pg 173)

'Methylcrotonyl-CoA carboxylase (EC 6.4.1.4) catalyses an essential step in the degradation of the branched chain amino acid leucine… (pg 173)

Propionyl-CoA carboxylase (EC 6.4.1.3) catalyses the incorporation of bicarbonate into propionyl-CoA to form methylmalonyl-CoA, which undergoes isomerization to succinyl-CoA and enters the tricarboxylic acid cycle.' (pg 173)

Reference 3.13:
'In main biotin is an essential co-factor for four carboxylases which catalyse the incorporation of bicarbonate into a substrate and are involved in gluconeogenesis and provision of intermediates into the citric acid cycle (pyruvate carboxylase, PC, EC 6.4.1.1)... leucine catabolism (3-methylcrotonyl-CoA carboxylase, MCC EC 6.4.1.4) and propionate catabolism (propionyl-CoA carboxylase, PCC, EC 6.4.1.3). The propionate to be carboxylated has various sources: catabolism of valine, iosleucine, threonine, methionine, the side chain of cholesterol, odd-numbered saturated fatty acids, and metabolism of intestinal bacteria.' (pg 5)

Reference 4.12:
'Biotin acts as an essential cofactor for the… propionyl-CoA, β-methlcrotonyl-CoA, and pyruvate carboxylase (… PCC, MCC and PC) enzymes. These … enzymes catalyse critical steps in pathways of intermediary metabolism… PC catalyses the incorporation of bicarbonate into pyuvate to form oxaloacetic acid (OAA), a Kreb's tricarboxylic acid cycle intermediate. In gluconeogenic tissues, such as liver and kidney OAA can be converted into glucose. MCC catalyses a critical step in the degradation of the branch-chain amino acid, leucine. PCC catalyses the carboxylation of propionyl-CoA to form D-methylmalonyl-CoA. D-methylmalonyl-CoA is racemised to the L-isomer and subsequently undergoes isomerisation to form the tricarboxylic acid intermediate succinyl-CoA.' (pg 13, 14)

2) Fatty acids

Code　　**Proposed statement**
Bi2:　　*Biotin is necessary for the synthesis of fatty acids, which are important for the normal structure of cell membranes*

Reference 1.3:
'Acetyl-CoA carboxylase catalyzes the carboxylation of acetyl CoA to form malonyl CoA. Malonyl CoA then serves as a substrate for fatty acid elongation.' (pg 375)

Reference 2.0:
'Acetyl-CoA carboxylase (EC 6.4.1.2) catalyses the incorporation of bicarbonate into acetyl-Co-A to form malonyl-CoA. This three-carbon compound then serves as a substrate for the fatty acid synthetase complex; the net result is the elongation of the fatty acid substrate by two carbons and the loss of the third carbon as CO_2.' (pg 173)

'Odd-chain fatty acid accumulation is also a marker of biotin deficiency. The accumulation of odd-chain fatty acid is thought to result from PCC deficiency.' (pg 174)

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Reference 3.13:
'In main biotin is an essential co-factor for four carboxylases which catalyse the incorporation of bicarbonate into a substrate and are involved in …fatty acid synthesis (acetyl-CoA carboxylase, ACC, EC 6.4.1.2)…' (pg 5)

Reference 4.12:
'Biotin acts as an essential cofactor for … acetyl-CoA…ACC catalyses the incorporation of bicarbonate into acetyl-CoA to form malonyl-CoA. Malonyl-CoA, in turn, serves as the substrate for the fatty acid synthetase complex, donating two of its carbons to the fatty acid elongation process with the loss of the third as carbon dioxide.' (pg 14)

3) Cell proliferation and growth

Code   **Proposed statement**
Bi3a: *Biotin is necessary for normal cell proliferation*
Bi3b: *Biotin is contributes to normal growth in the developing embryo and infant*

Reference 1.3:
'In infants on biotin-free TPN, symptoms of biotin deficiency begin to appear within 3 to 6 months after initiation of the TPN regimen, which is earlier than that seen in adults, probably because of the increased biotin requirement related to growth (Mock, 1996).' (pg 377)

Reference 2.0:
'In the normal turnover of cellular proteins, holocarboxylases are degraded to biocytin or biotin linked to an oligopeptide containing at most a few amino acid residues. Because the amide bond between biotin and lysine is not hydrolysed by cellular proteases, the specific hydrolase biotinidase (biotin-amide hydrolase, EC 3.5.1.12) is required to release biotin for recycling. The biotinidase gene is a single copy gene of 1629 bases encoding a 543 amino acid protein. Biotinidase mRNA is present in many tissues including heart, brain, liver, lung, skeletal muscle, kidney, pancreas and placenta. The greatest biotinidase activities are found in serum, liver, kidney and the adrenal gland. The observation that serum concentrations of biotinidase are decreased in patients with impaired liver function suggest that if the liver is the source of serum biotinidase.' (pg 173)

Reference 3.13:
'Biotinidase is able to recycle biotin bound to carboxylases and to cleave biotin bound to dietary proteins. Apart from this important function of biotinidase in providing biotin for intermediary metabolism, a function of this enzyme, is the transfer of biotin to nucleophilic acceptor proteins such as histones, thereby affecting gene expression (Hymes and Wolf, 1996) and e.g. embryological development (Bender, 1999; Zempleni and Mock, 2000b). Biotin is essential for cell proliferation. Its proliferative effect in immune cells can become of clinical relevance in biotin deficiency (Zempleni and Mock, 2001).' (pg 5, 6)

Reference 4.12:

'In addition to its role in the hydrolysis of biotin…biotinidase has been shown in vitro to catalyse the biotinylation of histone proteins…The specific transfer of biotin to histones may explain the presence of the vitamin inside the nucleus and suggests a role in the regulation of protein transcription.' (pg 14)

ANNEX 4.13

Vitamin C

Source documents for reviewing vitamin C

Reference 1.2:
Institute of Medicine Dietary Reference Intakes for Vitamin C, Vitamin E, selenium and carotenoids.
Washington D.C. National Academy Press, 2000.

Reference 2.0:
'Encyclopedia of Human Nutrition 2E'.
Editor-in-chief, Michele J. Sadler, editors, J.J. Strain, Benjamin Caballero. San Diego: Academic Press, c1999.

Reference 3.23:
Report of the Scientific Committee for Food (Thirty-first series) on 'Nutrient and energy intakes for the European Community' (1992).

Reference 4.13:
Revised review of Vitamin C. Expert Group on Vitamins and Minerals. August 2002.
(http://www.food.gov.uk/multimedia/pdfs/vitaminc.pdf).

1) Connective tissue

Code   **Proposed statement**
VC1:   *Vitamin C is necessary for the normal structure and function of connective tissue (such as that required for normal gums, skin, healing processes, bone and cartilage).*

Reference 1.2:
'It is a co-factor for enzymes involved in the biosynthesis of collagen...' (pg 95)

'Vitamin C is known to be an electron donor for eight human enzymes. Three participate in collagen hydroxylation ...' (pg 96)

'Evidence also suggests that ascorbate plays a role in or influences collagen gene expression, cellular procollagen secretion, and the biosynthesis of other connective tissue components besides collagen, including elastin, fibronectin, proteoglycans, bone matrix, and elastin-associated fibrillin.' (pg 98).

'Lack of ascorbate-related hydroxyproline and hydroxylysine formation needed for collagen cross-linking may explain many of the connective tissue and hemorrhagic manifestations of scurvy, however, the specific histologic defects have not been identified.' (pg 101)

'Ascorbic acid is required along with iron as a cofactor for the post-translation hydroxylation of proline and lysine to effect cross-linking of mature collagen… despite the important role of the vitamin in collagen formation, no collagen-related measures are available to use as a functional indicator for the dietary vitamin C requirement.' (pg 118-119)

Reference 2.0:
'Proline and lysine hydroxylases are required for the postsynthetic modification of collagen …' (pg 146)

'The best studied of this class of enzymes is procollagen proline hydroxylase; it is assumed that the others follow essentially the same mechanism. The first step in the reaction is an attack on the substrate by oxygen, followed by condensation with 2-oxoglutarate, the release of the hydroxylated substrate and decarboxylation to release succinate. There is oxidation of ascorbate during the reaction, but not stoichiometrically, with the decarboxylation of 2-oxoglutarate and hydroxylation of the substrate. The purified enzyme is active in the absence of ascorbate, but after some 5-10 seconds (about 15-30 cycles of enzyme action) the rate of reaction begins to fall. At this stage the iron in the catalytic site has been oxidized to Fe^{3+}, which is catalytically inactive. Activity is only restored by ascorbate, which reduces the iron back to Fe^{2+}. This oxidation of the catalytic iron in the enzyme is the consequence of a side reaction rather than the main reaction of the enzyme. Nevertheless, ascorbate is essential for the activity of these enzymes *in vivo.* (pg 146-147)

Reference 4.13:
'The functions of vitamin C include synthesis of collagen…' (pg 6)

'Clinical signs of scurvy are due to inhibition of collagen synthesis, this leads to failure to maintain the cellular structure of supporting tissues of mesenchymal origin, such as bone, dentine, cartilage and connective tissues.' (pg 7)

2) & 3) Wound healing & scar tissue formation

Code   **Proposed statement**
VC2:   *Vitamin C is necessary for the normal structure of wounds*
VC3:   *Vitamin C is necessary for the normal structure of scar tissue*

Reference 1.2:
'Clinical features of scurvy include … impaired wound healing' (pg 101)

Reference 4.13:
'Wound healing is impaired, since in deficiency, although fibroblasts proliferate they remain immature and fail to synthesise collagen.' (pg 7)

4) Gums

Code   **Proposed statement**
VC4:   *Vitamin C is necessary for the normal structure of gums*

Reference 1.2:
'In experimental subjects made vitamin C deficient,… gingival inflammation and fatigue were amongst the most sensitive markers of deficiency' (pg 101)

'Epidemiological studies have failed to demonstrate an association between Vitamin C intake and periodontal disease. Controlled experimental studies of patients with gingivitis and apparently healthy adults with vitamin C intakes of 5 to 1500 mg/day have shown mixed results with regards to the influence of vitamin C status on periodontal integrity. Other studies, with animals and humans, have shown that vitamin C intake can affect the structural integrity of gingivial tissue...' (pg 120)

'Overall, while evidence suggests that Vitamin C deficiency is linked to some aspects of periodontal disease, the relationship of vitamin C intake to periodontal health in the population is unclear.' (pg 120)

Reference 4.13:
'Specific signs [of scurvy] such as swollen, bleeding and sensitive gums,…' (pg 7)

5) Blood vessels

Code   **Proposed statement**
VC5:   *Vitamin C is necessary for the normal structure and function of blood
              vessels*

Reference 1.2:
'Clinical features of scurvy include … Ecchymoses …(*the skin discoloration caused by the escape of blood into the tissues from ruptured blood vessels).* Vitamin C deficiency in infants may result in … and hemorrhagic symptoms and resultant anaemia…. Oxidative degradation of some blood coagulation factors due to low plasma ascorbate concentrations may contribute to hemorrhagic symptoms.' (pg 101)

'Impaired vascular function is crucial to the clinical manifestation of atherosclerosis… numerous investigators have reported a beneficial effect of high dose *(up to 3g per day)* vitamin C administration…on vasodilation. Vitamin C improves endothelial function and vasodilation, possibly by scavenging superoxide radicals, conserving intracellular glutathione, or potentiating intracellular NO synthesis.' (pg 103)

Reference 4.13:
'Specific signs [of scurvy] such as,…petechial haemorrhages under the skin…A loss of blood may also be observed due to petechiae, perifollicular haemorrhages and bleeding gums.' (pg 7)

6) Skin

Code   **Proposed statement**
VC6:   *Vitamin C is necessary for the normal structure of skin*

Reference 1.2:
'Clinical features of scurvy include follicular hyperkeratosis … and coiled hairs'. (pg 101)

Reference 4.13:
'Specific signs [of scurvy] include hardening and roughness around hair follicles (hyperkeratosis),…' (pg 7)

7) & 8) Bone and joints

Code   **Proposed statement**
VC7:   *Vitamin C is necessary for the normal structure of connective tissue in bone*
VC8:   *Vitamin C contributes to the normal structure of joints*

Reference 1.2:
'Evidence also suggests that ascorbate plays a role in or influences…and the biosynthesis of other connective tissue components beside collagen, including, elastin, fibroconectin, proteoglycans, bone matrix and elastin-associated fibrillin.' (pg 98).

'Clinical features of scurvy include… joint effusions *(pouring out of fluid)*, arthralgia *(joint pain)*,…' (pg 101)

'Vitamin C deficiency in infants may result in bone abnormalities such as impaired bone growth and disturbed ossification,…' (pg 101)

Reference 2.0:
'… proline hydroxylase is also required for the postsynthetic modification of osteocalcin in bone and the C1q component of complement.' (pg 146)

Reference 3.23:
In childhood scurvy, the bone tissue is most obviously involved, especially in the breast cage and the stressed epiphyseal cartilage of the extremities. (pg 124)

Vitamin C status can be evaluated from signs of clinical deficiency such as joint pain. (pg 124)

Reference 4.13:
'Early signs of deficiency, that is scurvy, are relatively non-specific. They often include…, aching bones, joints,…As the dentine becomes porous, alveolar bone becomes osteoporotic, and the teeth loosen and fall out. The cartilage matrix of the epiphyseal plate builds up between long bones and can become calcified; this results in compressed and brittle bone.' (pg 7)

9) Iron absorption

Code   **Proposed statement**
VC9:   *Vitamin C contributes to the absorption of iron from food*

Reference 1.2:
'Ascorbic acid modulates iron absorption, transport and storage.' (pg 99)

Reference 2.0:
'Inorganic dietary iron is absorbed as Fe^{2+}, not as Fe^{3+}; ascorbic acid in the intestinal lumen will not only maintain iron in the reduced state but also chelate it, thus increasing absorption considerably. A dose of 25mg of vitamin C taken with a meal increases the absorption of iron by some 65%, while a 1g dose gives a nine-fold increase. This is an effect of ascorbic acid present together with the test meal; neither intravenous administration of vitamin C nor supplements several hours before the test meal have any effect on iron absorption. The endogenous vitamin C in foods has the same effect on iron absorption. This is not a specific effect of ascorbate; a variety of other reducing agents also enhance the absorption of inorganic iron.' (pg 147)

Reference 4.13:
'Ascorbic acid is a potent enhancer of non-haem iron absorption from food. Ascorbic acid in the intestine is thought to keep iron in its reduced form, preventing the formation of insoluble ferric hydroxide and hence aids absorption. Ascorbic acid may also be involved in the transfer of iron into the blood, as well as mobilising it from its stores.' (pg 7)

10) Antioxidant activity

Code   **Proposed statement**
VC10:   *Vitamin C contributes to cell protection from the damage caused by free radicals (such as during the immune response)*

Reference 1.2:
'Vitamin C functions physiologically as a water-soluble antioxidant by virtue of its very high reducing power… and facile regeneration via ubiquitous reductants such as glutathione…' (pg 43)

'Evidence for in vivo antioxidant functions of ascorbate include the scavenging of reactive oxidants in activated leukocytes, lung, and gastric mucosa, and diminished lipid peroxidation as measured by urinary isoprostane excretion.' (pg 95)

'Because of it's ability to donate electrons, ascorbic acid is an effective antioxidant. The vitamin readily scavenges reactive oxygen species (ROS) and reactive nitrogen species (RNS)… The relatively high tissue levels of ascorbate provide substantial antioxidant protection in the eye, … in neutrophils, … and in semen…' (pg 98).

'Ascorbic acid protects against plasma and low-density lipoprotein oxidation by scavenging ROS … and possibly by sparing or regenerating vitamin E. Evidence

suggests that ascorbate also provides antioxidant protection indirectly by regenerating other biological antioxidants such as glutathione and α −tocopherol back to their active state.'(pg 98)

'The most convincing evidence that vitamin C functions as an antioxidant in vivo is the study by Reilly et al. (1996), showing that supplementation of smokers with 2g/day for 5 days was associated with a significant reduction in urinary isoprostanes, an indicator of oxidative stress… Vitamin C supplementation (2g/day for 4-12 months) in 41 patients with non-atrophic gastritis decreased gastric mucosal nitrotyrosine, a measure of RNS activity Mannick et al, 1996. Thus, from this study and the study by Reilly et al. (1996) it can be concluded that supplementation with vitamin C results in an antioxidant effect in vivo because it significantly reduces nitrotyrosine and urinary isoprostanes. ' (pg 102).

'The content of vitamin C in leukocytes is especially important because the ROS generated during phagocytosis and neutrophil activation are associated with infectious and inflammatory stresses… Along with pituitary and adrenal glands and eye lens, leucocytes contain the highest vitamin C concentrations of all body tissues…' (pg 103).

'The high intra-cellular concentration of ascorbate in leukocytes provides cellular protection against oxidant damage associated with the respiratory burst.' (pg 108)

'The ratio of oxidised to reduced ascorbate was found to be increased in the knee synovial fluid of active rheumatoid arthritis patients, which suggests that ascorbate is acting to scavenge phagocyte-derived oxidants in this locally inflamed area'. (pg 108)

'Increased ascorbate oxidation in the plasma of patients with adult respiratory distress syndrome and in smokers indicates protection against oxidant damage from activated neutrophils and other sources in the lung. ….These results imply that ascorbate protects against inflammatory oxidative stress induced by ozone.' (pg 108)

'Ascorbate scavenging of myeloperoxidase-derived oxidants from phagocytic white cells may also be protective against in vivo LDL oxidation because HOCl oxidised proteins have also been identified in human atherosclerotic lesions.' (pg 109)

Reference 2.0:
'Ascorbic acid functions as a relatively nonspecific, radical-trapping antioxidant …' (pg 144)

'Ascorbate reacts with nitrite and other nitrosating reagents *in vitro,* forming nitric oxide, nitrous oxide and nitrogen. This may be important in preventing the formation of carcinogenic nitrosamines by reaction between nitrites and amines present in foods in the acid conditions of the stomach. Again, this is an effect of ascorbate present in the stomach together with the dietary nitrites and amines, rather than an effect of vitamin C nutritional status. However, while ascorbate can deplete nitrosating compounds under anaerobic conditions, the situation may be reversed in the presence of oxygen. Nitric oxide reacts with oxygen to form N_2O_3 and N_2O_4, both of which are nitrosating reagents, and can also react with ascorbate to form NO and monodehydroascorbate. It is thus possible for ascorbate to be depleted, with no

significant effect on the total concentration of nitrosating species. It remains to be determined whether or not ascorbate has any significant effect in reducing the risk of nitrosamine formation and carcinogenesis.' (pg 147)

'Ascorbate also acts nonenzymically to reduce oxidized vitamin E…Vitamin C thus has a vitamin E-sparing antioxidant action, coupling lipophilic and hydrophilic antioxidant reactions. The antioxidant efficiency of ascorbate is variable…it is only at very low concentrations of ascorbate that it tends towards the theoretical 2 : 1 ratio. This is probably because, as well as its antioxidant role, ascorbate can be a source of hydroxyl and superoxide radicals.' (pg 147)

Reference 4.13:
'Vitamin C plays a major role as an antioxidant and free-radical scavenger…Vitamin C is a strong reducing agent and hence has a general importance as an antioxidant, affecting the body's 'redox potential'…In fact it forms part of the body's antioxidant defences against reactive oxygen species and free radicals, thereby preventing tissue damage.' (pg 6)

'Regeneration of ascorbic acid from its oxidation products, by reducing agents such as glutathione and nicotinamide-adenine dinucleotide (NAD) potentiate its antioxidant potential.' (pg 6)

11) Carnitine

Code   **Proposed statement**
VC11:   *Vitamin C is necessary for the normal structure of carnitine*

Reference 1.2:
'It is a co-factor for enzymes involved in the biosynthesis of,… carnitine,...' (pg 95)

'Vitamin C is known to be an electron donor for eight human enzymes... two [participate] in carnitine biosynthesis.' (pg 96)

'…ascorbate is required along with iron at two steps in the pathway of carnitine biosynthesis' (pg 99)

Reference 2.0:
'Trimethyllysine and γ-butyrobetaine hydroxylases are required for the synthesis of carnitine.' (pg 146)

Reference 4.13:
The functions of vitamin C include the synthesis of…and carnitine.' (pg 6)

12) Neurological system

Code   Proposed statement
VC12:   Vitamin C is necessary for the normal neurological function

Reference 1.2:
'It is a co-factor for enzymes involved in the biosynthesis of …neurotransmitters in vitro,...' (pg 95)

'Vitamin C is known to be an electron donor for eight human enzymes. The three enzymes that participate in hormone [and amino acid] biosynthesis are dopamine β–hydroxylase, necessary for the biosynthesis of the catecholamines norepinephrine and epinephrine and; peptidyl-glycine monooxygenase, necessary for amidation of peptide hormones; and ….' (pg 96)

'Ascorbic acid is involved in the synthesis and modulation of some hormonal components of the nervous system. The vitamin is …involved in the biosynthesis of neuropeptides. Other nervous system components modulated by ascorbate concentrations include neurotransmitter receptors, the function of glutamatergic and dopaminergic neurons, and synthesis of glial cells and myelin.' (pg 98)

'The vitamin is involved in the biosynthesis of corticosteroids and aldosterone and…' (pg 99)

'Although vitamin C's role as an antioxidant and cofactor for catecholamines biosynthesis might suggest that it protects cognitive function, there is little valid, consistent evidence that it does.' (pg 127)

Reference 2.0:
'It also has a specific metabolic function as the redox coenzyme for dopamine β-hydroxylase and petpidyl glycine hydroxylase …' (pg 144)

'Dopamine β-hydroxylase (EC 1.14.17.1) is a copper-containing enzyme involved in the synthesis of the catecholamines, noradrenaline and adrenaline, from tyrosine in the adrenal medulla and central nervous system. The active enzyme contains Cu^+, which is oxidized to Cu^{2+} during the hydroxylation of the substrate; reduction back to Cu^+ specifically requires ascorbate, which is oxidized to monodehydroascorbate.' (pg 146)

Reference 4.13:
'The functions of vitamin C include the synthesis of…, neurotransmitters, …(pg 6)

'Additional clinical manifestations observed in vitamin C deficiency include behavioural changes, often apathy, depression and emotional disturbances. Such observations are thought to be the consequence of a depression in catecholamine synthesis.' (pg 7)

13) Metabolism of foreign compounds

Code **Proposed statement**
VC13: *Vitamin C contributes to the breakdown of undesirable chemicals*

Reference 1.2:
'Ascorbic acid functions as a reducing agent for mixed-function oxidases in the microsomal drug-metabolising system that inactivates a wide variety of substrates, such as endogenous hormones or xenobiotics (i.e., other chemical compounds such as drugs, pesticides, or carcinogens that are foreign to humans). The activity of both microsomal drug-metabolising enzymes and cytochrome P-450 electron transport is lowered by ascorbate deficiency.'(pg 99)

Reference 4.13:
'Vitamin C is… involved in the detoxification of many foreign compounds.' (pg 6)

14) Muscle function

Code **Proposed statement**
VC14: *Vitamin C is necessary for the normal function of muscles*

Reference 1.2:
'Muscle carnitine is significantly depleted in scorbutic guinea pigs, suggesting that loss of energy derived from carnitine-related β-oxidation of fatty acids may explain the fatigue and muscle weakness observed in human scurvy. However, neither guinea pig nor human studies show a consistent relationship between vitamin C status and carnitine levels. Although vitamin C deficiency appears to alter carnitine metabolism, the specific interactions and their relevance to functional carnitine status in humans are unclear.' (pg 120)

Reference 3.23:
'Prescorbutic symptoms include weakness, lassitude, fatigue'. (pg 124)

Reference 4.13:
'Early signs of deficiency, that is scurvy, are relatively non-specific. They often include…, aching bones, joints and muscles…'(pg 7)