1 **DRAFT SCIENTIFIC OPINION**

2 Scientific Opinion on Dietary Reference Values for choline[1]

3 **EFSA Panel on Dietetic Products, Nutrition, and Allergies (NDA)**[2, 3]

4 European Food Safety Authority (EFSA), Parma, Italy

5 **ABSTRACT**

6 Following a request from the European Commission, the EFSA Panel on Dietetic Products, Nutrition and
7 Allergies (NDA) derives Dietary Reference Values (DRVs) for choline. In this Opinion, the Panel considers
8 dietary choline or choline compounds (e.g. glycerophosphocholine, phosphocholine, phosphatidylcholine,
9 sphingomyelin). The Panel considers that none of the biomarkers of choline intake or status is suitable to derive
10 DRVs for choline. The Panel considers that Average Requirements and Population Reference Intakes for choline
11 cannot be derived for adults, infants and children, and therefore defines Adequate Intakes (AIs). For all adults,
12 the Panel sets an AI at 400 mg/day based on the average observed choline intake in healthy populations in the
13 European Union and in consideration of the amounts of choline needed to replete about 70% of depleted subjects
14 who showed signs of organ dysfunction in a depletion/repletion study. For all infants aged 7–11 months, the
15 Panel proposes an AI of 160 mg/day, based on upwards extrapolation from the estimated choline intake of
16 exclusively breastfed infants from birth to six months. For all children aged 1–17 years, the Panel proposes AIs,
17 based on downward extrapolation from the adult AI, applying growth factors. These AIs range from 140 mg/day
18 (1–3 years) to 400 mg/day (15–17 years). For pregnant women, the Panel derives an AI of 480 mg/day,
19 calculated by extrapolation from the AI for non-pregnant women and the mean gestational increase in body
20 weight. For lactating women, the amount of choline secreted per day in human milk during the first six months
21 of exclusive breastfeeding (120 mg/day) is added to the AI for non-lactating women, and an AI of 520 mg/day is
22 set.

24 **KEY WORDS**

25 choline, phosphatidylcholine, observed intake, depletion/repletion study, Adequate Intake, Dietary Reference
26 Value

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SUMMARY

Following a request from the European Commission, the EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) was asked to deliver a Scientific Opinion on Dietary Reference Values for the European population, including choline.

Choline is a quaternary amine (2-hydroxyethyl-N,N,N-trimethylammonium) present in food in free and esterified forms. The main forms present in foods are phosphatidylcholine (PC, lecithin), which is also the main form present in animal tissues, free choline, phosphocholine (PChol), glycerophosphocholine (GPC) and sphingomyelin (SPM), and minor amounts of cytidine-5-diphosphate-choline (CDP-choline) and acetylcholine. Choline, PChol and GPC are water-soluble choline compounds, whereas PC and SPM are lipid-soluble.

Although choline can be synthesised *de novo* by the human body, this synthesis may become insufficient, making choline an essential component of the diet. Choline is predominantly provided via the diet. The human body can form choline either *de novo* by methylation of phosphatidylethanolamine (PE) via the hepatic phosphatidylethanolamine N-methyltransferase (PEMT) pathway, or by hydrolysis of PC formed in the CDP-choline pathway in all cells of the body. The PC formed in the PEMT pathway contains substantial amounts of long-chain polyunsaturated fatty acids, like docosahexaenoic acid and arachidonic acid. Both pathways can be stimulated by dietary choline and the PEMT pathway is sensitive to the presence of oestrogens.

Choline is an integral part of some phospholipids, which play an important role in the structure and function of membranes. Choline (as PC) plays an important role in the metabolism and transport of lipids and cholesterol by lipoproteins and is needed for the assembly and secretion of very low density lipoproteins by the liver. Choline is a precursor of the neurotransmitter acetylcholine, and of betaine, an osmoregulator to which choline is irreversibly oxidised in the liver and kidney. Via betaine, choline is involved in the folate-dependent one-carbon metabolism. Dietary deficiency of choline can cause fatty liver or hepatic steatosis that can result in non-alcoholic fatty liver disease (NAFLD), and can cause liver and muscle damage. This indicates that *de novo* production can be insufficient.

Dietary free choline is quickly taken up by a carrier-mediated saturable transport system. PC and GPC from the diet or secreted in the bile, and dietary SPM are hydrolysed by phospholipases (PLs) to liberate choline. Choline and water-soluble choline compounds (PChol and GPC) are rapidly absorbed and appear in plasma predominantly as free choline. Phospholipids (PC and SPM) that have escaped PLs enter the lymph incorporated into chylomicrons. The available data do not allow defining the percentage of intestinal absorption of choline in humans, and the total amount of choline in the human body. Non-absorbed choline is a precursor of trimethylamine (TMA) produced in the gut by anaerobic symbiotic microbes. TMA is efficiently absorbed from the gastrointestinal tract and then converted in the liver to trimethylamine-oxide (TMAO), and both TMA and TMAO (i.e. total trimethylamine (TTMA)) are eliminated in the urine. Choline urinary excretion is low in relation to usual dietary intakes, while no human data are available on faecal excretion of choline or choline compounds in relation to dietary intake. Breast milk mainly contains PChol and GPC, besides free choline, PC and SPM, in concentrations depending on the progress of lactation, maternal diet and genotype.

The Panel reviewed possible biomarkers of choline intake and/or status. The Panel considers that the available data do not allow conclusions to be drawn on a dose-response relationship between choline intake or status and plasma choline concentration, and that plasma choline concentrations cannot be used to set DRVs for dietary choline. Plasma concentrations of choline, PC, betaine, dimethylglycine, total homocysteine or TMAO, erythrocyte PC concentration, or urinary betaine and TTMA urinary excretion also cannot used to set DRVs for dietary choline. The Panel also notes that single-nucleotide polymorphisms (SNPs) in genes coding for enzymes involved in choline metabolism, some of them present with high frequency in the population, can influence the dietary requirement for choline and determine the susceptibility to dietary choline deficiency, but data are insufficient to predict variations in individual choline requirements based on genetic polymorphisms. The Panel concludes that the

available data on choline intake and health consequences (NAFLD, cardiovascular disease, cancer, birth defects, cognition) are not suitable for the setting of DRVs for dietary choline.

The Panel considers that Average Requirements and Population Reference Intakes for choline cannot be derived for adults, infants and children, and therefore defines Adequate Intakes (AIs).

Dietary total choline intake was calculated based on individual food consumption data that were available to EFSA and classified according to EFSA's food classification system, from healthy populations investigated in 12 national surveys undertaken in nine countries of the European Union (EU), between 2000 and 2011. In the absence of food composition data with respect to choline in Europe, composition data on free choline and choline compounds from the US Department of Agriculture were used. The total choline intake mean estimates ranged from 75 to 127 mg/day in infants, from 151 to 210 mg/day in children aged 1–3 years, from 177 to 304 mg/day in children aged 3–< 10 years, and from 244 to 373 mg/day among children aged 10–< 18 years. The total choline intake mean estimate was 336 mg/day in pregnant adolescents, and 356 mg/day in pregnant women. The total choline intake mean estimates ranged from 269 to 444 mg/day and from 332 to 468 mg/day in women and men, respectively, i.e. for all adults: 269–468 mg/day.

The Panel reviewed 11 choline depletion/repletion studies with similar design. Only one reported the amounts of choline needed to replete depleted subjects who showed signs of organ dysfunction. The Panel concludes that choline depletion/repletion studies do not provide sufficient data to calculate average requirements for choline, but may be used to inform data on observed choline intakes to set AIs for choline.

For all adults, the Panel set an AI of 400 mg/day. This is based on the mid-point of the range of observed mean intakes in healthy populations in the EU (about 370 mg/day), and in consideration of the results of a depletion-repletion study in which about 70% of the depleted subjects who had developed signs of organ dysfunction were repleted with an intake of about 400 mg/70 kg body weight per day. Although premenopausal women may have a lower requirement for dietary choline in connection with a potential stimulation of the PEMT pathway by oestrogens, and ranges of estimated mean total choline intake in Europe are slightly lower in women than men, the Panel considered unnecessary to give sex-specific AIs for adults.

For all infants aged 7–11 months, the Panel set an AI of 160 mg/day, based on the estimated intake of choline of exclusively breastfed infants from birth to six months, and upwards extrapolation by allometric scaling (taking into account the difference in reference body weight).

For all children aged 1–7 years, the Panel set AIs ranging from 140 mg/day (1–3 years) to 400 mg/day (15–17 years). These were set by downward extrapolation from the adult AI, by allometric scaling (taking into account the difference in reference body weight), and applying growth factors. No data are available that would justify different AIs for boys and girls. These AIs are supported by total choline intake mean estimates in the EU.

For pregnant and lactating women, the Panel considered that, although the available intervention studies on choline supplementation in the second half of pregnancy or in lactating women indicate that pregnant or lactating women may need more choline than non-pregnant non-lactating women, the data are not sufficient to allow an estimate of the additional requirement for dietary choline in pregnant or lactating women (above that of non-pregnant non-lactating women).

For pregnant women, the Panel set an AI of 480 mg/day, calculated by isometric scaling from the AI for non-pregnant women, using the mean gestational increase in body weight. For lactating women, the AI for non-lactating women is increased to account for the secretion through breast milk. The Panel set an AI of 520 mg/day, considering an average concentration of choline in mature breast milk of 145 mg/L, and a mean milk transfer during the first six months of lactation in exclusively breastfeeding women (0.8 L/day).

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

The scientific advice on nutrient intakes is important as the basis of Community action in the field of nutrition, for example such advice has in the past been used as the basis of nutrition labelling. The Scientific Committee for Food SCF (1993) report on nutrient and energy intakes for the European Community dates from 1993. There is a need to review and if necessary to update these earlier recommendations to ensure that the Community action in the area of nutrition is underpinned by the latest scientific advice.

In 1993, the SCF adopted an opinion on the nutrient and energy intakes for the European Community[4]. The report provided Reference Intakes for energy, certain macronutrients and micronutrients, but it did not include certain substances of physiological importance, for example dietary fibre.

Since then new scientific data have become available for some of the nutrients, and scientific advisory bodies in many European Union Member States and in the United States have reported on recommended dietary intakes. For a number of nutrients these newly established (national) recommendations differ from the reference intakes in the SCF (1993) report. Although there is considerable consensus between these newly derived (national) recommendations, differing opinions remain on some of the recommendations. Therefore, there is a need to review the existing EU Reference Intakes in the light of new scientific evidence, and taking into account the more recently reported national recommendations. There is also a need to include dietary components that were not covered in the SCF opinion of 1993, such as dietary fibre, and to consider whether it might be appropriate to establish reference intakes for other (essential) substances with a physiological effect.

In this context the EFSA is requested to consider the existing Population Reference Intakes for energy, micro- and macronutrients and certain other dietary components, to review and complete the SCF recommendations, in the light of new evidence, and in addition advise on a Population Reference Intake for dietary fibre.

For communication of nutrition and healthy eating messages to the public it is generally more appropriate to express recommendations for the intake of individual nutrients or substances in food-based terms. In this context the EFSA is asked to provide assistance on the translation of nutrient based recommendations for a healthy diet into food based recommendations intended for the population as a whole.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

In accordance with Article 29 (1)(a) and Article 31 of Regulation (EC) No. 178/2002,[5] the Commission requests EFSA to review the existing advice of the Scientific Committee for Food on population reference intakes for energy, nutrients and other substances with a nutritional or physiological effect in the context of a balanced diet which, when part of an overall healthy lifestyle, contribute to good health through optimal nutrition.

In the first instance the EFSA is asked to provide advice on energy, macronutrients and dietary fibre. Specifically advice is requested on the following dietary components:

- Carbohydrates, including sugars;
- Fats, including saturated fatty acids, polyunsaturated fatty acids and monounsaturated fatty acids, *trans* fatty acids;

[4] Scientific Committee for Food, Nutrient and energy intakes for the European Community, Reports of the Scientific Committee for Food 31st series, Office for Official Publication of the European Communities, Luxembourg, 1993.

[5] Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. OJ L 31, 1.2.2002, p. 1–24.

- Protein;
- Dietary fibre.

Following on from the first part of the task, the EFSA is asked to advise on population reference intakes of micronutrients in the diet and, if considered appropriate, other essential substances with a nutritional or physiological effect in the context of a balanced diet which, when part of an overall healthy lifestyle, contribute to good health through optimal nutrition.

Finally, the EFSA is asked to provide guidance on the translation of nutrient based dietary advice into guidance, intended for the European population as a whole, on the contribution of different foods or categories of foods to an overall diet that would help to maintain good health through optimal nutrition (food-based dietary guidelines).

ASSESSMENT

1. Introduction

Choline is a water-soluble organic compound needed for normal functioning of the body. Although choline can be synthesised *de novo* by the human body, this synthesis may become insufficient, making choline an essential component of the diet (Ueland, 2011).

In 1993, the Scientific Committee for Food (SCF) adopted an opinion on nutrient and energy intakes for the European Community and considered that there was no evidence for the necessity of an intake of choline via the diet for persons older than six months (SCF, 1993). Since it was unclear if young infants depend on exogenous sources of choline and because choline is an integral component of human milk, the addition of choline to infant formula with a minimum level of 7 mg of choline/100 kcal was made mandatory.[6]

The purpose of this Opinion is to review the available evidence to assess whether it might inform the setting of Dietary Reference Values (DRVs) for choline. The Panel focuses in this Scientific Opinion on dietary choline including choline containing compounds.

2. Definition/category

2.1. Chemistry

Choline, 2-hydroxyethyl-N,N,N-trimethylammonium (2-Hydroxy-*N*,*N*,*N*-trimethylethanamonium, IUPAC, molar mass 104.17 g/mol) is a quaternary amine. In foods, it is present in free and esterified forms, mainly as phosphatidylcholine (PC, lecithin), free choline, phosphocholine (PChol), glycerophosphocholine (GPC) and sphingomyelin (SPM) (Figure 1), and minor amounts of cytidine-5-diphosphate-choline (CDP-choline) and acetylcholine (Ueland, 2011). PC accounts for approximately 95% of total choline found in animal tissues. Choline, PChol and GPC are water-soluble choline compounds, whereas PC and SPM are lipid-soluble.

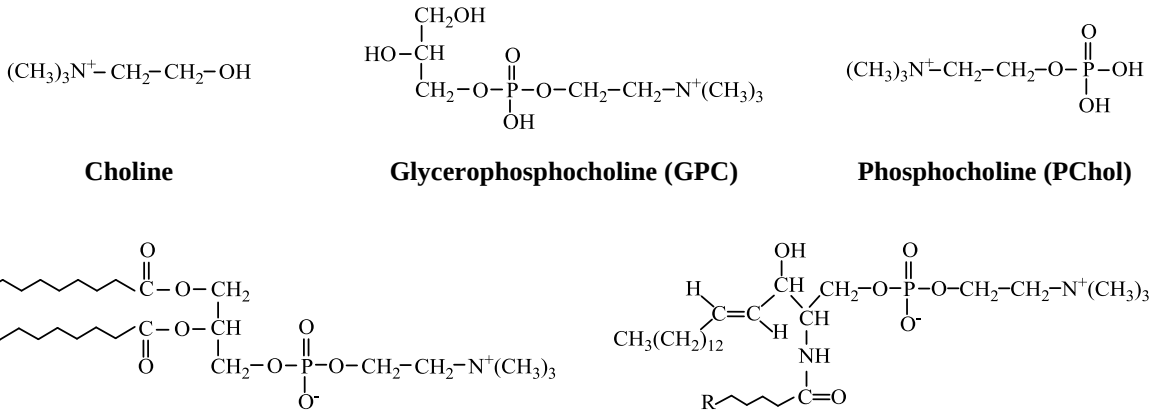

Figure 1: Chemical formulas of choline, glycerophosphocholine, phosphocholine, phosphatidylcholine and sphingomyelin

Choline is a component of some phospholipids. Phospholipids are derived from either glycerol or sphingosine, an amino alcohol with a long unsaturated hydrocarbon chain (C 18). Phosphoglycerides consist of a glycerol of which the hydroxyl groups at C1 and C2 are esterified to the carboxyl groups of two fatty acids, whilst the hydroxyl group at C3 is esterified to PChol (or other phosphorylated

[6] Commission Directive 2006/141/EC of 22 December 2006 on infant formulae and follow-on formulae and amending Directive 1999/21/EC, OJ L 401, 30.12.2006, p.1.

alcohols derived from ethanolamine, serine or inositol). SPM consists of sphingosine, which amino group is linked to a fatty acid by an amide bond and which primary alcohol group is esterified to PChol.

2.2. Function of choline

2.2.1. Biochemical functions

Choline has a number of important functions: it is a precursor for the phospholipid PC (Section 2.1.), it is involved in the metabolism and transport of lipids and in the folate-dependent one-carbon metabolism, and it is a precursor of acetylcholine and of betaine.

Choline is an integral part of some phospholipids (Section 2.1). Phospholipids are abundant in all biological membranes (40–50% of phospholipids of cellular membranes consist of PC (Zeisel, 2006)), where they play an important role in the structure and function of membranes, including signalling and transport, and they are also a constituent of the surfactant complex in the lung (Dushianthan et al., 2014).

Choline plays an important role in the metabolism and transport of lipids and cholesterol. PC makes up 70–95% of phospholipids in lipoproteins (Zeisel, 2006) and is needed for normal assembly and secretion of very low density lipoproteins (VLDL) in the liver (Vance et al., 2007).

Choline is acetylated in cholinergic neurons to form acetylcholine, a key neurotransmitter involved in functions like memory storage and muscle control (IOM, 1998; Ueland, 2011). Pre- and post-natal choline availability has been shown to be important for neurodevelopment in animals (Meck and Williams, 2003).

In the liver and kidney, choline is irreversibly oxidised, by a mitochondrial choline oxidase (also called choline dehydrogenase CHDH) and betaine aldehyde dehydrogenase, to betaine (Lin and Wu, 1986) (Sections 2.3.5.2.1. and 2.3.6.1.2.). Betaine serves as an osmoregulator and is a substrate in the betaine-homocysteine methyltransferase (BHMT) reaction. This reaction links choline and betaine to the folate-dependent one-carbon metabolism (Figure 2, Sections 2.3.5. and 2.3.7.). Choline and betaine are important sources of one-carbon units, in particular during folate deficiency (Ueland, 2011). In remethylating homocysteine (Hcy) to methionine, choline contributes, via betaine, to the availability of S-adenosyl-methionine (SAM) as the universal methyl-group donor (Figure 2, Section 2.3.5.). For example, the methyl-group of SAM can be transferred to cytosine residues adjacent to guanine (CpG) of DNA or to histones at specific lysine sites, thereby contributing to epigenetic modification and potentially exert effects on gene expression (Mehedint and Zeisel, 2013).

2.2.2. Health consequences of deficiency and excess

2.2.2.1. Deficiency

Dietary deficiency of choline can cause fatty liver (hepatic steatosis, which can result in non-alcoholic fatty liver disease (NAFLD)) (Buchman et al., 1995), and liver damage (Zeisel et al., 1991) and muscle damage as indicated by an increase of creatine phosphokinase (CK) concentration in serum (Fischer et al., 2007). Hepatic steatosis may be due to impaired triacylglycerol (TAG) transport out of the liver. Since PC is an essential component of VLDL, the lipoprotein responsible for transporting TAG out of the liver (Section 2.2.1.), TAG cannot be exported in case of choline deficiency and accumulates in the hepatocytes (Cole et al., 2012). Hepatic steatosis can progress to liver damage with release of liver enzymes into the blood. This release of enzymes from the liver into the blood may follow induction of apoptosis and cell membrane fragility (da Costa et al., 2006b; Fischer et al., 2007). In serum of 41 long-term parenterally fed subjects, both alanine amino transferase (ALT) and aspartate amino transferase (AST) concentrations were significantly and negatively associated with the concentration of free choline ($r = -0.34$, $p = 0.03$, $r = -0.37$, $p = 0.02$ respectively), but not with that of phospholipid-bound choline (Buchman et al., 1993). In this study, the concentration of free choline

in serum was low, i.e. one third of the reference values used by the authors, whilst that of PC was normal.

The susceptibility to develop NAFLD was found to be related to polymorphisms of the gene for phosphatidylethanolamine N-methyltransferase (PEMT) (Song et al., 2005) with loss of oestrogen receptor binding (Resseguie et al., 2007; Resseguie et al., 2011), as well as to polymorphisms of other enzymes involved in choline metabolism (CHDH and 5,10-methylenetetrahydrofolate dehydrogenase 1 (MTHFD1)) (Section 2.5 and Appendix C). Premenopausal women developed signs of choline deficiency less commonly than postmenopausal women or men, possibly as a consequence of up-regulation of hepatic PEMT by oestrogen, leading to an increase in the endogenous synthesis of PC (Fischer et al., 2007; Zeisel, 2007). PEMT is important for this endogenous synthesis of PC in case of insufficient dietary choline intake (Figure 2, Section 2.3.5.). The amount of dietary choline to prevent organ damage or to maintain normal organ function varies between people (Section 5.1.2). In addition, there is some evidence that the susceptibility to develop fatty liver with choline deficiency is influenced by the gastrointestinal microbiome (Spencer et al., 2011).

Zeisel (2012) reviewed the potential effects of choline deficiency on gene expression via epigenetic marks and DNA integrity that could result in increased mutation rates and thereby increased risks of certain cancers. An influence on the risk of breast cancer of single nucleotide polymorphisms (SNPs) of several genes involved in choline metabolism and enhancing the requirement for dietary choline has been observed in large epidemiological studies (Xu et al., 2008; Xu et al., 2009) (Appendix C and Section 2.5.).

In subjects that received a choline diet providing < 50 mg choline/70 kg body weight per day, fasting plasma concentration of total homocysteine (tHcy) significantly increased among those with clinical expression of choline deficiency, compared to baseline (da Costa et al., 2005; Fischer et al., 2007) (Section 5.1.1. and Appendix D). However, many factors besides dietary or endogeneous choline determine tHcy concentration in plasma (Section 2.4.3.) (EFSA NDA Panel, 2014a, 2015).

2.2.2.2. Excess

The SCF did not consider choline when setting Tolerable Upper Intake Levels (ULs) for vitamins and minerals. The US Institute of Medicine (IOM, 1998) defined a UL for adults based on a study in seven patients with Alzheimer dementia, where the oral administration of 7.5 g/day of choline (as chloride) had a hypotensive effect accompanied by nausea and diarrhoea (Boyd et al., 1977). Similar gastrointestinal effects and a fishy body odour were observed in therapeutic studies with choline (8–20 g/day) on individuals with tardive dyskinesia and Huntington's disease (Growdon et al., 1977; Gelenberg et al., 1979; Lawrence et al., 1980). IOM considered 7.5 g/day of choline as the Lowest Observed Adverse Effect Level (LOAEL), and after the application of an uncertainty factor of 2 and rounding, set a UL of 3.5 g choline/day for adults. No UL was established for infants and ULs for children were derived from the adult value by allometric scaling (exponent 0.75) according to reference body weights.

An association between an increased risk of cardiovascular diseases (CVD) and 'higher intake' of choline, which possibly exceeds the intestinal absorption capacity for dietary free choline, has been suggested by a metabolomic study (Wang et al., 2011), which investigated the relationship between plasma choline and TMAO concentrations and risk of CVD. Non-absorbed choline will become available to microbial degradation, predominantly to trimethylamine (TMA) (Sections 2.3.1. and 2.3.5.2.2.), which is metabolised in the liver to trimethylamine-N-oxide (TMAO). TMA has been found to promote atherosclerosis in animals (Wang et al., 2011; Bennett et al., 2013; Tang et al., 2013; Wang et al., 2014). TMA has also been suggested to be involved in depression, neurological symptoms, teratogenic effects in humans as well as in the potential formation of the carcinogen N-nitrosodimethylamine (for a review, see Bain et al. (2005)). These are indirect adverse effects of choline, depending both on a 'high' dietary amount and a specific gut microbiome (Wang et al., 2011). However, the dietary intake of choline was not reported in these studies.

2.3. Physiology and metabolism

2.3.1. Intestinal absorption

Dietary free choline is quickly taken up by the enterocytes, mediated by the saturable organic cation transporters (OCTs) (choline transporter-like protein 1 (CTL1) or solute carrier 44A1 (SLC44A1)) (Section 2.3.3.), which rely on facilitated diffusion governed by the choline concentration gradient and the electrical potential across the membrane, then free choline is cleared from the plasma within about three hours (Zeisel et al., 1980; Jope et al., 1982). Dietary PC increases plasma choline concentration for 8–12 hours, without a significant rise in PC concentration in plasma (Zeisel et al., 1980; Jope et al., 1982). PChol and GPC are rapidly absorbed and appear in plasma predominantly as free choline.

PC and GPC from the diet or secreted in the bile are hydrolysed by phospholipases (PLs) to liberate choline (Zeisel and Blusztajn, 1994). Water-soluble choline compounds (PChol and GPC) can also enter the portal circulation of the liver intact. Lipid-soluble compounds (PC and SPM) are either hydrolysed by PLs or enter the lymph incorporated into chylomicrons.

Unabsorbed choline is catabolised by the intestinal microbiota to TMA (Sections 2.2.2.2. and 2.3.5.2.2.). TMA is absorbed from the gastrointestinal tract and converted to TMAO in the liver.

The Panel notes that the amount of choline absorbed is restricted by the capacity of the transport system via the saturable CTL1 or SLC44A1. The Panel notes that the available data do not allow defining the percentage of intestinal absorption of choline in humans.

2.3.2. Transport in blood

Free choline is transported in the aqueous phase of plasma, whereas phosphorylated choline compounds (i.e. PC, PChol, GPC, SPM) are associated with or are part of lipoproteins.

2.3.3. Distribution to tissues

Since choline is a charged hydrophilic cation, it needs transport mechanisms to cross biological membranes. Three transport mechanisms are known (Fagone and Jackowski, 2013).

The first is a sodium- and chloride-dependent high-affinity (K_m < 10 µM) (Okuda and Haga, 2000) carrier-mediated saturable uptake system in presynaptic cholinergic nerve terminals, that is linked to acetylcholine synthesis (Section 2.2.1.). The transporter is the high-affinity choline transporter (CHT; (solute carrier family 5 member 7 encoded by *SLC5A7*) that needs adenosine triphosphate (ATP) hydrolysis. Disturbing the integrity of the cell membrane can reduce choline availability for acetylcholine synthesis and diminish cholinergic transmission (Cuddy et al., 2014).

The second transport mechanism is a sodium-independent low-affinity carrier-mediated saturable mechanism (CTL1 or SLC44A1) in all tissues. This mechanism is energised by ATP hydrolysis, with an average affinity (K_m) for choline of > 20–200 µM. It is present in enterocytes, hepatocytes, kidneys, placental tissue, mitochondria, and synaptosomes, and supplies choline for the synthesis of PC and SPM as well as of betaine (Sections 2.2.1. and 2.3.5.). This uptake is stereospecific and can be inhibited by similar nitrogen-methyl compounds and by high concentrations of choline (Michel and Bakovic, 2012).

The third transport mechanism is a sodium–independent saturable uptake mechanism (a member of the solute carrier 22 family), for choline to cross the blood-brain barrier and erythrocyte membranes by facilitated diffusion. Its affinity to choline is similar to the high-affinity mechanism, but it is not linked to acetylcholine synthesis (Cornford et al., 1980; Lockman and Allen, 2002).

Choline uptake by the mammary epithelium occurs by an energy-dependent saturable transport system, but with higher maternal choline supply non-saturable transport can also occur. Choline is metabolised within the mammary epithelium to PChol and other choline compounds, to a lesser extent

via degradative pathways (Fischer et al., 2010b; Davenport et al., 2015) (Sections 2.3.6., 2.4.1.2. and 5.1.3.4.). The size of the efflux of choline compounds from the mammary epithelium occurs via exocytosis or as a component of the milk fat globule (Davenport and Caudill, 2013).

Choline crosses the placenta via a specific transport system on both the maternal and fetal side of the syncytiotrophoblast, with an apparent small excess (about 4%) preferential towards the fetal circulation, as demonstrated in perfusion studies with [^{3}H]-choline (Sweiry et al., 1986). Umbilical cord blood free choline concentration is about three times that of maternal blood (Visentin et al., 2015) (Section 2.4.1.2.).

2.3.4. Storage

Choline is stored in tissues either as membrane-bound phospholipids or as intracellular PC or GPC (Zeisel and Blusztajn, 1994). Choline is stored in the brain as membrane-bound phospholipids, which are hydrolysed by choline acetyltransferase to provide choline for acetylcholine synthesis (Section 2.2.1.). In most animal tissues, PC accounts for 95% of the total choline content, the remaining 5% are choline, PChol, GPC, CDP-choline and acetylcholine (Li and Vance, 2008).

The content of choline and its metabolites in the body is balanced by two pathways of acquisition, either diet and the CDP pathway, or the PEMT pathway (Sections 2.2.2.1. and 2.3.5.), and two pathways of depletion, either choline oxidation or the secretion of PC in the bile, and to a lesser extent, by the intestinal mucosa (Li and Vance, 2008; Ehehalt et al., 2010) (Sections 2.3.5.2.1. and 2.3.6.2.). Choline imbalances can be compensated by adaptive increases in PEMT activity, by recycling of choline, decreased oxidation of choline, reabsorption of biliary PC (95% of bile phospholipids is PC, of which about 40% return to the liver), and by redistribution of tissue choline to maintain homeostasis particularly in the brain and liver (Li et al., 2007; Li and Vance, 2008).

Regarding the choline content of adult tissues, the choline content of human liver has been measured *in vivo* to be on average 8.6 mmol/kg or 894 mg/kg wet weight (range 3.8–17.6 mmol/kg) (n = 44 including 24 women, mean age 46 ± 17 years), using proton (hydrogen 1 [^{1}H]) magnetic resonance spectroscopy (MRS) (Ouwerkerk et al., 2012). The choline content of quadriceps muscle was in the range 6.7-13 mmol/kg or 697–1 352 mg/kg (n = 7 including 4 women, mean age 37.7 years, range 28-50 years) (Fayad et al., 2010). The choline content in parietal white matter of the brain was (mean ± SD) 1.73 ± 0.24 mmol/L or 180 ± 25 mg/L (n = 20 including 11 women, mean age 29.4 ± 7.4 years) (Mazzetti et al., 2013). All these data were done with proton MRS. This method measures, besides choline as such, primarily GPC and PChol, but also includes phosphatidylethanolamine (PE), glyceroPE, betaine, myo-inositol and taurine; however, it does not include all choline lipids in membranes.

Regarding the fetus, infant and young child, phospholipids in the brain increase two-fold in the cortex (and three-fold in the white matter) from the 10th week of gestation to the age of two years (Svennerholm and Vanier, 1972). This study shows a relative continuous decrease of choline phosphoglycerides, from 50% of total phospholipids in the cerebral cortex of the fetus to 45% in infants at term and 38% in children at two years of age. In this study, SPM shows a continuous increase, from 3% of total phospholipids in the cerebral cortex of the fetus to 5% in infants at term and 10% in children at two years of age.

Regarding the placenta, placental total lipid content is 14 ± 1.0 mg/g dry tissue at term, and is rich in phospholipids (about 80% of total lipids), of which 42.1 ± 7.3% were choline glycerophospholipids. The long-chain polyunsaturated fatty acids (LC-PUFAs) arachidonic acid (ARA) and docosahexaenoic acid (DHA) are found in high proportion (about 40% of the phospholipid fatty acids) in all phospholipid classes (Bayon et al., 1993; Bitsanis et al., 2005). The placenta is one of the human organs most rich in free choline (14.6 mg/100 g wet weight) and this concentration decreases by 50% in (pre)eclampsia (Mischel, 1956).

The Panel notes that no data are available on the total amount of choline in the human body. The Panel also notes that there is a lack of data on the choline accretion in the fetus and placenta during the duration of pregnancy.

2.3.5. Metabolism

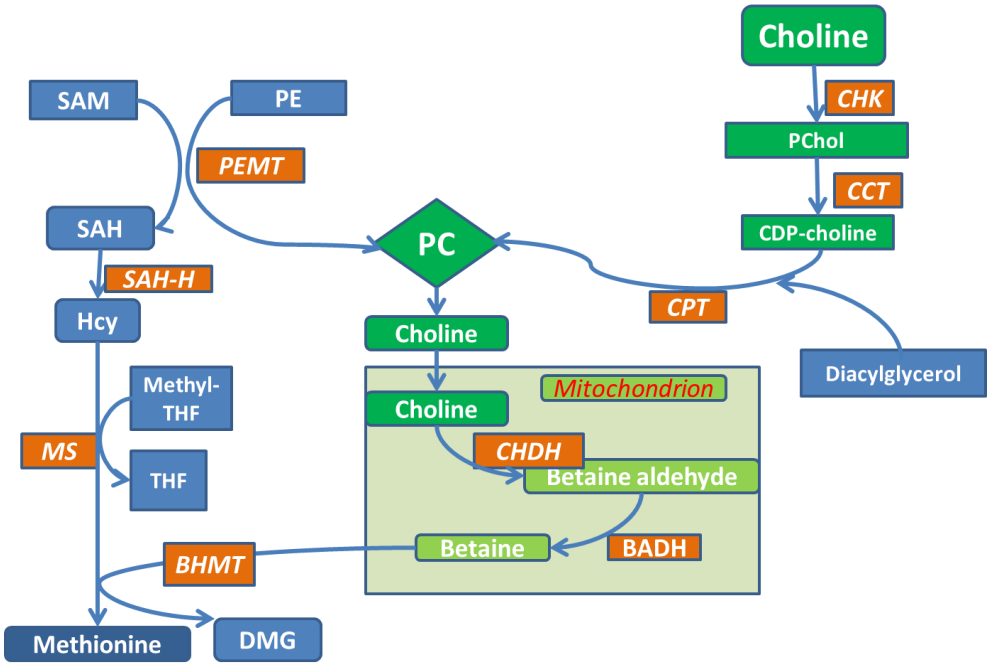

Figure 2: PC synthesis and choline metabolism and its involvement in folate-dependent one-carbon metabolism.

Left shows the endogeneous synthesis of PC; right the synthesis of PC from (dietary) choline.
Abbreviations: BADH, betaine aldehyde dehydrogenase; BHMT, betaine homocysteine methyltransferase; CCT, phosphocholine cytidyltransferase; CDP-choline, cytidine diphosphocholine; CHK, choline kinase; CHDH, choline oxidase (or dehydrogenase); CPT, CDP-choline diacylglycerol cholinephosphotransferase; DMG, dimethylglycine; Hcy, homocysteine; methyl-THF, methyltetrahydrofolate; MS, methionine synthase; PChol, phosphocholine; PE, phosphatidylethanolamine; PEMT, phosphatidylethanolamine N-methyltransferase; PC, phosphatidylcholine; SAH, S-adenosylhomocysteine; SAH-H, S-adenosylhomocysteine hydrolase; SAM, S-adenosylmethionine; THF, tetrahydrofolate.

2.3.5.1. Metabolism of choline and synthesis of phosphatidylcholine (PC)

Besides dietary intake, choline in the body can be generated *de novo* via the hepatic PEMT pathway. Both dietary and endogenous choline sources are incorporated into PC. PC is synthesised in all cells from choline (Li and Vance, 2008).

The predominant pathway of PC synthesis in all cells is via the CDP-choline pathway. Choline, taken up into cells or generated by hydrolysis of choline compounds (Figure 2, right side), is phosphorylated by choline kinase (CHK) to PChol or oxidised to betaine in some cell types like liver and kidney. PChol reacts with cytidine triphosphate (CTP) to form cytidine 5-diphosphate choline (CDP-choline) (by phosphocholine cytidyltransferase CCT). CDP-choline is esterified with diacylglycerol by choline phosphotransferase (CPT) or the choline/ethanolaminephosphotransferase (CEPT) to form PC (Li and Vance, 2008).

The other pathway of PC synthesis in the human body *de novo* starts from 3-phosphoglycerate, which receives two acyl groups from acyl-coenzyme A and is converted to a phosphatidate (not shown in Figure 2). Phosphatidate can react with CTP to form cytidine diphosphate-diacylglycerol, whose hydroxyl group can react with serine to form phosphatidylserine that is decarboxylated to PE. PE can then be methylated in the liver to synthesise PC (Figure 2, left side). This reaction is catalysed by

PEMT, which is dependent on SAM, and consumes three molecules of SAM while releasing three molecules of S-adenosylhomocysteine (SAH) per molecule of formed PC. Quantitatively, this appears to be the most important SAM-dependent transmethylation reaction and source of Hcy in mammals (Stead et al., 2006). The PEMT pathway is mostly active in the liver, but some low activity has been described in other tissues, e.g. in adrenal medulla, mammary gland and adipose tissue at about 0.1% of the hepatic activity (Vance, 2014).

The PEMT pathway accounts for 30% of hepatic PC synthesis in rodents, whilst 70% are produced from choline via the CDP-choline pathway (Reo et al., 2002; Li and Vance, 2008). The gene for PEMT has multiple oestrogen-responsive elements and its transcription is enhanced by oestradiol *in vitro*. Oestrogen enhanced activity of PEMT can provide for the increased demand for choline during pregnancy when oestrogen concentrations are high (Resseguie et al., 2007) (Sections 2.2.2.1. and 5.1.3.).

PC derived via the two different pathways apparently enters separate pools. PC formed in the hepatic PEMT pathway differs from that generated via the CDP-choline pathway, in that it contains primarily LC-PUFAs like DHA and ARA instead of medium-chain, mono- and bi-unsaturated and saturated fatty acids. This has been demonstrated in studies with deuterated choline and ethanolamine in rat and mouse liver and in mice and humans after parenteral administration of methyl-D_9-choline[7] (DeLong et al., 1999; Pynn et al., 2011) and using multiple isotopomer distribution analysis (MIDA) (Pynn et al., 2011). In addition, in 21 healthy non-pregnant women randomised to consume for 12 weeks either 480 or 930 mg choline/day (about 20% of which was provided as methyl-D_9-choline for the last six weeks), Yan et al. (2013) demonstrated that the higher choline intake (930 mg/day) favours the use of the PEMT pathway (relative to CDP-choline pathway), and yielded a significantly higher isotope enrichment in plasma PC-DHA (West et al., 2013) (Section 5.1.3.).

The ratio of PC-DHA to total PC in plasma is considered a surrogate measure for hepatic PEMT activity (3% of total plasma PC is PC-DHA). It is significantly greater ($p < 0.01$) in premenopausal women than in men or in postmenopausal women. It is significantly lower ($p < 0.05$) in premenopausal women homozygous for the loss-of-function rs12325817 SNP of the *PEMT* gene than in women with the wildtype of PEMT. This has been confirmed by measuring PEMT activity in liver biopsies together with the PC-DHA concentration (da Costa et al., 2011) (Appendix D).

The Panel notes that the PC required by the body can be derived from dietary choline and from endogenous synthesis, but is distributed into different pools and carries different fatty acids. The PC formed in the PEMT pathway contains substantial amounts of LC-PUFAs, like DHA and ARA, whilst the PC formed in the CDP-choline pathway does not. The PEMT pathway is mostly active in the liver, but some low activity has been described in e.g. in adrenal medulla, mammary gland and adipose tissue. The CDP-choline pathway is present in all cells of the body. Both can be stimulated by dietary choline. Moreover, the PEMT pathway is sensitive to the presence of oestrogens.

2.3.5.2. Degradation

Catabolism of phospholipids is initiated by PLs hydrolysing their respective bonds: i.e., PLA1 and PLA2 hydrolyse fatty-acyl bonds (e.g. PC to lysophosphatidylcholine (lyso-PC)), PLC glycerophosphate bond, and PLD choline phosphate ester bonds. Further, lysoPL degrades lysophosphatidylcholine, which is subsequently converted to GPC and further hydrolysed to choline by a phosphodiesterase (Lockman and Allen, 2002).

[7] Methyl-D_9-choline, with fully deuterated methylgroups, can either be converted via the CDP-choline pathway to D_9-PC or by oxidation to D_9-betaine that will transfer D_3-methyl groups to homocysteine via BHMT, forming D_3-methionine and D_6-DMG. D_3-methionine can transfer deuterated methyl groups to PE via PEMT, forming predominantly D_3-PC and D_6-PC. By estimating the enrichment of the different metabolites and the ratios of deuterated isotopomers, an assessment of the metabolic fluxes is possible (Pynn et al., 2011).

568 *2.3.5.2.1. Choline oxidation to betaine*

569 Oxidation of choline in the liver and kidney produces, in a two-step enzymatic reaction, first betaine
570 aldehyde by mitochondrial CHDH, and then betaine by mitochondrial or cytoplasmic betaine aldehyde
571 dehydrogenase (BADH) (Lin and Wu, 1986) (Figure 2). Mitochondrial betaine synthesis from choline
572 is controlled by choline transport across the mitochondrial membrane (O'Donoghue et al., 2009). The
573 formation of betaine links choline to the folate-dependent one-carbon metabolism, because betaine is
574 the methyl-group donor in the BHMT reaction (Sections 2.2.1., 2.3.6.1.2. and 2.3.7.). This reaction
575 converts Hcy in the liver and kidney to methionine and releases dimethylglycine (DMG), which is
576 converted into sarcosine and methylene-tetrahydrofolate with tetrahydrofolate (THF) as methyl group
577 acceptor. The resultant sarcosine can be degraded into glycine or be excreted in the urine, whilst
578 methylene-THF can be reduced to methyl-THF by methylene-THF reductase (MTHFR) (Ueland et al.,
579 2005) (Section 2.5.).

580 *2.3.5.2.2. Microbial choline degradation to trimethylamine (TMA)*

581 Non-absorbed choline is one of the precursors of TMA produced in the gut by anaerobic symbiotic
582 microbes (Zhang et al., 1999; Craciun and Balskus, 2012) (Section 2.2.2.2.). TMA is efficiently
583 absorbed from the gastrointestinal tract (Al-Waiz et al., 1987), and then converted in the liver to
584 TMAO by the flavin-containing monooxygenase isoform 3 enzyme (FMO3) (Lang et al., 1998). Both
585 TMA and TMAO are eliminated in the urine (urinary total TMA i.e. TTMA = TMA plus TMAO).

586 TMA has an unpleasant fishy odour and can result in a corresponding fishy body odour when either
587 choline intake is 'high' (Section 2.2.2.2.), the intestinal microbiota is disturbed or the subjects suffer
588 from autosomal-recessive trimethylaminuria due to defects in FMO3 (Mitchell and Smith, 2001;
589 Zeisel et al., 2003).

590 On 'normal' diets, only milligram amounts of TMA were excreted in the urine of healthy subjects and
591 subjects with liver cirrhosis, but when single choline doses of 2–8 g as bicarbonate were given on
592 separate occasions, about 69% of choline nitrogen was excreted in the urine as TMA nitrogen (De la
593 Huerga and Popper, 1951).

594 In a study in six healthy males, measuring the conversion of single oral doses of 15 mmol of choline or
595 PC (i.e. 2.1. and 11.65 g, respectively, given on separate occasions at least two weeks apart) into
596 urinary TTMA, about 63% of choline appeared as urinary TTMA within three days after ingestion
597 (Zhang et al., 1999). In this study, PC did not lead to similar increases in urinary TTMA concentration
598 (0.5–2 % of the administered dose).

599 However, a double-blind randomised controlled trial (RCT) in six healthy volunteers (four women),
600 consuming single increasing amounts of PC separated by two to four weeks (119 up to 714 mg/day of
601 choline, mainly as PC, in the form of egg yolk(s)) in addition to a low-choline diet[8], demonstrated that
602 an intake of increasing amounts of PC resulted in a rise in TMAO concentrations in both plasma and
603 urine (Miller et al., 2014). TMAO concentration in plasma increased in five of six subjects after egg
604 ingestion, with a peak after six to eight hours; however, there was great interindividual variability.
605 TMAO concentration in urine in the 24 hours after egg yolk ingestion increased in proportion to the
606 amount of PC ingested (11 to 15% of the total ingested choline). The authors also found differences in
607 the profile of the faecal microbiome and in the gene for the FMO3 enzyme (the SNP *FMO3* G566A,
608 rs2266782 is associated with a 25% reduction in the enzyme activity) between the study participants.
609 This may explain the variable responses of plasma and urinary TMAO concentrations to PC intake.

610 The Panel notes a relationship between dietary choline, microbial metabolism of choline to TMA,
611 hepatic TMAO production and urinary TTMA excretion. The Panel notes as well an influence of other
612 dietary, genetic and environmental factors on TMA production. The Panel concludes that a dose-
613 response relationship between dietary choline and hepatic TTMA production cannot be established.

[8] 11 mg choline/1 000 kcal per day, i.e. about 2.6 mg choline/MJ per day

2.3.6. Elimination

2.3.6.1. Urine

The kidneys accumulate choline via the sodium-independent low-affinity carrier-mediated saturable mechanism described in Section 2.3.3.

2.3.6.1.1. Choline and trimethylamine-N-oxide (TMAO)

Excretion of choline in the urine is low in relation to usual dietary intakes. De la Huerga and Popper (1951) (Section 2.3.5.2.2.) determined the excretion of choline and TMA in the urine in four healthy adult subjects after single oral doses of 2–8 g of choline (as choline bicarbonate). The authors detected no or negligible choline in urine at baseline and not more than 0.3% of the administered dose thereafter. Within 24 hours, two thirds of the administered dose were excreted as TMA and TMAO, which suggests that unabsorbed choline was metabolised by the intestinal microbiota.

In pregnant and non-pregnant women (consuming either 480 or 930 mg of choline/day for 12 weeks), the (geometric) mean of the excretion of choline in the urine throughout the 12-week study was 10.7 (95% CI: 8.1–14.1) and 3.2 (95% CI: 2.3–4.4) mg/day, respectively ($p \leq 0.001$), and did not change significantly with choline intake (Yan et al., 2012) (Sections 2.3.6.1.2., 2.4.1.2. and 5.1.3.). In lactating and non-lactating women (from the study by Yan et al. (2012)), mean excretion of choline in the urine throughout the study (10–12 weeks) did not differ (Davenport et al., 2015) (Sections 2.3.3., 2.3.6.1.2., 2.3.6.3., 2.4.1.2. and 5.1.3.4.).

2.3.6.1.2. Betaine and dimethylglycine (DMG)

Betaine in the urine originates either from the diet or is formed in the kidney (and liver) via CHDH and BADH from choline. In this reaction, betaine is a methyl group donor for Hcy remethylation (Figure 2 and Sections 2.2.2.1. and 2.3.5.2.1.). BHMT demonstrates saturation kinetics, its activity increases in rat liver when the diet is low in methionine but contains choline or betaine (Park and Garrow, 1999) and its activity is inhibited by DMG, which is the product of BHMT activity. Moreover, oxidative demethylation of DMG to sarcosine is the rate-limiting step in betaine metabolism. Betaine normally accumulates in the kidney medulla, where its release into the urine is controlled by intracellular tonicity.

While the betaine plasma concentration remains almost stable on a habitual diet, it increases rapidly about 30-fold following one oral dose of about 50 mg betaine/kg body weight in 12 healthy males and has an elimination half-life of around 14 hours (Schwahn et al., 2003a). In this study, on average, 4 % of the ingested dose was excreted as betaine in the 24-hour urine; the renal clearance[9] was in the range of 0.4–13.9 mL/hour per kg body weight and about 5% of the apparent total plasma clearance. Betaine is freely filtered in the kidney, but normally almost completely reabsorbed in the proximal tubule (Lever et al., 2007).

In a randomised cross-over study on eight healthy males consuming five different intervention meals, including one high-choline meal (564 mg) or a single dose of choline supplement (500 mg), compared to a low-choline meal (< 1 mg choline), urinary betaine excretion was not significantly different between groups (Atkinson et al., 2008). In contrast, in this study, urinary DMG excretion peaked at 4-6 hours ($p < 0.005$ compared to control), but was still higher than baseline 24 hours after the high-choline meal ($p < 0.05$).

In pregnant and non-pregnant women (consuming either 480 or 930 mg of choline/day for 12 weeks), the (geometric) mean of the excretion of betaine in the urine throughout the 12-week study was 12.9 (95% CI: 10.0–16.6) and 8.1 (95% CI: 6.1–10.8) mg/day, respectively ($p \leq 0.05$) (Yan et al., 2012) (Sections 2.3.6.1.1., 2.4.1.2. and 5.1.3.). Lactating women (versus control women) (from the study by Yan et al. (2012)) had a lower excretion of choline metabolites (betaine: –3 mg/day, $p = 0.001$;

[9] Defined as the ratio of 24h urinary excretion (mmol/kg body weight) to the respective area under the curve (in mmol/L per hour).

DMG: -2.3 mg/day, $p < 0.001$) in the urine throughout the study period (Davenport et al., 2015) (Sections 2.3.6.1.1., 2.3.6.3., 2.4.1.2. and 5.1.3.4.).

Infants excrete high amounts of betaine in their urine, up to 1.5 mmol/mmol creatinine (1.55 g/g creatinine) during the first year of life, with a maximum at the age of two to three months and a decrease to 0.2 mmol/mmol creatinine at one year (Holmes et al., 1996). During the first ten days of life, a urinary excretion of betaine of 27.4 ± 2.8 µmol/kg body weight per day (3.2 ± 0.3 mg/kg per day; mean ± SEM) was reported in 27 infants. At that age, no dietary source of betaine is available (Holmes et al., 1996). In the newborn period, urinary excretion of betaine may be higher than choline intake (Davies et al., 1992).

2.3.6.1.3. Conclusion on urinary excretion

The Panel notes that choline excretion in the urine is low in relation to usual dietary intakes (and 0.3% of the administered dose of 2-8 g choline). A study showed that pregnant women have higher urinary excretion of choline and betaine than non-pregnant women. The Panel notes that excretion of betaine in urine may be of dietary origin or produced from choline. The rise in urinary DMG concentration, the second product of BHMT activity, after a choline supplement or a high-choline meal, suggests that choline-derived betaine is primarily used for Hcy remethylation in the liver (rather than fulfilling the other functions of betaine in the body).

2.3.6.2. Faeces

Hepatic PC synthesised either from dietary choline via the CDP-choline pathway or via the PEMT pathway (Figure 2) is used for secretion of VLDL or formation of HDL, or secretion into the bile. In mice, PC secretion into the bile was equivalent to the entire hepatic PC pool, of which 95% is reabsorbed (Li and Vance, 2008). In addition, PC is secreted by the intestinal mucosa, according to data in animals and patients (Ehehalt et al., 2010).

No human data are available on faecal excretion of choline or choline compounds in relation to dietary choline intake. Depending on the composition of the gut microbiome, non-absorbed choline in the gut can be converted to TMA (Sections 2.2.2.2 and 2.3.5.2.2.).

2.3.6.3. Human milk

Choline is found in milk predominantly as PChol and GPC, together with free choline, PC, SPM. Its concentration changes during the progress of lactation, and is influenced by maternal diet (Fischer et al., 2010b; Davenport et al., 2015). Apart from choline and choline containing compounds, milk also contains betaine.

In an RCT in 103 pregnant (then lactating) women (94 completers), Fischer et al. (2010b) (Sections 2.3.3., 2.4.1.2., 2.5.1., 5.1.3. and 5.2.5.) investigated the response of maternal plasma and breast milk choline concentrations to a PC supplement (750 mg/day choline, n = 48, from the 18th gestational week to 90 days post partum), compared to placebo (n = 46). The supplement was consumed in addition to a mean dietary choline intake of about 350 mg/day (measured by a three-day food record at 45 days post partum). Breast milk (and maternal plasma) concentrations were measured at 45 days post partum. There was a significant linear correlation between total choline intake (from foods and supplements, range about 150 to > 750 mg/day) and breast milk concentrations of PChol, PC, free choline and betaine ($R^2 = 0.16$ and $p = 0.0001$, $R^2 = 0.07$ and $p = 0.02$, $R^2 = 0.08$ and $p = 0.001$, $R^2 = 0.13$ and $p = 0.0003$, respectively), when all subjects were taken into account. Mean (± SE) breast milk concentrations of PChol (722 ± 39 vs 553 ± 27 µmol/L) and free choline (106 ± 10 vs 83 ± 8 µmol/L) were significantly higher ($p < 0.001$) in the supplemented group than in the placebo group, whereas PC, GPC and SPM were not significantly different.

In a controlled feeding study, Davenport et al. (2015) (Sections 2.3.3., 2.3.6.1.1., 2.3.6.1.2., 2.4.1.2. and 5.1.3.4.) investigated the response of breast milk choline concentration to different choline intakes. In this study, lactating (n = 28, five weeks post partum) and control (n = 21, non-pregnant non-lactating) women were randomised to consume 480 mg/day (15 lactating women and 10 controls)

or 930 mg choline/day (13 lactating women and 10 controls), from food and supplements[10], for 10 (lactating women) or 12 weeks (control women). Lactating women consuming 930 mg/day choline had a significantly higher concentration of total choline in breast milk (sum of all choline compounds) at the end of the study compared to those consuming 480 mg/day (mean ± SD: 1 200 ± 60 vs 1 000 ± 50 µmol/L, p = 0.041). They also had higher concentrations of PChol (392 ± 26 vs 285 ± 24 µmol/L, p = 0.008) and GPC (471 ± 36 vs 346 ± 33 µmol/L, p = 0.031), but their free choline concentration in breast milk did not differ (148 ± 13 vs 158 ± 12 µmol/L). During the last four to six weeks, 20% of the total choline intake was provided as deuterium labelled choline (methyl-D_9-choline). Women consuming the higher choline intake (930 mg/day) during lactation had in their breast milk, at the end of the study, a significantly higher enrichment of the metabolites generated endogenously via the hepatic PEMT pathway, but not of the metabolites generated from intact exogenous choline via the CDP-choline pathway (Figure 2, Section 2.3.5.). The Panel notes that the higher choline intake during lactation (930 mg/day, compared to 480 mg/day) significantly increased the concentration of total choline in breast milk, and increased the supply of PEMT-derived choline metabolites in breast milk.

The content of PC and SPM in breast milk was reported to remain constant from day zero to 85 of lactation, whilst the content of GPC, PChol and, to a lesser extent, free choline, in breast milk increased significantly after the first week after birth (Zeisel et al., 1986), but only free choline content decreased significantly with time.

A search of the literature published after January 2000 was performed as preparatory work to this assessment, in order to identify breast milk composition data for choline (LASER Analytica, 2014). This search was completed with two additional papers (Holmes-McNary et al., 1996; Davenport et al., 2015). Appendix A reports data from six studies (Holmes-McNary et al., 1996; Holmes et al., 2000; Ilcol et al., 2005; Fischer et al., 2010b; Ozarda et al., 2014; Davenport et al., 2015) conducted in the UK, Turkey and the USA, on the mean/median free and total choline concentrations of human milk from healthy lactating mothers. Either the infants were full-term (Holmes-McNary et al., 1996; Ozarda et al., 2014), or there was a mixed population of full-term and pre-term infants or it was unclear whether the infants were born at term or not.

Stages of lactation varied between birth and 180 days post partum. Mean maternal choline intake was not reported in four studies (Holmes-McNary et al., 1996; Holmes et al., 2000; Ilcol et al., 2005; Ozarda et al., 2014), while one study compared choline supplemented versus non-supplemented women (Fischer et al., 2010b) and the other compared two doses of choline supplementation (Davenport et al., 2015). Three studies (Ilcol et al., 2005; Fischer et al., 2010b; Davenport et al., 2015) reported information on maternal plasma choline concentration (considered by the authors as an indication of maternal status). The mean/median concentration of total choline in mature milk ranged from 120 to 160 mg/L (see Appendix A).

Based on the two studies on full-term fully breast-fed infants (Holmes-McNary et al., 1996; Ozarda et al., 2014) in the US and Turkey (n = 70 women in total), an average total choline concentration (free choline and choline compounds) of about 145 mg/L in mature breast milk can be calculated. Assuming a mean milk transfer of 0.8 L/day during the first six months of lactation in exclusively breastfeeding women (Butte et al., 2002; FAO/WHO/UNU, 2004; EFSA NDA Panel, 2009), the estimated secretion of choline into milk during lactation would be 116 mg/day, rounded to 120 mg/day.

The Panel notes that breast milk mainly contains PChol and GPC, besides free choline, PC and SPM, in concentrations depending on the progress of lactation and maternal diet/supplementation. The Panel also notes that increased maternal choline intake enhances the concentration of total choline in breast milk and increases the supply of PEMT-derived choline metabolites in breast milk. The Panel considers that secretion of choline into breast milk during the first six months of exclusive breastfeeding is about 120 mg/day.

[10] Diet provided an average of 380 mg/day of choline, and supplemental choline was 100 or 550 mg/day.

2.3.7. Interaction with other nutrients: folate

The interrelationship between folate and choline metabolism, both involved in the remethylation of Hcy to methionine, the first using 5-methyl-THF, the latter using betaine, has been demonstrated in animal studies (Varela-Moreiras et al., 1992; Kim et al., 1994) (Section 2.3.5.2.1.). In the first case, Hcy is methylated to methionine by the ubiquitous methionine synthase (MS, Figure 2), which requires methyl-THF as methyl-group donor and cobalamin as cofactor (Ueland et al., 2005). In the second case, Hcy is methylated to methionine by BHMT (Figure 2), which requires betaine as methyl-group donor. Choline insufficiency, with consequently low betaine formation, increases the requirement for methyl-THF for the remethylation of Hcy and, therefore, the requirement for dietary folate. Vice versa, in folate depletion, methyl groups from choline and betaine are increasingly used for Hcy remethylation, thereby increasing the requirement for choline. Methyl-THF and choline/betaine can be considered as partially exchangeable sources of methyl groups (Kim et al., 1994).

Jacob et al. (1999) investigated the effect of folate depletion and repletion on choline status and the *in vivo* methylation capacity in humans residing in metabolic units. Following a baseline period of six to nine days on a diet sufficient in energy and all nutrients including folate (440 µg/day), 11 healthy men (aged 33 to 46 years) and ten healthy women (aged 49 to 63 years) consumed, for 4-5 weeks, a low folate (average of 25 µg/day and 56 µg/day for men and women, respectively) and low choline diet (average of 238 mg/day and 147 mg/day for men and women, respectively). Two to six weeks of folate repletion followed (440 and 516 µg folate/day for men and women, respectively, partially supplied as folic acid) without change in the choline intake. Variation in the methionine content of the diet in men (400 mg or 1 400 mg/day in the first half of the study period with cross-over thereafter) had no effect on the outcomes (this was not investigated in women). No functional deficiencies of organs were noted in any subject. Methylation capacity, as assessed by the urinary excretion of creatinine and of methylated nicotinamide breakdown products after ingestion of 1 g of nicotinamide, was not diminished. At the end of the folate depletion phase, plasma choline (and folate) concentrations were significantly lower in both men and women compared with baseline, and plasma tHcy concentration was significantly higher whilst PC concentration was decreased in men compared to baseline (PC concentration was not investigated in women). At the end of the folate repletion phase, plasma choline concentrations increased significantly in both sexes compared to the folate depletion phase ($p < 0.05$), in women to even higher values than at baseline ($p < 0.05$), with no significant change in plasma tHcy concentration compared to the folate depletion phase. No changes in choline, folate and SAM concentrations in red blood cells were noted throughout the study. The Panel notes that, in this study, an adequate folate intake maintained plasma choline concentration despite a low choline intake of about 150–250 mg/day on average, whilst plasma choline and PC concentrations decreased and tHcy concentration increased when both folate and choline intakes were low.

In 43 premenopausal Mexican-American women, folate intake was restricted for seven weeks to 135 µg dietary folate equivalent (DFE) per day, followed by seven weeks of randomisation to either 400 or 800 µg DFE/day, whilst choline intake was kept constant at 349 mg/day (including 250 mg/day of a choline supplement) (Abratte et al., 2008). In this study, plasma PC concentration decreased during dietary folate restriction compared to baseline ($p = 0.001$), presumably due to the unfulfilled demand of folate-derived one-carbon units for PC synthesis. Plasma PC concentration increased again after administration of 800 µg DFE/day ($p = 0.03$) (but not significantly with 400 µg DFE/day). The Panel notes that, in this study, folate intake was shown to influence plasma PC concentration.

Changes in the activity of enzymes involved in folate and choline metabolism, due to polymorphisms of genes for enzymes of this metabolism, can be expected to have an impact on the status of folate and choline. An example is the C677T genotype of the MTHFR (Sections 2.3.5.2.1. and 2.5.), which has a strong influence on folate status (Abratte et al., 2008).

Ivanov et al. (2009) examined the potential influence of polymorphisms of two genes involved in choline metabolism (*MTHFD1* rs2236225 and *PEMT* rs12325817 and rs7946) (Section 2.5.) on

plasma PC and tHcy concentrations in the presence of folate restriction, in the same Mexican-American women studied by Abratte et al. (2008). These polymorphisms are functional in that they impair the activity of the two enzymes (PEMT and MTHFD1) and thereby possibly increase choline requirement and compromise the production of methyl-THF. The *PEMT* and *MTHFD1* polymorphisms did not modify the small negative response of plasma PC concentration to folate restriction, except in case of homozygosity for *PEMT* rs1232587 that attenuated the decline in plasma PC concentration. Homozygosity for *PEMT* rs7946 and *MTHFDH1* rs2236225 SNPs was associated with a greater increase ($p < 0.001$) in plasma tHcy concentration during folate restriction than in subjects homozygous for the wildtype.

The Panel notes that low folate intake has a negative impact on plasma PC concentration in the presence of 'adequate' choline intake, and that the impact of SNPs of genes of some enzymes involved in metabolic pathways of choline may result in increased tHcy concentrations in plasma during folate restriction. These changes are not predictable, due to compensatory changes in other parts of those pathways. The Panel, moreover, notes the small number of subjects investigated and stratified for genetic polymorphisms that limits the generalisation of these studies.

2.4. Biomarkers

2.4.1. Plasma/serum concentration of choline and choline-compounds

2.4.1.1. Adults

Fasting plasma free choline concentrations usually range between 7 and 20 µmol/L, with most subjects having a concentration of 10 µmol/L (IOM, 1998). Plasma choline concentrations are regulated and remain around 10 µmol/L in humans. However, some variability in plasma concentrations occurs with changes in choline intake. Choline-deficient diets, as applied in depletion/repletion studies (Section 5.1.2.) and consumed over weeks, can reduce plasma concentrations by approximately 50%, and ingestion of choline-rich foods (e.g. ≥ 500 mg/day) can increase plasma concentrations beyond 20 µmol/L (Zeisel et al., 1991). Plasma choline concentration was found not to decrease beyond 50% of the initial normal value even after one week of total fasting, presumably because of release of choline from membrane phospholipids (Savendahl et al., 1997).

Fasting plasma PC concentration varied between adults (1.5–2.5 mmol/L) and decreased by 30% after three weeks on a low choline diet, while erythrocyte PC concentration decreased by 10% (Zeisel et al., 1991).

2.4.1.2. Pregnancy and lactation

During pregnancy, serum free and phospholipid-bound choline concentrations increase, compared to non-pregnant women (Ozarda Ilcol et al., 2002).

The controlled feeding study by Yan et al. (2012) (Sections 2.3.6.1. and 5.1.3.) compared the effects of two doses of choline supplementation (480 or 930 mg of choline/day from food and supplements) in healthy pregnant (recruited at 27 weeks gestation) and non-pregnant women. In this study, pregnant women had similar mean plasma free choline concentration as non-pregnant women at recruitment, but significantly higher concentration (by 30 %) than non-pregnant women throughout the 12-week study (geometric means, (95% CI): 8.2 (7.6–8.7) vs 6.3 (5.6-6.9) µmol/L, respectively, $p < 0.001$). Pregnant women had lower mean plasma concentrations of the three methyl-group donors (betaine, DMG, sarcosine) as well as methionine and Hcy at recruitment, and this persisted throughout the study (lower by 13–55%, $p < 0.001$). The lower circulating concentrations of choline-derived methyl-group donors in pregnant women, than in non-pregnant women, throughout the study, was possibly a consequence of the greater use of these molecules in both maternal and fetal compartments. Pregnant women consuming 930 mg choline/day had higher mean plasma concentration of free choline than those consuming 480 mg choline/day (13% higher, $p = 0.021$).

In a prospective observational study, choline intake of 154 pregnant women, estimated by a food frequency questionnaire (FFQ), was weakly correlated to their natural log-transformed plasma concentration of free choline at 16 and 36 weeks of gestation (16 weeks: r = 0.20, p = 0.013, range of intake read on figure: 150–700 mg/day) (Wu et al., 2012).

In a prospective cohort study on 368 Canadian pregnant women recruited at 12–16 weeks of gestation, Visentin et al. (2015) investigated the relationship between maternal choline intake and concentrations of choline and its metabolites in maternal and umbilical cord plasma. Mean maternal choline intake (total of all compounds), as estimated by a semiquantitative FFQ, was 306 ± 127 and 302 ± 122 mg/day in early (0–16 weeks) and late (23–37 weeks) pregnancy, respectively. Mean maternal plasma free choline (95% CI) was 7.2 (7.1–7.4) µmol/L. The mean concentrations of free choline, DMG and TMAO in maternal plasma increased significantly ($p \leq 0.005$) between recruitment in pregnancy and delivery by 49%, 17%, and 13% respectively, whereas that of betaine decreased by 21% ($p \leq 0.005$). Maternal dietary intake (total or free) was not associated with these maternal plasma concentrations. The mean concentrations of free choline, betaine and DMG in cord plasma were 3.2, 2.0 and 1.3 times the concentrations in maternal plasma at delivery, whereas the mean concentration of TMAO cord plasma was lower by 12%. Maternal dietary choline intake (or fetal genetic variants in genes involved in choline metabolism[11]) was not associated with cord plasma concentrations of free choline and its metabolites. In contrast, maternal plasma concentrations of betaine, DMG and TMAO at delivery strongly influenced umbilical cord plasma concentrations (r^2 between 0.19 and 0.51, all $p < 0.0001$, after adjustment for potential confounders). There was only a weak correlation between the concentration of free choline in maternal and umbilical cord plasma ($r^2 = 0.12$, $p = 0.06$).

Results are indicative of an active transport of choline from the mother to placental tissue (Section 2.3.3.) and/or an uptake and metabolism of choline by the fetus reflecting a demand of the fetus for choline and methyl group donors.

In lactating women, serum free and phospholipid-bound choline concentrations were significantly higher than in non-lactating women ($p < 0.05$), and gradually decrease until 180 days after the birth of the child (Ilcol et al., 2005).

In the lactating women of the RCT by Fischer et al. (2010b) (Sections 2.3.3., 2.3.6.3., 2.5.1., 5.1.3. and 5.2.5.), there was a significant correlation between total choline intake (from foods and supplements) and maternal plasma concentration of free choline (R^2 of 0.15 in the supplemented group, and 0.55 in all subjects combined, $p = 0.03$ and $p = 0.0001$, respectively). Choline supplementation increased mean maternal plasma concentration of free choline compared to placebo (mean ± SE: 13.7 ± 0.6 vs 7.7 ± 0.3 nmol/mL at 45 days post partum, $p < 0.001$).

In addition, in the controlled feeding study by Davenport et al. (2015) (Sections 2.3.3., 2.3.6. and 5.1.3.4.), lactating women showed higher (+27 %, $p < 0.001$) plasma free choline concentrations than non-pregnant non-lactating women throughout the study period. Lactating women who consumed 930 mg/day choline had significantly higher plasma free choline concentration (+16%, $p = 0.012$) compared to those consuming 480 mg/day.

2.4.1.3. Infants

In newborns, serum free choline concentrations were significantly higher (> twice maternal values) and phospholipid-bound choline concentrations were significantly lower (by about 40%) than in their mothers (Holmes et al., 2000). Phospholipid-bound choline plasma concentrations in the infants rose by 40% starting from day 5–15 after birth to reach adult levels by the age of about ten years. Plasma free choline concentration of newborns remained high for two weeks after birth, was still slightly higher than adult levels at the age of two years and remained stable at around 10 µmol/L at the age 3-12 years. This high newborn's plasma concentration possibly reflects the increase of choline in

[11] Ten SNPs in seven candidate genes.

breast milk in the second week of life (Section 2.3.6.3.). There was no correlation between maternal and newborn plasma phospholipid-bound choline (Buchman et al., 2001; Ilcol et al., 2005).

2.4.1.4. Conclusion on plasma/serum concentration of choline and choline-compounds

The Panel notes age-related changes in choline concentrations in plasma, with higher values in infants and young children than in adults.

The Panel also notes that pregnancy and lactation are associated with higher free choline concentrations in plasma than in the non-pregnant non-lactating state, and that choline supplementation increases maternal plasma concentration of free choline in pregnancy or lactation. However, the Panel considers that the maternal intake of choline cannot be deduced from the choline concentration in maternal plasma during early and late pregnancy or lactation, nor from the choline concentration in venous umbilical cord plasma.

No relationship between choline intake and plasma concentration of free choline (or of PC, betaine, DMG or TMAO, or erythrocyte PC) can be deduced from the available data and, therefore, the Panel considers that plasma concentrations of choline and choline compounds cannot be used for setting DRVs for dietary choline.

2.4.2. Total trimethylamine (TTMA) hepatic production

The Panel concludes that TTMA hepatic production and excretion in urine are not predictably related to dietary choline intake and cannot be used for setting DRVs for dietary choline (Section 2.3.5.2.2.).

2.4.3. Plasma total homocysteine

Appendix B compiles the results of six studies on adults (19–82 years of age) investigating the influence of choline intake on plasma tHcy concentrations. Three studies were RCTs (Olthof et al., 2005; Atkinson et al., 2008; Wallace et al., 2012), with choline given as supplements (500 to 2 600 mg/day of choline) for 2–12 weeks or just once a week. Three others were cross-sectional studies within long-term cohorts (Cho et al., 2006; Chiuve et al., 2007; Lee et al., 2010a), involving 6 069 subjects of which 1 325 were men. The results from RCTs with supplements are inconsistent. RCTs with choline doses of 500 and 1 000 mg/day showed no decrease in plasma tHcy concentration (Atkinson et al., 2008; Wallace et al., 2012). However, a dose of 2 600 mg/day (as PC) over two weeks resulted in a significant decrease of fasting plasma tHcy concentration (mean ± SD: 15.6 ± 4.0 vs 13.6 ± 2.5 µmol/L; $p < 0.0001$) and, compared to placebo, a significantly lower rise ($p < 0.0001$) in plasma tHcy concentration following a methionine load (0.1 g/kg body weight) (Olthof et al., 2005). The cross-sectional studies showed an inverse relationship between dietary choline intake (that ranged in quintiles from around 230 to 400 mg/day) and fasting plasma of tHcy concentrations.

The Panel notes that many factors besides dietary or endogenous choline determine the tHcy concentration in plasma. The Panel concludes that neither fasting nor post-methionine load tHcy concentrations in plasma can be used for setting DRVs for dietary choline.

2.4.4. Urinary betaine excretion

The Panel notes that betaine in urine may be of dietary origin or produced in the body from choline (Section 2.3.6.1.2.). The rise in urinary DMG concentration, the second product of BHMT activity (Figure 2 and Section 2.3.6.1.3.), after a choline supplement or a high-choline meal, suggests that choline-derived betaine is primarily used for Hcy remethylation in the liver (rather than fulfilling the other functions of betaine in the body).

The Panel concludes that urinary betaine excretion is not predictably related to dietary choline intake and, therefore, cannot be used for setting DRVs for dietary choline.

2.4.5. Conclusions on biomarkers

The Panel considers that the available data do not allow concluding on a dose-response relationship between choline intake or choline status and plasma choline concentration. The Panel also considers that plasma choline concentrations are not suitable to derive DRVs for dietary choline. Plasma concentrations of PC, betaine, DMG, tHcy or TMAO, erythrocyte PC concentration, or urinary betaine and TTMA excretion can neither be used to set DRVs for dietary choline.

2.5. Effects of genotypes involved in choline metabolism

Several SNPs in genes coding for enzymes in choline metabolism and in methyl-group metabolism can alter the requirement for choline and determine the likelihood of developing signs of choline deficiency (Section 2.2.2.1.) with low dietary choline intakes. For example, MTHFD1 (Sections 2.2.2.1. and 2.3.7.) is a trifunctional enzyme responsible for generating and interconverting 1-carbon-substituted THF cofactors from formate. *MTHFD1* mutations can impact both Hcy remethylation and thymidylate (dTMP) biosynthesis.

Genetically modified mice with defective MTHFR activity become choline deficient (Schwahn et al., 2003b) and 15–30% of humans have genetic polymorphisms that alter the activity of this enzyme resulting in a higher requirement for folate, and potentially indirectly for choline if folate intake is lower than the requirement (Rozen, 1996; Wilcken et al., 1996).

Da Costa et al. (2006b) (Section 5.1.1.3. and Appendix D) performed a controlled trial in 57 subjects (26 men and 31 women), aged 18-70 years, to determine whether susceptibility to develop organ dysfunction due to choline deficiency was influenced by common genetic polymorphisms. The choline depletion/repletion study design is described in Section 5.1.2. Sixty-eight percent of the subjects (n = 39) developed organ dysfunction on the low-choline diet, which was resolved during choline repletion. Mean plasma choline concentrations decreased by almost 30% (from 9.8 to 7.1 µmol/L), irrespective of development of organ dysfunction. Susceptibility to choline deficiency was not affected by *BHMT +742G→A* SNP (rs3733890) in this study.

Niculescu et al. (2007) (Section 5.1.1.3. and Appendix D) performed a study in 33 subjects (14 men and 19 women), aged 20 to 67 years, to examine the effects of a low-choline diet on gene expression in subjects who developed organ dysfunction due to low choline intake, those who did not, and the potential role of four SNPs in genes involved in folate and choline metabolism (*PEMT* rs12325817, *MTHFD1*rs2236225, *CHDH* rs9001 and rs12676). The choline depletion/repletion study design is described in Section 5.1.2. Blood was collected after the baseline diet and after the low-choline diet, and peripheral lymphocytes were used to measure gene expression and for SNP genotyping. The low-choline diet resulted in underexpression of 152 genes and overexpression of 107 genes. Differences in gene expression changes were noted between those who developed organ dysfunction and those who did not. Analyses using group clustering and gene ontology showed that changes in gene expression related to the experimental diets were significantly altered by the SNPs examined.

Appendix C lists the enzymes (PEMT, MTHFD1, CHDH, BHMT, choline kinase isoform A or B (CHKA or CHKB), CCT, SLC44A1, MTHFR), which have SNPs with known qualitative impact on choline requirement and/or are associated with an increased risk of developing organ dysfunction or other health outcomes, including birth defects, when consuming a low-choline diet. In particular, some specific polymorphisms of the genes for PEMT, CHDH and MTHFD1 were shown to increase the dependency on dietary choline intake (Appendix C).

According to the review by Au et al. (2010), it may not be accurate to include or exclude risk contribution of the tested genes investigated in epidemiological studies on neural tube defects (NTDs), some of them having limitations in study design, that potentially affect the power of statistical analysis, thus providing conflicting conclusions. For complex diseases like NTDs, it is anticipated that the risk of a disease-associated allele is between 1 and 2, and over 2 000 samples (cases plus controls) would be needed to provide statistical power of 80% to assess a risk of 1.8–2 of a disease locus with a

SNP allele frequency of 0.1. Double or quadruple the controls would be needed if unmatched controls are used, to adjust for confounding factors.

The Panel notes that many SNPs have been described for genes coding for eight enzymes (Appendix C) involved in choline or methyl-group metabolism, and that carrier frequency in mixed populations can be up to about 70%. Kohlmeier et al. (2005) mention a personal communication by K. Meyer and P.M. Ueland that the distribution of polymorphic variants of *MTHFR* and *MTHFD1* in North Carolina (Appendix C) largely agreed with that of North European populations (Norwegian Colorectal Cancer Prevention (NORCCAP) study).

The effects of the *PEMT* polymorphism rs12325817, on the likelihood of development of signs of organ dysfunction (mainly liver) when choline intake is experimentally restricted to ≤ 50 mg/70 kg body weight per day, have been investigated most often. The risk of organ dysfunction is higher in postmenopausal than in premenopausal women and is increased by simultaneous restriction in folate intake. Due to the experimental design of choline depletion/repletion studies with a low choline intake during the depletion period (≤ 50 mg/70 kg body weight per day) (Section 5.1.1.), and because of the lack of data on the relationship between habitual choline intakes and signs attributable to choline deficiency in populations, the Panel notes that the amount of dietary choline needed to prevent such signs cannot be predicted with confidence.

2.5.1. Influence of polymorphisms in pregnancy and lactation

Polymorphisms in the *MTHFD1* gene and the *BHMT* gene, coding for enzymes involved in choline metabolism, were identified as potential candidates for association with choline concentrations in maternal plasma and breast milk (Fischer et al., 2010b) (Sections 2.3.3., 2.3.6.3., 2.4.1.2., 5.1.3., 5.2.5.).

In the RCT by Fischer et al. (2010b), the authors also investigated whether maternal polymorphisms (370 SNPs in 10 genes involved in choline metabolism) modified the response of maternal plasma and breast milk choline concentrations (measured at 45 days post partum) to choline supplementation (compared with placebo). These SNPs were tested in linear regression models, with choline metabolites as the response and homozygous wild-type, heterozygous wild-type and homozygous variant alleles of SNPs, as well as choline intake (from food and supplements), as predictors. In these models, five SNPs in the *MTHFR* gene were identified in the placebo group that, for most of them, reduced the slope of the response curve of free choline concentration in breast milk to choline intake ($p < 0.05$). In addition, outliers previously identified by the authors (in a first analysis of the relationship between intake and concentrations in breast milk or plasma) were tested for combinations of shared SNPs. In this analysis, three subjects of the placebo group were identified with five SNPs in common in the *MTHFD 1* gene and who had exceptionally high breast milk choline concentrations (in relation to choline intake). Five participants were also identified with two SNPs in common in the *BHMT* gene, and four of these subjects had lower-than-average plasma free choline concentrations (in relation to choline intake).

Besides the choline intake of the mother (Section 2.3.6.3. and Appendix A), the Panel notes that polymorphisms in genes coding for enzymes involved in choline and methyl-group metabolism, particularly if they occur in combinations, can influence the amount of choline secreted into breast milk. The Panel considers that the available data on polymorphisms in genes are insufficient to predict choline concentrations in breast milk.

2.5.2. Conclusion on effects of genotypes

The Panel concludes that SNPs can enhance or reduce the function of enzymes involved in choline metabolism. This can influence the requirement for choline and, moreover, can determine the susceptibility to dietary choline deficiency. The Panel considers that particularly some specific polymorphisms of the genes for the enzymes PEMT, CHDH and MTHFD1 are known to increase the dependency on dietary choline intake. Since their frequency in populations vary and their impact on

dietary choline requirement may be influenced by dietary habits, no conclusions can be drawn from available studies on predictable variations in individual choline requirements.

3. Dietary sources and intake data

3.1. Dietary sources

Total choline content is highest in eggs (raw egg yolk: about 670 mg/100 g food, whole raw fresh egg: about 290 mg/100 g food) followed by meats and fish, whole grains, vegetables and fruit, and fats and oils (median content of fats and oils: about 5 mg/100 g food) (USDA, 2015). The proportion of different choline compounds in food can change by preparation. For example, cooking decreases the concentration of free choline and increases the content of PC per 100 g food, whilst mincing of raw vegetables decreases the content of PC by activating phospholipase D with the release of free choline and phosphatidic acid (Zeisel et al., 2003). The implications of such changes in choline compounds for human nutrition are unknown.

Human milk is rich in choline (Section 2.3.6.). Ilcol et al. (2005) showed that the distribution of choline compounds in human milk, and bovine-derived and soy-protein based formulae from different manufacturers differed considerably, e.g. soy-derived formulas had much less sphingomyelin than human milk.

In the EU the addition of choline to infant formula is mandatory with a minimum level of 7 mg and a maximum level of 50 mg of choline/100 kcal and the total phospholipid concentration must be not higher than 2 g/L[12].

Currently, choline, choline chloride, choline bitartrate and choline citrate may be added to food intended for infants and young children, food for special medical purposes, and total diet replacement for weight control in the EU[13]. CDP-choline (citicoline) has been evaluated as novel food by EFSA and no safety concerns were raised (EFSA NDA Panel, 2013a).[14] Choline and choline compounds can be found in dietary supplements.

3.2. Dietary intake

3.2.1. Dietary intake in EU countries

The Panel notes that no food composition data with respect to choline are available at the European level, and that there is a lack of reliable measurements of choline content in foods in the EU. The Panel refers to the study by Vennemann et al. (2015), which used, with the aim at assessing choline intake in the EU, the total choline composition data from the release n°26 of the the National Nutrient Database for Standard Reference from the US Department of Agriculture (USDA database) (issued in November 2013) (USDA, 2013) (Section 3.1.). Total choline content of US foodstuffs was calculated by USDA as the sum of five choline-contributing metabolites, the water-soluble free choline, GPC and PChol, and for the lipid-soluble PC and SPM.

In the assessment by Vennemann et al. (2015), food consumption data from the EFSA Comprehensive European Food Consumption Database (EFSA, 2011), classified according to FoodEx2 classification, were used. This assessment includes food consumption data from 12 dietary surveys from nine EU

[12] Commission Directive 2006/141/EC of 22 December 2006 on infant formulae and follow-on formulae and amending Directive 1999/21/EC, OJ L 401, 30.12.2006, p.1.

[13] Regulation (EU) No 609/2013 of the European Parliament and of the Council of 12 June 2013 on food intended for infants and young children, food for special medical purposes, and total diet replacement for weight control and repealing Council Directive 92/52/EEC, Commission Directives 96/8/EC, 1999/21/EC, 2006/125/EC and 2006/141/EC, Directive 2009/39/EC of the European Parliament and of the Council and Commission Regulations (EC) No 41/2009 and (EC) No 953/2009, OJ L 181, 29.6.2013, p. 35.

[14] Commission Implementing Decision 2014/423/EU of 1 July 2014 authorising the placing on the market of citicoline as a novel food ingredient under Regulation (EC) No 258/97 of the European Parliament and of the Council, OJ L 196, 3.7.2014, p. 24

countries (Finland, France, Germany, Ireland, Italy, Latvia, the Netherlands, Sweden and the United Kingdom). These surveys used 3–7-day food records, 24-h recalls performed on at least two days or 48-h recalls. Individual data from these nationally representative (except for the Finnish surveys in children) surveys undertaken between 2000 and 2011 were available to EFSA. In this assessment by Vennemann et al. (2015), the nutrient composition data was obtained for 2 684 food items by re-coding the USDA nutrient composition food list (based on the LanguaL food description thesaurus) to FoodEx2 classification (used for the food consumption data). Nutrient intake calculations were performed only on subjects with at least two reporting days. Choline intake from dietary supplements was not assessed. Mean, medians, 5^{th} and 95^{th} percentiles of intake of the population, per survey, age, class and sex, were calculated.

Data were available from four surveys for children aged 1–3 years, from seven surveys for older children, and from eight surveys for adults (including one survey during pregnancy). Total choline intake mean estimates ranged from 151 to 210 mg/day in children aged 1–3 years, 177 to 304 mg/day in children aged 3–< 10 years, 244 to 373 mg/day in children aged 10-< 18 years. Total choline intake mean estimates ranged from 269 to 468 mg/day in adults aged 18–≥ 75 years, i.e. from 332 to 468 mg/day in men and from 269 to 404 mg/day in women of this age range, respectively. From one survey in Latvia, the choline intake mean estimate was 336 mg/day in pregnant adolescents and 356 mg/day in pregnant women.

Data on infants (< 1 year old) were available from three out of the seven surveys, namely from Finland, Germany and Italy[15] (data not shown in the study by Vennemann et al. (2015)). The total choline intake mean estimates in infants ranged from 75 to 127 mg/day. The Panel notes the limitations in the methods used for assessing breast milk consumption in infants and related uncertainties in the choline estimates for infants.

Choline intake estimates are also available from a convenience sample of Flemish women (aged 18-35 years) (Pauwels et al., 2015). In this study, food consumption was assessed by FFQs covering 51 food items that had been selected because they were part of the Belgian diet and/or were the main contributors for one of four methyl-group donors (including choline), and the USDA database was also used as food composition database for choline. Despite important methodological differences with the intake assessment described above from the study by Vennemann et al. (2015), and the specific population group investigated, choline intake estimates in Flemish women (mean ± SD: 286.6 ± 105.1 mg/day) were in the same order of magnitude of the estimates produced by Venneman et al (2015) for several EU countries.

3.2.2. Dietary intake in non-EU countries

In view of the limited data on choline intake published in the EU, the Panel again refers to the study by Vennemann et al. (2015), which compared their estimates with four studies carried out in non-EU countries in adult men and women in the USA, New Zealand and Taiwan (Chu et al., 2012; USDA, 2012; Mygind et al., 2013), and pregnant and lactating women in Canada, followed from the first or second trimester to three months post partum (Lewis et al., 2014). Two of these studies used nationally representative data (Chu et al., 2012; USDA, 2012), all studies used 24-h recalls or three-day food records as dietary assessment methods (but not FFQs), were cross-sectional (apart from the study on pregnant and lactating women) and used the same composition database (USDA database) as Vennemann et al. (2015) although from different releases.

The mean choline intake estimates in adults was 415 and 279 mg/day in US men and women, respectively, (USDA, 2012), 316 mg/day in women aged 18–40 years in New Zealand (Mygind et al.,

[15] The proportions of breast-fed infants were 58 % in the Finnish survey, 40 % in the German survey, 44 % in the Italian survey. Most infants were partially breast-fed. For the Italian and German surveys, breast milk intake estimates were derived from the number of breastfeeding events recorded per day multiplied by standard breast milk amounts consumed on an eating occasion at different ages. As no information on the breastfeeding events was reported in the Finnish survey, breast milk intake was not taken into consideration in the intake estimates of Finnish infants.

2013) and 372 and 265 mg/day in men and women aged 18–64 years, respectively, in Taiwan (Chu et al., 2012). The mean (± SD) choline intake in pregnant and lactating women in Canada ranged between 340 ± 148 in the second trimester and 346 ± 151 mg/day at three months post partum (Lewis et al., 2014).

3.2.3. Conclusion on dietary intake

The Panel notes that mean choline intake estimates in adults ranged from 269 to 468 mg/day in national surveys from seven EU countries (Vennemann et al., 2015), was about 290 mg/day in one EU country (Pauwels et al., 2015), and were between 265 and 415 mg/day in three studies conducted in non-EU countries (Chu et al., 2012; USDA, 2012; Mygind et al., 2013). The Panel also notes that mean choline intake was about 350 mg/day in the only EU survey on pregnant women considered in Vennemann et al. (2015), as well as in one study on pregnant or lactating women in one non-EU country (Lewis et al., 2014). The Panel concludes that the choline intake data resulting from the assessment by Vennemann et al. (2015) in EU countries are generally of the same magnitude as the intakes of the published studies available in adults in EU (Pauwels et al., 2015) and non-EU countries (Chu et al., 2012; USDA, 2012; Mygind et al., 2013; Lewis et al., 2014).

4. Overview of dietary reference values and recommendations

To date, DRVs for choline have only been proposed by the IOM (1998).

4.1. Adults

The IOM (1998) set Adequate Intakes (AIs), since data were not sufficient for deriving an Estimated Average Requirement (EAR) and a Recommended Dietary Allowance (RDA). The AIs for choline are based on data on the prevention of liver damage, as assessed by measuring serum ALT concentrations. The estimate is considered by the IOM as being uncertain because it was based on a single RCT by Zeisel et al. (1991) (depletion/repletion study, Section 5.1.2. and Appendix D). This study examined serum ALT activity in 16 healthy male hospitalised volunteers. They were supplemented with 500 mg choline/day for one week, then randomised to receive for three additional weeks either the choline-supplemented diet (control group, n = 7) or the same diet without choline but with cellulose as placebo (n = 8), then all subjects consumed the choline-supplemented diet during the fifth week of the study. A choline intake of 500 mg/day, which is approximately 7 mg/kg body weight per day using the mean body weight for the control group, i.e. 74.4 kg, prevented alanine aminotransferase abnormalities in these healthy men. Thus, the AI was set at 550 mg/day after rounding, considering the US reference weight of 76 kg for men (NHANES III, 1988–1994).

The IOM noted that, at that time, no studies undertaken in healthy women following a choline deficient diet were available. However, from an intervention study (Buchman et al., 1995) on one man and three women with hepatic steatosis receiving total parenteral nutrition containing 1 to 4 g/day of choline chloride for six weeks, the IOM concluded that women were just as likely as men to develop low plasma choline concentrations and fatty liver. To set an AI for women, the IOM assumed that the data used to set an AI for men could be used, even though women may use choline more efficiently, thus the derived AI for women was set at 425 mg/day based on the US reference weight of 61 kg for women (NHANES III, 1988–1994). IOM noted some evidence that transport across the blood-brain barrier is diminished in older adults (60-85 years, compared to younger adults aged 20-40 years), suggesting the possibility of a higher requirement than for younger adults (Cohen et al., 1995). Nevertheless, for older adults, no adjustment was made to the AI.

4.2. Infants and children

For breastfed infants from birth to six months, IOM (1998) set an AI of 125 mg/day. This AI was based on an average breast milk consumption of 0.78 L/day (Hofvander et al., 1982; Butte et al., 1984; Chandra, 1984; Neville et al., 1988; Allen et al., 1991) and an average choline concentration of 160 mg/L. This average choline concentration was obtained from 15 healthy US mothers exclusively breastfeeding and followed from 30 days up to 85 days post partum (Zeisel et al., 1986) and 33 healthy

US mothers participating in the study during postnatal days 27–32 (Holmes et al., 1996). For older infants aged 7–12 months, the AI was extrapolated upward from the AI for infants from birth to six months by allometric scaling and using US reference weights (NHANES III, 1988–1994), and was set at 150 mg/day. This value was confirmed by the downward extrapolation from the AI for adults by allometric scaling using a growth factor, which gave the same result.

In the absence of data on which to base an EAR or AI for choline for children, IOM (1998) extrapolated the AIs for children aged 1 to 18 years from adult values, by allometric scaling using growth factors.

4.3. Pregnancy

IOM (1998) concluded that an increase in the AI to support pregnancy should be based on the fetal and placental accumulation of choline. The IOM took into account animal data on choline concentration in adult tissues (Pomfret et al., 1989), organ weight in the human fetus (Widdowson, 1963) and human data (n = 7) on choline concentration in placental tissue (Welsch, 1976), and considered an average choline concentration of 321 mg/kg of fetal and placental tissue combined. The IOM assumed that there is no extra choline synthesis by the mother during pregnancy, and that there is no choline synthesis by the placenta or fetus. Thus, the required additional dietary intake of choline for 10 kg of tissue, that comprises the fetus (3 kg) and organs of pregnancy (7 kg), was calculated to be approximately 11 mg/day throughout pregnancy. The AI for choline was thus set at 450 mg/day (after rounding) for pregnant adolescent and adult women.

4.4. Lactation

The IOM (1998) proposed an additional intake of 125 mg/day for lactating women aged 14 to 50 years, considering an average breast milk production of 0.78 L/day (Hofvander et al., 1982; Butte et al., 1984; Chandra, 1984; Neville et al., 1988; Allen et al., 1991) and an average choline concentration of breast milk of about 160 mg/L.

An overview of DRVs for choline for infants, children, adults, pregnant or lactating women is presented in Table 1.

Table 1: Dietary Reference Values for choline for infants, children, adults, pregnant or lactating women

	IOM (1998) [(a)]
Age (months)	7–12
All (mg/day)	150
Age (years)	1–3
All (mg/day)	200
Age (years)	4–8
All (mg/day)	250
Age (years)	9–13
All (mg/day)	375
Age (years)	14–18
Boys (mg/day)	550
Girls (mg/day)	400
Age (years)	≥ 19
Men (mg/day)	550
Women (mg/day)	425
Pregnancy (mg/day)	450
Lactation (mg/day)	550

(a): AI.

5. Criteria (endpoints) on which to base Dietary Reference Values

5.1. Indicators of choline requirement

Plasma choline concentration may increase when intake is increased, and decreases by up to 50% when dietary intake is severely restricted (Zeisel et al., 1991) (Section 2.4.1.). However, plasma choline concentration of healthy subjects is determined not only by diet, but also by endogenous choline synthesis, potential release of choline from tissue phospholipids, microbial metabolism of dietary choline in the gut and degradation of choline via betaine. The result of these different influences on plasma choline concentration is unpredictable. As indicated in Section 2.4.5., the Panel concludes that the available data do not allow on the conclusion of a dose-response relationship between choline intake or choline status and plasma choline concentration. The Panel also concludes that plasma concentrations of choline, PC, betaine, DMG, tHcy or TMAO, erythrocyte PC concentration, or urinary betaine and TTMA excretion cannot be used to set DRVs for dietary choline (Section 2.4.5.).

5.1.1. Adults

Zeisel and co-workers performed 11 choline depletion/repletion studies in different groups of both women and men that all followed a similar design. For this reason, the characteristics of these studies are summarised below, while detailed information is available in Appendix D.

5.1.1.1. Study goals

The goals differed between the studies. The first study evaluated the changes in choline status and liver function of healthy humans fed a choline-deficient diet (Zeisel et al., 1991). Another study assessed whether choline deficiency decreases the capacity to methylate homocysteine (da Costa et al., 2005) (Section 2.2.2.1.). One study investigated the influence of genetic variants of folate metabolism on susceptibility to choline deficiency symptoms (Kohlmeier et al., 2005). Another assessed whether SNPs in genes coding for enzymes involved in choline metabolism influence the dietary requirement for choline and whether choline deficiency is associated with apoptosis and DNA damage (da Costa et al., 2006a). One study investigated the influence of sex and menopausal status on dietary requirement of choline (Fischer et al., 2007). Another investigated the influence of genetic polymorphisms in *PEMT*, *MTHFD1*, *CHDH* on susceptibility for organ dysfunction in choline deficiency (Niculescu et al., 2007). One study estimated whether the risk for choline deficiency induced organ dysfunction in premenopausal women is dependent on the number of variant *PEMT* rs12325817 alleles in premenopausal women and whether oestrogen can decrease the risk in postmenopausal women (Fischer et al., 2010a). One study assessed whether metabolomic profiling of plasma can predict organ dysfunction in choline deficiency (Sha et al., 2010). One study investigated how diet and choline deficiency influence the human gastrointestinal tract microbiome and the development of liver steatosis (Spencer et al., 2011). One study assessed whether plasma PC-DHA concentration is a non-invasive marker for liver PEMT activity (da Costa et al., 2011). Finally, one study identified effect alleles in a number of SNPs of genes known to be of influence on the dietary requirement of choline (da Costa et al., 2014). Characteristics and outcomes of these 11 studies are compiled in Appendix D.

5.1.1.2. Study design

The design was similar in all studies (Appendix D), and was the following: a 7–10 day baseline diet, followed by a 42-day choline depletion diet, and then a choline-repletion diet (3–40 days). During the ten-day baseline diet, the subjects received normal foods providing 550 mg choline and 50 mg betaine/70 kg body weight per day. During the choline-depletion diet, the subjects received foods providing < 50 mg choline and 6 mg betaine/70 kg body weight per day for up to 42 days (with or without a folic acid supplement (100 or 400 µg/day according to study objective)), or until they were deemed choline-deficient and/or developed signs of organ dysfunction. In some studies, the participants were randomised into a depletion group and a control group that continued on the baseline diet. More details on the design per study are provided in Appendix D.

Muscle and liver dysfunction associated with choline deficiency was defined by the authors as a five-fold or greater increase in serum creatine phosphokinase (CK) activity, a 1.5-fold or greater increase in AST, ALT, γ-glutamyltransferase (GGT), or lactate dehydrogenase (LDH), and/or a 28% or greater increase in liver fat content measured by computerised tomography (CT) or magnetic resonance imaging (MRI) compared to baseline and, depending on the study, estimated on day 21 and 42 of depletion. The same parameters were measured to assess reversion of the damage.

Those who completed the 42-day depletion phase without the development of hepatic steatosis were put on a diet providing 550 mg choline/70 kg body weight per day for three days and then discharged. Choline deficient subjects were put on a diet with stepwise increases in choline intake, in sequential 10-day periods of 137.5, 275, 412.5, or 550 mg choline/70 kg body weight per day. Those who showed signs of organ damage with increases of CK activity > 10 000 U/L were immediately switched to the choline-repletion diet or directly to 850 mg choline/70 kg body weight per day or to an ad libitum diet. Status was monitored regularly using blood and urine samples (at screening, day 1, at the end of each dietary phase, and every three to four days during the intervention).

5.1.1.3. Number of subjects and choline intake

Per study, the number of subjects ranged from 8 to 72 and some of the subjects participated in several studies (Appendix D). The total number of subjects (in all studies) investigated is not quite clear, because subjects recruited in 2001 and 2007 (approximately 150–160) were investigated in different studies. The susceptibility for organ dysfunction by choline depletion was significantly influenced by polymorphisms of the *MTHFD1* (e.g. rs2236225), *CHDH*, *CHK*, *CLCA441* and *PEMT* (e.g. rs12325817) genes (Section 2.5.), by being male or postmenopausal and not receiving oestrogen therapy. Folic acid supplementation (400 µg/day) did not prevent the development of organ dysfunction during choline depletion (Kohlmeier et al., 2005).

In the depletion/repletion study that investigated the influence of sex and menopausal status on choline requirement (Fischer et al., 2007) in 57 healthy adult subjects (26 males, 16 premenopausal and 15 postmenopausal women), aged 18–70 years, 20 of 26 (77%) men developed choline deficiency signs, six already in the baseline phase with 550 mg choline/70 kg body weight per day. In this study, 12 of 15 (80%) postmenopausal women and 7 of 16 (44%) premenopausal women developed choline deficiency signs on the low-choline diet. In total n = 39 of 57 male or female subjects developed signs of choline deficiency, or 68%. In the same study, the authors also looked for differences in clinical chemistry data between subjects who developed choline deficiency and subjects who did not (apart from the parameters used to define choline deficiency-related organ dysfunction). Between sexes and life-stage groups, there were no significant differences in plasma concentrations of free choline, betaine, DMG, tHcy, which all decreased upon depletion, and of SAM and SAH, which did not change. Plasma PC concentrations, however, decreased only in subjects who developed organ dysfunction.

The amount of choline needed to replete subjects with signs of organ dysfunction differed between subjects (Fischer et al., 2007) as shown in Table 2. In all the other studies mentioned in Appendix D, this was not reported. Disregarding missing data as well as sex differences because the numbers are too small, 10 of 39 choline deficient subjects were repleted with 137.5 mg/70 kg body weight per day, three with 275, five with 412.5, and 13 needed 550 or more than 550 mg/70 kg body weight per day (or an *ad libitum* diet) including the six men with signs of choline deficiency already on the baseline diet with 550 mg choline/70 kg of body weight, while the data from eight subjects were completely missing.

Table 2: Amount of choline needed to replete subjects after experimental choline depletion (Fischer et al., 2007).

Study subjects		No signs of choline deficiency with low-choline diet	Signs of choline deficiency, with choline intake (mg/70 bw × d^{-1}) of		Choline needed for repletion, total mg/70 kg bw × d^{-1}				Missing data for repletion
	n	n	550 mg, n	50 mg, n	137.5, n	275, n	412.5, n	≥ 550, n	n
Men	26	6	6*	14	6	2	3	7*	2
Premenopausal women	16	9	-	7	1	-	-	2	4
Postmenopausal women	15	3	-	12	3	1	2	4	2
Total	57	18	6*	33	10	3	5	13	8

*Six men showed already signs of choline deficiency with 550 mg choline/70 kg body weight (bw) per day and consequently needed more than that amount for repletion.

Out of 25 subjects[16] who showed signs of choline deficiency after experimental choline depletion and for whom the amount of choline needed to replete them was available, the Panel notes that 18 i.e. about 70%, needed up to about 400 mg choline/70 kg body weight per day for repletion. The Panel also notes that this percentage decreased to 58% when the six men with signs of choline deficiency already during the baseline period with 550 mg choline/day (and therefore presumably with a higher choline requirement) were taken into account (Fischer et al., 2007). The Panel did not consider the 18 individuals who did not show signs of choline deficiency with 50 mg/70 kg body weight per day. It is not known if they would have developed signs of choline deficiency with a longer period of choline depletion (> six weeks). The Panel notes that data are missing for the precise amount of choline needed for repletion in eight subjects.

The Panel notes that the subjects of this trial (Fischer et al., 2007) were classified according to polymorphisms in genes coding for PEMT, CHDH, BHMT (da Costa et al., 2006b) and for MTFHR, MTFHD1 and the reduced folate carrier 1 (RFC1) (Kohlmeier et al., 2005) (Appendices C and D). The susceptibility to develop organ dysfunction on the low-choline diet was significantly increased ($p = 0.002$, odds ratio (OR): 25; 95% CI: 2–256) (18 of 23 carriers of the C allele) in women carriers of the *PEMT* promoter SNP rs12325817 (-744 G→C), and specifically in postmenopausal women ($p = 0.03$, OR: 42; 95% CI: 1–1 348). In contrast, being a carrier of the *CHDH* gene SNP rs9001, +318 A→C) had a protective effect on the susceptibility to develop organ dysfunction ($p = 0.03$, OR: 0.2; 95% CI: 0.05–0.7), whilst the *CHDH* SNP rs12676 (+432 G→T) did not, except in premenopausal women. The SNPs *PEMT* rs7946 (+5465 G→A) and *BHMT* rs3733890 (+742 G→A) were not associated with susceptibility to organ dysfunction on a low-choline diet. Only the *MTHFD1* SNP (1958G→A) rs2236225 carriership increased the susceptibility to develop signs of choline deficiency when the choline intake was very low, and that only in premenopausal women (OR: 85, 95% CI: 3–2 418), and this susceptibility was attenuated by folate supplementation.

There are indications that choline deficiency during depletion repletion studies (da Costa et al., 2006b; Niculescu et al., 2007) (Appendix D) may increase cell apoptosis and induce DNA damage (assessed *ex vivo/in vitro*), for which the carriers of certain polymorphisms of *PEMT* and *MTHFD1* were more susceptible (Section 2.5). The Panel considers that the significance of these studies is unclear.

There are also indications (Appendix D) that metabolomic profiling of the plasma of subjects on baseline diet can predict susceptibility to develop organ dysfunction when deprived of dietary choline

[16] i.e. 10+3+5+13-6, indicated in Table 2.

(Sha et al., 2010) and that host factors and the gut microbiota (Spencer et al., 2011) both respond to dietary choline intake and choline deficiency (Section 2.2.2.1).

5.1.1.4. Summary

Eleven available depletion/repletion studies in adults have demonstrated that dietary choline can become insufficient, e.g. within six weeks of a depletion phase with ≤ 50 mg choline/70 kg body weight per day (Appendix D). Only one of these studies reported the amount of choline needed to replete subjects with signs of organ dysfunction (Fischer et al., 2007).

The Panel notes that experimental dietary depletion of choline led, in most (70–80%) of the male and postmenopausal female subjects, to signs of organ dysfunction involving liver and muscle, but only in 44% of premenopausal women (Fischer et al., 2007). These signs can be mild with biochemical alterations only or can be severe with liver steatosis and muscle function impairment developing rapidly. The susceptibility to develop organ dysfunction differs between subjects and is influenced by genetics, sex, possibly the intestinal microbiome, and hormonal status (Section 2).

In addition, it is not known if the 18 subjects who have not developed signs of organ dysfunction within six weeks would have done so in the long term, when their endogenous choline (PC) synthesis would become insufficient (Fischer et al., 2007). It is not known, but can be assumed, that the factors that have an impact on the development of organ dysfunction also determine the amount of choline needed to replete the body and reverse the signs of organ dysfunction and the requirement for dietary choline.

According to the study by Fischer et al. (2007) described above, this requirement for dietary choline in adults lies between about 130 and 500 mg choline/day, with most subjects needing more than 130 mg/day and some needing 500 mg/day or more (Table 2). From the 39 subjects who became deficient either with 550 or with 50 mg choline/70 kg body weight per day, the data from 14[17] are missing. From the remaining 25, ten needed 137.5, three 275, five 412.5 and seven 550 mg choline/70 kg body weight per day or more. An intake of 412.5 mg choline/70 kg body weight per day (i.e. 5.9 mg/kg body weight per day) was sufficient to replete 18 of 25 deficient subjects, that is about 70% or two thirds.

The Panel considers that reliable markers of intake and status are not available (Section 2.4) and that the study by Fischer et al. (2007) is too small and insufficient to draw firm conclusions on the Average Requirement (AR) for dietary choline in adults. However, as supportive evidence, it may contribute to inform an Adequate Intake (AI) that covers most of the population.

5.1.2. Infants and children

The Panel is unaware of any data in infants aged 7–11 months and children on indicators of choline requirement.

5.1.3. Pregnancy and lactation

The Panel considered whether the calculation of choline transfer from the mother to the fetus and of choline accretion in the fetus and placenta during pregnancy could be used to calculate the additional need for dietary choline during pregnancy. However, a review of the available evidence (Sections 2.3.3. and 2.3.4.) showed that this was not feasible due to a lack of data.

The Panel then considered the available intervention studies on choline supplementation in pregnant women in the second half of pregnancy. Although none of the biomarkers in plasma, urine or erythrocyte previously reviewed by the Panel are suitable biomarkers to set DRVs for choline (Section 2.4.5.), the Panel considers that they may be useful to assess potential changes in choline metabolism in intervention studies in pregnant women.

[17] 8+6 (Table 2).

5.1.3.1. Effect of total choline intake in pregnant (versus non pregnant) women and the offspring

As described already in Sections 2.3.6.1. and 2.4.1.2, Yan et al. (2012) reported on plasma and urine choline concentrations in 26 healthy pregnant women (third trimester) and 21 non-pregnant controls who were randomly assigned to consume either 480 or 930 mg of choline/day from food[18] and supplements for 12 weeks (or until delivery). Pregnant women had higher free choline concentration in plasma and urinary excretion of choline and betaine than non-pregnant women throughout the study (Sections 2.3.6.1. and 2.4.1.2.). Also, pregnant women consuming 930 mg of choline/day had higher plasma concentrations of free choline than pregnant women consuming 480 mg of choline/day. The lower circulating concentrations of choline-derived methyl-group donors (betaine, DMG and sarcosine) observed in pregnant women compared with non-pregnant women were suggestive of a greater use of these molecules in both maternal and fetal compartments (Section 2.4.1.2.).

This study also provided additional results. Plasma concentrations of the three methyl-group donors (betaine, DMG and sarcosine) over the duration of the study were higher in pregnant women consuming 930 mg choline/day compared with pregnant women consuming 480 mg of choline/day ($p < 0.016$, $p < 0.012$, and $p < 0.07$, respectively), but without achieving the concentrations measured in non-pregnant women consuming 480 mg choline per day. Urinary excretion of choline, betaine or DMG in pregnant women was not different between the choline intake groups. However, urinary excretion of sarcosine, methionine and Hcy were higher (46% higher, $p = 0.029$; 37% higher, $p = 0.02$; 45% higher, $p = 0.06$, respectively) in the pregnant women consuming 930 mg/day, compared with 480 mg/day. The results described above in plasma and urine suggest that the higher choline intake (930 mg/day) was predominantly used by the pregnant women, and not excreted. However, in pregnant women, mean concentration of free choline in the placenta (915 ± 231 vs 941 ± 309 nmol/g tissue) or in cord plasma (37.3 ± 13 vs 32.5 ± 7.5 µmol/L), and anthropometric parameters or Apgar scores of the newborns did not differ between the lower and the higher choline intake groups.

5.1.3.2. Effect of total choline intake in pregnant (versus non pregnant) women on the dynamics of choline-related metabolic pathways

As indicated previously, the PC formed in the PEMT pathway contains substantial amounts of LC-PUFAs, like DHA and ARA, whilst the PC formed in the CDP-choline pathway does not (Section 2.3.5.).

Yan et al. (2013) investigated the effect of pregnancy on the dynamics of choline-related metabolic pathways (Figure 2, Section 2.3.5.) in the same study cohort of pregnant (third trimester) and non-pregnant women investigated by Yan et al. (2012) who had received, after six weeks, 100 mg (of the 480 mg/day choline) and 200 mg (of the 930 mg/day choline) as deuterated choline (methyl-D_9 choline). In pregnant women (compared with non-pregnant women), the total plasma PC pool was about 50% greater (Yan et al., 2013).

With regard to the CDP-pathway, the analysis of the different isotopomers of deuterated choline, betaine and PC in plasma showed that, in pregnant women (compared with non-pregnant women), dietary choline was used more for PC production via the CDP-choline pathway than oxidised to betaine. The higher choline intake (930 mg choline/day) in pregnant women restored the distribution of dietary choline between PC synthesis via the CDP-choline pathway versus oxidation to betaine, to the levels observed in non-pregnant women consuming 480 mg choline/day. With regard to PEMT pathway, the analysis of the different isotopomers also showed that, in pregnant women (compared with non-pregnant women), PC produced via PEMT is more catabolised to free choline (and this may contribute to explain the rise in plasma choline in pregnancy), which is preferentially transferred to the fetus. The higher choline intake (930 mg choline/day) enhanced the PEMT-mediated PC synthesis relative to the CDP-choline pathway, compared to pregnant women consuming 480 mg choline/day.

[18] Diet provided an average of 380 mg/day of choline, and supplemental choline was 100 or 550 mg/day. In addition to the strictly controlled diet, all subjects received 600 µg folic acid, 2.6 µg cobalamin, 1.9 mg vitamin B6 and 200 mg DHA per day.

West et al. (2013) investigated the effect of different choline intakes on choline-related lipid metabolism in a separate analysis of the same study cohort of pregnant (third trimester) and non-pregnant women investigated by Yan et al. (2012). At baseline, pregnant women had a greater proportion of PC-DHA (% of total fatty acids) in both plasma ($p = 0.01$) and erythrocytes ($p = 0.001$) than non-pregnant women. The higher choline intake (930 mg/day) did not affect the proportion of PC-DHA in erythrocytes in pregnant women compared with an intake of 480 mg/day (whereas this was the case in non-pregnant women, as described in Section 2.3.5.1.). However, the higher choline intake (930 mg/day) lowered the proportion of PC-ARA in erythrocytes in pregnant women ($p = 0.02$), compared with an intake of 480 mg/day. The PC:PE ratio (Section 2.3.5.1.) in plasma and erythrocytes was not influenced by choline intake in pregnant or non-pregnant women.

5.1.3.3. *Ex-vivo* studies in placental samples

From 24 subjects from the study by Yan et al. (2012) (twelve each from the two choline groups), placental tissue, cord blood leukocytes and maternal fasting venous blood at delivery were investigated *ex vivo* by Jiang et al. (2012) and Jiang et al. (2013). In the group that consumed 930 mg/day choline compared with the group that consumed 480 mg/day choline, the authors found that: (i) placental global DNA methylation, histone methylation and the expression of a histone methyltransferase were higher; (ii) placental methylation of the promoters of two cortisol-regulating genes, corticotropin releasing hormone (*CRH)* and glucocorticoid receptor (*NR3C1*), was higher; (iii) placental *CRH* transcript abundance was lower (about 40%, read on figure, concentration of the protein was not reported); (iv) methylation of the *CRH* and *NR3C1* promoter in cord blood leukocytes was lower*;* (v) the maternal blood concentration of the protein antiangiogenic factor fms-like tyrosine kinase (sFLT1) at delivery was lower (by about 30%, estimated from the figure); (vi) placental *sFLT1* mRNA abundance was lower (by about 30%, estimated from the figure, concentration of the protein was not reported).

5.1.3.4. Effect of choline total intake on maternal plasma and breast milk during lactation

The RCT by Fischer et al. (2010b) (Sections 2.3.3., 2.3.6.3, 2.4.1.2., and 5.1.1.1.) demonstrated that total choline intake (from foods and supplements) is positively associated with the concentration of free choline and choline-compounds in plasma of these lactating women (Section 2.4.1.2.) and in breast milk (Section 2.3.6.3.). This study also showed that supplemental choline (750 mg/day choline, in addition to a mean dietary choline intake of about 350 mg/day) compared with placebo increased the mean concentration of free choline in plasma (Section 2.4.1.2.) and in breast milk (Section 2.3.6.3.).

In the previously described controlled feeding study by Davenport et al. (2015) (Sections 2.3.3., 2.3.6. and 2.4.1.2.), lactating and control non-lactating women (from the study by Yan et al. (2012)) were randomised to consume 480 mg choline/day or 930 mg choline/day from food and supplements[19] for 10–12 weeks, and they all received, during the last four to six weeks, 20% of the total choline intake as deuterium labelled choline. Lactating (versus control) women showed a statistically lower expression of three of the five genes investigated that code for enzymes/receptor involved in choline metabolism, in leukocytes at baseline (mRNA abundance, $p \leq 0.05$). They also showed a higher plasma free choline concentration (Section 2.4.1.2.) and lower urinary excretion of choline metabolites (Section 2.3.6.1.2.) throughout the study period. Lactating (versus control) women tended to have a decreased oxidation of choline to betaine (Figure 2, Section 2.3.5.), which would allow an increase in the supply of intact choline to the mammary epithelium. The higher choline intake during lactation (930 mg/day, compared to 480 mg/day) significantly increased the concentration of total choline in breast milk and increased the supply of PEMT-derived choline metabolites in breast milk (Section 2.3.6.3.), as well as in blood.

5.1.3.5. Conclusion on pregnancy and lactation

In pregnant women (compared to non-pregnant women) (Yan et al., 2012; West et al., 2013; Yan et al., 2013), the available studies:

[19] Diet provided an average of 380 mg/day of choline, and supplemental choline was 100 or 550 mg/day.

- show increased urinary losses of choline and betaine;
- suggest a greater use of choline-derived methyl-group donors (DMG, betaine and sarcosine) in both maternal and fetal compartments;
- suggest an enhanced PEMT activity to facilitate the transfer of LC-PUFA to the fetus via PC in lipoproteins.

These studies on choline supplementation also suggest that a choline intake of 930 mg/day (from food and supplements) in pregnant women (from the 27th week of gestation):

- increases (compared to 480 mg/day) maternal plasma choline concentration;
- increases maternal plasma concentrations of the three methyl-group donors (DMG, betaine and sarcosine) compared with pregnant women consuming 480 mg/day, but without achieving the concentrations measured in non-pregnant women consuming 480 mg/day;
- restored the distribution of dietary choline between PC synthesis via the CDP-choline pathway versus oxidation to betaine, to the levels observed in non-pregnant women consuming 480 mg choline/day;
- enhanced (compared to 480 mg/day) the PEMT-mediated PC synthesis versus the CDP-choline pathway-mediated PC synthesis;
- had no impact (compared to 480 mg/day) on maternal urinary excretion of choline and betaine, placental choline concentration, cord plasma choline concentration.

These results may indicate a higher choline requirement in pregnancy than in non-pregnant women, which would have to be supplied by additional dietary choline.

In lactating women, the available studies on choline supplementation on women either supplemented from the 18th gestational week to 45 days post partum (Fischer et al., 2010b) or recruited at five weeks post partum (Davenport et al., 2015), suggest that increased maternal choline intake enhances the concentration of total choline in breast milk and increased the supply of PEMT-derived choline metabolites in breast milk. Since PEMT generates PC molecules enriched in DHA, the supply of DHA from the lactating women to the infant might be facilitated. However, the fatty acid composition of breast milk was not measured in these studies.

The Panel notes that no maternal clinical signs of choline deficiency (as described in Sections 2.2.2.1. and 5.1.1.4.) or no adverse outcomes in the offspring were reported in these studies with a total choline intake from foods and supplements of 480 mg choline/day in pregnant women, or of about 350-480 mg choline/day in lactating women.

The Panel notes that these studies used high choline intakes (930 vs 480 mg/day from foods and supplements in pregnant and lactating women; about 1 100 mg/day from food and supplements vs about 350 mg/day from foods in lactating women). The Panel also notes that the interpretation of the biochemical outcomes investigated is difficult with the aim of defining choline insufficiency/adequacy in pregnancy.

The Panel notes that the *ex-vivo* studies suggest that different maternal choline intakes during pregnancy may induce epigenetic modifications of genes, and changes in genes involved in hormonal and vascular physiology. However, such changes are difficult to interpret and further research is required.

The Panel concludes that calculation of the additional need for dietary choline during pregnancy based on a calculation of choline transfer from the mother to the fetus and choline accretion in the fetus and placenta during the duration of pregnancy is not feasible due to a lack of data (Sections 2.3.3. and 2.3.4.). The Panel concludes that, taken together, the studies on choline supplementation provide evidence that pregnant or lactating women may need more choline than non-pregnant non-lactating women. However, the data are not sufficient to allow an estimate of the additional requirement for

dietary choline in pregnant or lactating women (above that of non-pregnant non-lactating women). The Panel considers, however, that the additional intake of choline required to compensate for the amount of total choline secreted in breast milk during the first six months of exclusive breastfeeding (Section 2.3.6.3.) can be calculated.

5.2. Choline intake and health consequences

Since the report by SCF (1993), more data have become available on the relationship between choline intake and NAFLD, CVD, different types of cancer, neural tube defects (NTD), and cognition. A comprehensive search of the published literature, without time limit, was performed in August 2012 as preparatory work to this Opinion in order to identify relevant health outcomes possibly associated with choline intake through diet or supplementation, and which may inform the setting of DRVs for choline (El-Sohemy et al., 2012). The main results of the preparatory work, together with new evidence from studies subsequently published (in Pubmed) until November 2015 are summarised below.

Of the available RCTs investigating the health effects of choline, the results only of one RCT was considered in this section, which reported dietary choline intake in addition to choline supplements. The relationship between choline intake and chronic disease outcomes has been investigated mainly in observational (prospective cohort, case-control) studies, where a positive, an inverse, or a lack of an association between choline intake and disease outcomes might be confounded by uncertainties inherent to the methodology used for the assessment of choline intakes, and by the effect of other dietary, lifestyle, or undefined factors on the disease outcomes investigated. Taking into account the uncertainty about the relationship between choline intake and biomarkers (Section 2.4), the Panel only considered observational studies that include an assessment of choline intake, whereas studies on the relationship of plasma choline concentrations (or those of choline compounds) and health outcomes with no quantitative data on choline intake (Wang et al., 2011) are not described below. In observational studies, habitual dietary choline intake was generally estimated using a FFQ (filled-in either once at baseline or at several time points, in prospective cohort studies) and composition data from the USDA database (Section 3) and/or from the literature (Zeisel et al., 2003). For some observational studies, choline intake from supplements was also assessed.

5.2.1. Non-alcoholic fatty liver disease

Dietary deficiency of choline can cause fatty liver (hepatic steatosis), which can result in NAFLD (Section 2.2.2.1.), which can be of different aetiologies and is the most common chronic liver disease in developed countries. It is often associated with insulin resistance and dyslipidaemia, is a risk factor for CVD and may progress to irreversible liver damage and liver cancer (Corbin et al., 2013; Lazo et al., 2013; Byrne and Targher, 2014).

In two population-based prospective cohorts, Yu et al. (2014) investigated the association between habitual dietary choline intake and risk of NAFLD in 56 195 women (recruited in 1997–2000 and followed-up through 2004–2007) and men (recruited in 2002–2006 and followed-up through 2008-2011), aged 40–75 years and free of hepatitis at baseline. NAFLD was diagnosed by sonography (self-report). Mean daily choline intake was 412 mg (women) and 452 mg (men) in the highest quintile, and 179 mg (women) and 199 mg (men) in the lowest quintile. After adjustment for potential confounders[20], women and men in the highest quintile had a significantly lower risk of NAFLD than those in the lowest quintile, but not after further adjustments. In stratified analysis, the highest quintile of choline intake remained inversely associated with risk of NAFLD compared with the lowest quintile (OR: 0.72; 95% CI: 0.57–0.91, p trend: 0.007) only in women with a BMI < 25 kg/m^2 (but not in women with a BMI ≥ 25 kg/m^2).

[20] Including age, total energy intake, education, income, physical activity, smoking, alcohol consumption, intake of protein, saturated fat, polyunsaturated fat. Further adjustments for menopause, hypertension, diabetes mellitus, gallstones, dyslipidemia, BMI.

The Panel notes that, in one prospective cohort study, a lower choline intake was associated with a higher risk of developing NAFLD in normal-weight women in adjusted stratified analysis. The Panel concludes that the data on choline intake and risk of NAFLD are limited and cannot be used to derive DRVs for choline.

5.2.2. Cardiovascular disease

A prospective cohort study, with an average follow-up of 8.1 years, investigated the association between habitual dietary intake of choline and risk of CVD, in 16 165 postmenopausal women aged 49–70 years and without prior CVD at baseline (Dalmeijer et al., 2008). After adjustment for potential confounders, comparing the highest quartile of choline intake (> 329 mg/day) with the lowest (< 266 mg/day) did not show a significant relationship between choline intake and risk of total CVD, coronary heart disease (CHD) or cerebrovascular accidents (CVA).

A prospective cohort study, with an average follow-up of 14 years, investigated the association between habitual dietary intake of choline and risk of CHD, in 14 430 men and women without prior CHD at baseline (mean age at baseline: about 54 years) (Bidulescu et al., 2007). After adjustment for potential confounders, comparing the highest quartile of choline intake (> 363 mg/day) with the lowest (< 217 mg/day) did not show a significant relationship between choline intake and risk of CHD.

The Panel notes that two large prospective observational studies on populations free of CVD at baseline did not show a significant association between choline intake and risk of CVD. The Panel concludes that the data on choline intake and risk of CVD cannot be used to derive DRVs for choline.

5.2.3. Cancer

Choline is a methyl group donor involved in the folate-dependent one-carbon metabolism (Sections 2.2.1. and 2.3.5.). Disturbances in this function that affect methylation or synthesis of DNA may contribute to carcinogenesis (Section 2.2.2.1.).

5.2.3.1. Colon/rectum

In a US prospective cohort study, Cho et al. (2007b) examined the relationship between total intake of choline (via food and supplements) and risk of colorectal adenoma, in 39 246 women free of cancer or polyps at baseline and who underwent at least one endoscopy in the 18 years of follow-up. After adjustment for potential confounders, a choline intake in the highest quintile (median: 383 mg/day) was associated with a higher risk of colorectal adenomas compared with the lowest quintile (median: 261 mg/day) (relative risk (RR):1.45; 95% CI: 1.27-1.67; p trend < 0.001).

In a US prospective cohort study, Lee et al. (2010b) investigated the relationship between total intake of choline (via food and supplements) and risk of colorectal cancers (CRCs), in 47 302 men (40-75 years at baseline) free of cancer at baseline and with 18 years of follow-up. After adjustment for potential confounders, a choline intake in the highest quintile, from either food or supplements, was not associated with a higher risk of CRC compared with the lowest quintile.

In a case-control study, Lu et al. (2015) investigated the relationship between habitual dietary intake of choline and risk of CRC, in 890 cases (aged 30–75 years) diagnosed up to three months previously, compared with 890 age- and sex-matched controls. Choline intake (median, 25^{th}, 75^{th} percentiles) was higher in controls (158, 120, 202 mg/day) than in cases (133, 100 and 176 mg/day) ($p < 0.01$). After adjustment for potential confounders, a choline intake in the highest quartile was inversely associated with risk of CRC compared with the lowest quartile (OR: 0.54; 95 % CI: 0.37–0.80; p trend < 0.01). The Panel notes that the diet in this population provided about half of the dietary choline and folate intake, and less red meat, poultry, eggs and milk than in the USA (Cho et al., 2007b).

The Panel notes the inconsistent results from observational studies on the association between choline intake and risk of colorectal cancer.

5.2.3.2. Breast cancer

In a prospective cohort study with a follow-up of 12 years, Cho et al. (2007a) examined the relationship between total intake of choline (via food and supplements) and risk of breast cancer in 90 663 premenopausal women, aged 26–46 years and free of cancer at baseline. Median intake per quintile ranged between 263 and 397 mg/day. After adjustment for potential confounders, choline intake was not associated with breast cancer risk.

In a prospective cohort study, Cho et al. (2010) investigated the relationship between habitual dietary intake of choline and risk of breast cancer in 74 584 women, who were either postmenopausal in 1984 or became postmenopausal during 20 years of follow-up (mean age of about 62 years at 10-year follow-up). Median intake per quintile ranged between 260 and 396 mg/day. After adjustment for potential confounders, choline intake was not associated with breast cancer risk.

In a population-based case-control study, Xu et al. (2009) investigated the relationship between total intake of choline (via foods and supplements) and risk of (and mortality from) breast cancer and all-cause mortality, in 1 508 cases of breast cancer (diagnosed in 1996–1997 and followed through 2005) and 1 556 controls. After adjustment for age, choline intake (sum of all forms, ranging from < 123 mg/day to > 247 mg/day) was not associated with risk of breast cancer. In addition, choline intake (sum of all forms, ranging from < 142 to > 205 mg/day) was not associated with all-cause or breast cancer mortality (while an inverse significant relationship for both types of mortality was observed comparing intake of free choline above about > 57 mg/day with that < 40 mg/day).

The Panel notes that three observational studies did not show a significant association between choline intake and risk of breast cancer. The Panel concludes that the data on choline intake and risk of breast cancer cannot be used to derive DRVs for choline.

5.2.3.3. Other cancers (oesophageal, prostate and ovarian cancers)

In two population-based case-control studies, Ibiebele et al. (2011) evaluated the association between habitual dietary intake of choline and risk of Barrett's oesophagus (BE) and oesophageal cancers. The first study compared eligible cases (n = 367), diagnosed with BE or BE with dysplasia, with 577 controls. The second study compared eligible cases (n = 881), diagnosed with oesophageal carcinoma of different types and location, with 1 507 controls. Median intake of choline in each quartile in controls ranged between 380 and 1 171 mg/day. After adjustment for potential confounders, choline intake was not associated with risk of BE or oesophageal cancers.

In a prospective cohort study with a follow-up of 22 years, Richman et al. (2012) examined the association between total intake of choline (via foods and supplements) and risk of fatal prostate cancer, in 47 896 men aged 40–75 years and free of cancer diagnosis at baseline. After adjustment for potential confounders, the highest quintile of choline intake (median 509 mg/day) was positively associated with risk of fatal prostate cancer (hazard ratio (HR): 1.70; 95% CI: 1.18–2.45, p trend = 0.005).

In two large prospective cohorts with a follow-up of up to 22 years, Kotsopoulos et al. (2010) investigated the relationship between total intake of choline (via foods and supplements) and risk of ovarian cancer, among 159 957 women, aged 25–55 years at enrollement. In both cohorts, choline cutpoints ranged between about 250–270 mg/day (lowest quintile) and 339–367 mg/day (highest quintile). After adjustment for potential confounders, choline intake was not associated with risk of ovarian cancer.

The Panel notes that choline intake was not associated with risk of oesophageal cancer in one reference on two case-control studies or with risk of ovarian cancer in two cohorts followed prospectively, while it was positively associated with risk of prostate cancer in one large prospective cohort study.

5.2.3.4. Conclusions

The Panel concludes that the available data on associations between choline intake and cancers of various sites are either inconsistent or limited and cannot be used to derive DRVs for choline.

5.2.4. Neural tube defects

In a US population-based case-control study, Shaw et al. (2004) investigated the relationship between periconceptional intake of choline and risk of NTDs, in 653 cases (liveborn, stillborn or electively terminated) identified from hospital and medical records (in 1989–1991), compared with 644 controls randomly selected from the same geographical area. Dietary choline intake of the mothers (not taking supplements with choline) in the three months before conception was estimated retrospectively. The authors analysed 424 FFQs from mothers of NTD cases (161 with anencephaly, 242 with spina bifida, 21 with other NTD phenotypes) and 440 FFQs of controls. After adjustments for potential confounders, a significantly decreased risk of all NTDs was found for quartiles 2–4 of periconceptional intake of choline compared to the lowest quartile (< 290 mg/day), e.g. for the fourth quartile (> 498 mg choline/day) OR: 0.49; 95% CI: 0.27–0.90.

In another US population-based case-control study, (Carmichael et al., 2010) investigated the relationship between periconceptional intake of choline and risk of NTDs, in 189 cases of spina bifida and 141 cases of anencephaly (liveborn, stillborn, electively terminated) identified from hospital and medical records (in 1999–2003), compared to 625 controls randomly selected from the same geographical area. Dietary choline intake of the mothers in the two months before/after conception was estimated retrospectively (8-10 months after delivery). After adjustments for potential confounders, periconceptional intake of choline (supplements excluded) below the 25th percentile (< 293 mg/day) and above the 75th percentile (> 506 mg/day) was not associated with a higher or lower risk for anencephaly and spina bifida, compared to a choline intake between the 25th and 75th percentiles.

Polymorphisms in genes for enzymes (CHKA, MTHFD1 and CCT) involved in choline metabolism may influence the risk of NTDs independently of maternal choline intake (Appendix C and Section 2.5), but that such information is not available for the studies cited above.

The Panel notes that the association between choline intake and risk of NTDs was inconsistent in the two case-control studies available, and that such association may be influenced by the intake of other nutrients and the genotype of the mother. The Panel concludes that the data on choline intake and risk of NTDs cannot be used to derive DRVs for choline.

5.2.5. Cognition

The only RCT, then the prospective observational studies (first in adults, then in children) are described below.

In a double-blind RCT, Cheatham et al. (2012) investigated the relationship between maternal PC supplementation during and after pregnancy (in women that, for most of them, had been investigated by Fischer et al. (2010b)) and several measures of cognition in the infants. From 18 weeks of gestation to 90 days post partum, 140 healthy women (Section 2.3.3., 2.3.6.3., 2.4.1.2, 2.5.1, 5.1.3) received either 750 mg/day of choline (as PC, n = 49 included in the analysis) or a placebo (n = 50 included in the analysis), in addition to a diet providing a mean of about 360 mg/day choline (assessed at 30 weeks of gestation and 45 days post partum). Infants (n = 99) were breastfed for at least 45 days, and were assessed for short-term visuospatial memory (with a Delayed Response Task), long-term episodic memory (with a deferred imitation task), language development (with the Mac-Arthur Bates Short Form Vocabulary Checklist) and global development (with the Mullen Scales of Early Learning) at ten and twelve months of age. There were no significant differences between the groups on any of the cognitive assessments at either age.

In a prospective cohort study, Poly et al. (2011) investigated the association between habitual dietary intake of choline and performance at a neuropsychological test battery or brain morphology, assessed by magnetic resonance imaging, in 1 391 men and women (aged 36–83 years) without dementia at baseline. Choline intake was estimated in 1991–1995 with the Harvard FFQ, and again in 1998–2001 when a neuropsychological test battery and a brain MRI scan were also administered. Factor analysis was used to identify four cognitive factors (verbal memory, visual memory, verbal learning and executive function) from the numerous individual neuropsychological tests. Mean choline intake was about 322 mg/day in both periods. After adjustment for potential confounders, performance on the verbal memory and visual memory factors were significantly better with higher choline intake in 1998–2001 ($p < 0.01$) but there were no significant effects for verbal learning and executive function. No significant association between choline intake (either period) and total cranium brain volume was found.

In a prospective pre-birth cohort in 2 128 pregnant women included at less than 22 weeks of gestation, Villamor et al. (2012) investigated the relationship between maternal intake of choline (via foods and supplements), assessed with an FFQ during the first and second trimesters of pregnancy, and performance on cognitive tests in their children (n = 1 210) at three years of age. The cognitive tests included the Peabody Picture Vocabulary Test III and the Wide Range Assessment of Visual Motor Abilities. Maternal intake of choline (mean ± SD) was 332 ± 63 and 325 ± 64 mg/day in the first and second trimesters, respectively. There was no association between maternal choline intake at either trimester and cognitive outcomes, after adjustment for potential confounders.

However, in this same cohort, Boeke et al. (2013) assessed 890 children with complete data at the age of seven years for visual memory (measured with the Wide Range Assessment of Memory and Learning Second Edition (WRAML2), Design and Picture Memory subtests) and both verbal and non-verbal intelligence, measured with the Kaufmann Brief Intelligence Test, Second Edition (KBIT-2)). The top quartile of second trimester maternal choline intake (median (range): 392 (364–806) mg/day) was significantly associated with a WRAML2 score 1.4 points higher (95% CI: 0.5–2.4, p trend = 0.003) than the bottom quartile (median (range): 260 (141–288) mg/day), after adjustment for potential confounders. The association was not statistically significant for the first trimester maternal choline intake. Comparing the top quartile of second trimester maternal intake with the first quartile, the effect estimate for the child non-verbal KBIT-2 score was 3.5 (95% CI: 0.1–6.9; p trend = 0.06).

The Panel notes that one RCT found no difference in four cognitive parameters investigated in infants, at ten and twelve months of age, whose mothers had consumed 750 mg/day choline or placebo in addition to their choline intake from the diet during the third trimester. The Panel also notes that available data on the relationship between choline intake and cognition in adults are limited. The Panel also notes the discrepancy in the results of a prospective cohort study, investigating the relationship between maternal choline intake during the first and second trimesters of pregnancy and cognitive outcomes in the children, when these children were aged three or seven years. The Panel considers that this might suggest that, to investigate the effects of prenatal choline supply on visual memory of the children, long-term observations are needed, and that the available evidence is insufficient to demonstrate a causal relationship. The Panel concludes that the data on choline intake and cognition cannot be used to derive DRVs for choline.

5.2.6. Conclusion on choline intake and health consequences

In studies pointing to an association of higher choline intake with a reduced risk for a certain outcome (i.e. risk of liver steatosis or of NTDs, one study each), the beneficial effect was associated with choline intakes between about 400 and 500 mg/day. However, one adverse health outcome (higher risk of prostate cancer in one study) was associated with similar choline intakes (Section 5.2.). The Panel concludes that the data on choline intake and health outcomes are either limited or inconsistent or do not show a significant association, and, therefore, cannot be used to derive DRVs for choline. There is a lack of data on choline intake in infants in the second half year of life and children and on

associations between choline intake and health outcomes in children that could be used to set requirement for choline in these age groups.

6. Data on which to base dietary reference values

6.1. Adults

Mean observed intakes of healthy adults of all ages in Europe ranged from about 270 to 470 mg choline/day (Section 3.2.1.), and the mid-point of this range is around 370 mg/day.

The Panel notes that choline depletion/repletion studies (Section 5.1.1.) indicate large variability in dietary choline requirement. The Panel also notes that the variability in choline requirement due to differences in sex, polymorphisms of genes coding for enzymes involved in choline and folate metabolism, nutritional and hormonal status, and likely the composition of the gut microbiome, pose a difficulty for dose-finding studies in a sufficiently large sample of the population (Section 2). The Panel concludes that choline depletion/repletion studies do not provide sufficiently precise data to calculate Average Requirements (ARs) and Population Reference Intakes (PRIs) for dietary choline.

The Panel also notes that there is only one depletion/repletion study that reports the choline amounts that were needed/sufficient to reverse the signs of choline deficiency in a small number of subjects (Fischer et al., 2007). In this study, out of 25 subjects who showed signs of choline deficiency after experimental choline depletion and for whom the amount of choline needed to replete them was available, about two thirds (or about 70%) of subjects needed up to about 400 mg choline/70 kg body weight per day for repletion (Table 2, Section 5.1.2.).

Finally, the Panel chose to set an AI for choline for adults based on data on observed mean intakes in healthy populations, investigated in 12 national surveys undertaken in nine countries in the EU between 2000 and 2011 (Section 3.2.1.), and in consideration of the amount of choline needed to replete about two thirds (or about 70%) of choline-depleted subjects who showed signs of organ dysfunction and for whom data on the amount of choline needed for repletion were available. The Panel is aware of the inherent uncertainty of the chosen value. However, assuming that the choline requirement of the 18 subjects of this study who did not show signs of choline deficiency after a restriction of the choline intake to 50 mg/70 kg body weight per day for six weeks, will also be covered by an intake of 400 mg/day, the Panel considers this choice of 400 mg/day to be a safe and conservative approach.

Although premenopausal women may have a lower requirement for dietary choline than postmenopausal women, in connection with a potential stimulation of the PEMT pathway by oestrogen, the Panel is not aware of quantitative data with regard to the enhanced activity of the PEMT. Although ranges of estimated mean observed choline intake in healthy populations in the EU are slightly lower in women than men (Section 3.2.1.), and considering that the data from the one depletion/repletion study (Fischer et al., 2007) are insufficient to conclude on sex-specific DRVs, the Panel considered unnecessary to give sex-specific AIs for adults.

The Panel proposes an AI of 400 mg/day for all adults.

6.2. Infants

Considering that there is no evidence for an insufficient choline intake of fully breast-fed infants during the first six months of life, the amount of choline provided in human milk is considered to be adequate. Considering a choline concentration of 145 mg/L (mean of two studies on full-term infants) and assuming a mean milk transfer of 0.8 L/day during the first six months of lactation in exclusively breastfeeding women (Butte et al., 2002; FAO/WHO/UNU, 2004; EFSA NDA Panel, 2009), the estimated choline intake of a fully breast-fed infants during the first six months of life would be 116 mg/day, rounded up to 120 mg/day (Section 2.3.6.3.).

In order to estimate the AI of infants aged 7–11 months by upwards extrapolation from the calculated choline intake for exclusively breastfed infants from birth to six months, allometric scaling was applied. The Panel calculated averages of the median weights of male and female infants, aged three months (6.1 kg) and nine months (8.6 kg); the median weight-for-age data came from the WHO Growth Standards (WHO Multicentre Growth Reference Study Group, 2006).

$$AI_{\text{infants 7-11 months}} = \text{choline intake}_{\text{infants 0-6 months}} \times (\text{weight}_{\text{infants 7-11 months}} / \text{weight}_{\text{infants 0-6 months}})^{0.75}$$

This calculation yields a value of 155, which gives an AI of 160 mg/day after rounding (Table 3).

Table 3: Reference body weights and Adequate Intake (AI) of choline for infants aged 7-11 months

Age	Reference body weight (kg)	AI (mg/day)
7–11 months	8.6 [(a)]	160

(a): Average of the median weight-for-age of male or female infants, respectively, aged nine months according to the WHO Growth Standards (WHO Multicentre Growth Reference Study Group, 2006)

6.3. Children

The Panel recognises the limited number of data on age-specific choline intake in European children and uncertainty surrounding these data (Section 3.2). The Panel chose to derive AIs for all children by downward extrapolation from the AI for adults (400 mg/day) (Section 6.1.), taking into account that this AI for adults was based on data on observed intakes in the EU, and the amounts of choline needed to replete about two thirds (or about 70 %) of choline-depleted adults who had developed signs of organ dysfunction and for whom data on choline amounts needed for repletion were available. This downward extrapolation was carried out based on reference body weights using allometric scaling with age dependent growth factors, and applying the 0.75 power of body mass to correct for differences in the metabolically active body mass of subjects of different sizes. Whilst it is not known if the choline requirement is related to energy metabolism, the Panel considers that allometric scaling, which results in a higher percentage of the adult AI than when the actual body weight is used, is justified to cover the need for choline in the development of organs and their composition.

No data are available that would justify different AIs for boys and girls.

The AIs were calculated by using the following equation

$$AI_{\text{child}} = AI_{\text{adults}} \times (\text{weight}_{\text{child}}/\text{weight}_{\text{adults}})^{0.75} \times (1 + \text{growth factor})$$

For the calculations (Table 4), median body weights of boys and girls (van Buuren et al., 2012) and median body weights of 18- to 79-year-old men and women were used, based on measured body heights of 16 500 men and 19 969 women in 13 EU Member States and assuming a body mass index of 22 kg/m^2 (see Appendix 11 in (EFSA NDA Panel, 2013b)). The following growth factors have been applied: 0.25 for boys and girls aged 1–3 years, 0.06 for boys and girls aged 4–6 years, 0.13 for boys and girls aged 7–10 years, 0.11 for boys and 0.08 for girls aged 11–14 years and 0.08 for boys and 0.03 for girls aged 15–17 years. Growth factors were calculated as the proportional increase in protein requirement for growth relative to the maintenance requirement at the different ages (EFSA NDA Panel, 2012). The value for each age group corresponds to the mean of values for the years included (EFSA NDA Panel, 2014b). Calculated AIs were rounded to the nearest 10. Although the calculations yielded an AI for children aged 15–17 years that was higher (i.e. 410 mg/day) than the value set for adults (i.e. 400 mg/day), the Panel considered that there was no reason for such a difference, thus decided to set the same AI for children aged 15–17 years and adults.

The AIs for children are supported by total choline intake mean estimates in the EU (Section 3.2.1.), i.e. estimates ranging from 151 to 210 mg/day (mid-point: 180 mg/day) in children aged 1–<3 years,

from 177 to 304 mg/day (mid-point: 240 mg/day) in children aged 3-< 10 years, from 244 to 373 mg/day (mid-point: 308 mg/day) among children aged 10–< 18 years.

The Panel is aware that the AI for children aged 1–3 years (140 mg/day) is lower than the AI for infants aged 7–11 months (160 mg/day, Section 6.2.). This difference is due to the approaches used for calculation (upward extrapolation from the high choline intake of breastfed infants from birth to six months, for infants aged 7–11 months, versus downward extrapolation from the AI for adults, for children aged 1–17 years). The Panel considers this higher AI for infants aged 7–11 months compared with children aged 1-3 years to be justified by a high demand for choline for phospholipid synthesis by the developing brain of infants (Section 2.3.4).

Table 4: Reference body weights and Adequate Intake (AI) of choline for children aged 1–17 years

Age (years)	Reference body weights (kg)		Growth factors		Calculated AIs (mg/day)		Calculated average AI (mg/day)	Proposed AIs (mg/day)
	Boys	Girls	Boys	Girls	Boys	Girls		
1–3	12.2 [(a)]	11.5 [(a)]	0.25	0.25	137.68	147.61	142.65	140
4–6	19.2 [(b)]	18.7 [(b)]	0.06	0.06	164.05	180.25	172.15	170
7–10	29.0 [(c)]	28.4 [(c)]	0.13	0.13	238.27	262.88	250.58	250
11–14	44.0 [(d)]	45.1 [(d)]	0.11	0.08	319.97	355.42	337.70	340
15–17	64.1 [(e)]	56.4 [(e)]	0.08	0.03	412.83	400.86	406.84	400[(f)]

(a): Average of the median weight-for-age of male or female children aged 24 months according to the WHO Growth Standards (WHO Multicentre Growth Reference Study Group, 2006).
(b): Average of the median weight of male or female children aged 5 years (van Buuren et al., 2012).
(c): Average of the median weight of male or female children aged 8.5 years (van Buuren et al., 2012).
(d): Average of the median weight of male or female children aged 12.5 years (van Buuren et al., 2012).
(e): Average of the median weight of male or female children aged 16 years (van Buuren et al., 2012).
(f): The Panel decided to set the same AI for children aged 15-17 years and for adults.
Adult body weight used for calculations: 68.1 kg for men and 58.5 kg for women (Median body weight of 18 to 79-year-old men and women, respectively, based on measured body heights of 16 500 men and 19 969 women in 13 EU Member States and assuming a BMI of 22 kg/m^2, see Appendix 11 in EFSA NDA Panel (2013b)).

6.4. Pregnancy

The Panel concludes that calculation of choline transfer from the mother to the fetus and choline accretion in the fetus and placenta during the duration of pregnancy is not feasible to set DRVs for dietary choline during pregnancy due to a lack of data (Sections 2.3.3., 2.3.4. and 5.1.3.5.). Although the available intervention studies on choline supplementation in the second half of pregnancy indicate that pregnant women may need more choline than non-pregnant women (Section 5.1.3.5.), the data are not sufficient to allow an estimate of the additional requirement for dietary choline in pregnant women (above that of non-pregnant women).

Therefore, the Panel proposes to calculate the additional choline intake needed by pregnant woman, by isometric scaling from the AI of non-pregnant women (400 mg/day, Section 6.1.), using the reference body weight for non-pregnant women, and the mean gestational increase in body weight. The reference body weight of 18 to 79 year-old women (58.5 kg) was previously calculated from the measured body heights of 19 969 women in 13 EU Member States and assuming a BMI of 22 kg/m^2 (see Appendix 11 in (EFSA NDA Panel, 2013b). A mean gestational increase in body weight of 12 kg, for women with a singleton pregnancy and a pre-pregnancy BMI in the range between 18.5 and 24.9 kg/m², was also previously considered (EFSA NDA Panel, 2013b). Thus, the calculation was based on the equation below:

$$AI_{pregnant} = AI_{non\text{-}pregnant} \times (70.5\ kg / 58.5\ kg) = 480\ mg/day.$$

The Panel notes that the calculation by allometric scaling (as applied in Section 6.3.) would lead to a value of 460 mg/day. The Panel however notes that the amount obtained by isometric scaling

(480 mg/day) is the same as the lower dose in one intervention study on pregnant women (recruited at 27 weeks of gestation) (Yan et al., 2012). In view of the weak evidence and the minimal differences between the two scaling approaches, the Panel chose the value of 480 mg/day.

The Panel notes that this AI is higher than the mean choline intake of pregnant women (around 350 mg/day), observed either in the Latvian survey for which individual data were available to EFSA (Section 3.2.1.) or in another publication outside the EU (Canada, Section 3.2.2.).

The Panel proposes an AI of pregnant women of 480 mg choline/day. The Panel points out that this AI applies to the whole duration of pregnancy.

6.5. Lactation

The Panel concludes that the available intervention studies in lactating women (Sections 2.3.6.3. and 5.1.3.5.) provide evidence that increased maternal choline intake enhances the concentration of choline in breast milk and that lactating women may need more choline than non-lactating women, but the data are not sufficient to allow an estimate of the additional requirement for dietary choline in lactating women (above that of non-lactating women).

For lactating women, the Panel decides to set a higher AI than for non-lactating women, by compensating for the secretion of choline in breast milk. Approximately 120 mg choline is secreted per day in human milk during the first six months of exclusive breastfeeding, considering an average concentration of total choline (free choline and choline compounds) in mature breast milk from mothers of full-term infants of 145 mg/L and a mean milk transfer during the first six months of lactation in exclusively breastfeeding women of 0.8 L/day (Section 2.3.6.3.). The Panel proposes an additional AI of 120 mg/day above the AI for non-lactating women (400 mg/day), without correcting for intestinal absorption due to lack of data (Section 2.3.1.). Thus, the Panel sets an AI of 520 mg/day for lactating women.

CONCLUSIONS

The Panel considers that none of the biomarkers of choline intake or status is suitable to derive DRVs for choline. The Panel concludes that ARs and PRIs for choline cannot be derived for adults, infants and children, and therefore defines AIs. For all adults, the Panel sets an AI based on the mid-point of the range of observed mean choline intakes in healthy populations in the EU (about 370 mg/day), and in consideration of the results of a depletion-repletion study in which about 70% of the depleted subjects who had developed signs of organ dysfunction were repleted with an intake of about 400 mg/70 kg body weight per day. For all infants aged 7–11 months, the Panel proposes an AI based on upwards extrapolation by allometric scaling from the estimated choline intake of exclusively breastfed infants from birth to six months. For all children aged 1–17 years, the Panel derives AIs by downward extrapolation from the adult AI, by allometric scaling, applying growth factors. These AIs are supported by estimated mean total choline intake in Europe. When applying allometric scaling, differences in reference body weight were taken into account. The Panel considers unnecessary to give sex-specific AIs for adults, infants or children. For pregnant women, the Panel derives an AI by extrapolation from the AI for adults using isometric scaling and the mean gestational increase in body weight. For lactating women, the amount of choline secreted per day in human milk during the first six months of exclusive breastfeeding is added to the AI for non-lactating women.

1902 **Table 5:** Summary of dietary reference values for choline

Age	Adequate Intakes (mg/day)
7–11 months	160
1–3 years	140
4–6 years	170
7–10 years	250
11–14 years	340
15–17 years	400
Adults	400
Pregnancy	480
Lactation	520

1903 ## RECOMMENDATIONS FOR RESEARCH

1904 The Panel suggests to undertake further research on:

1905 - the identification of frequency of SNPs in genes coding for enzymes involved in choline
1906 metabolism that change the requirement for dietary choline in the EU;

1907 - the quantification of the extent of increased choline requirement in carriers of alleles with
1908 increased need for choline;

1909 - choline content of EU foods, to obtain better quantitative data on choline intake in Europe;

1910 - biomarkers of choline status;

1911 - criteria on which to base choline sufficiency in different populations;

1912 - the consequences of the epigenetic modifications of genes involved in hormonal and vascular
1913 physiology and their expression following changes in choline intake during pregnancy;

1914 - quantitative assessment of choline transfer from mother to fetus;

1915 - quantification of the incorporated choline compounds in the body or in different organs during
1916 development.

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2414 **APPENDICES**

2415 **Appendix A. Concentrations of free and total choline in breast milk of healthy lactating mothers**

Reference	n (number of samples)	Country	Maternal dietary intake (mg/day) mean ± SE (range)	Stage of lactation	Choline concentration (mg/L) mean ± SE	Choline concentration (mg/L) median	Analytical method	Comments
Holmes-McNary et al. (1996)	16(16)	US	Not reported	27–32 days post partum	Free choline 12.1 ± 2.3 Total choline [(a)] 130.6 ± 25.3		Water soluble compounds extracted with $HClO_4$, HPLC after hydrolysis; phospholipid-bound choline separated by TLC and analysed after hydrolysis by GC–MS or phosphorus quantification	Hospital bank milk. Pumped milk samples. Full term infants. No information on polymorphism and supplementation of the mothers. Plasma choline concentration was not assessed.
Holmes et al. (2000)	8(8)	UK	Not reported	2–6 days post partum 7–22 days post partum	Free choline 11 ± 2 Total choline [(a)] 63 ± 9 Free choline 22 ± 5 Total choline [(a)] 133 ± 15		Nuclear magnetic resonance spectrometry (extraction with perchloric acid and chloroform of water soluble and phopholipid-bound choline, respectively).	Infants born at 28 to 38 weeks of gestation (preterm and term). No information on the supplementation of the mothers. Aliquots of expressed foremilk. Plasma choline concentration not reported.

Reference	n (number of samples)	Country	Maternal dietary intake (mg/day) mean ± SE (range)	Stage of lactation	Choline concentration (mg/L) mean ± SE	median	Analytical method	Comments
Ilcol et al. (2005)	(21)	Turkey	Not reported	Colostrum (0-2 days after birth)	Free choline 13.8 ± 2.2 Total choline [(a)] 70.4 ± 3.6		*__Free choline__ in milk: measured with a modification of the enzymatic radiochemical method. *__Phospholipid-bound choline, PC and SPM__ in milk: measured with an enzymatic colorimetric method. *__PChol and GPC:__ first hydrolyzed enzymatically to free choline then measured with high-performance liquid chromatography–electrochemical detection system.	116 breastfeeding women : 32 smokers No information about the term of the infants and supplementation of the mothers. For colostrum analyses 0–2 days, 57 full-term plus 24 pre-term infants were investigated; milks from day 12–180 were provided by 95 mothers with no indication of gestational age. Maternal plasma choline concentration reported and correlation with breast milk concentration investigated. Inverse linear relationship between free choline concentration in breast milk and lactating days of the mothers (r = -0.625; p < 0.001).
	(95)			12–180 days Post partum	Free choline 23.8 ± 1.04 Total choline [(a)] 153.8 ± 5.0			
	(14)			12–28 days post partum	Free choline 31.1 ± 3.8 Total choline [(a)] 166.2 ± 8.5			
	(12)			75–90 days postpartum	Free choline 29.8 ± 2.2 Total choline [(a)] 150.1 ± 8.8			
	(11)			165–180 days post partum	Free choline 13.8 ± 1.6 Total choline [(a)] 140.5 ± 10.9 All breast-milk free choline and total choline mean values were significantly higher than colostrum values, except free choline value for days 165–180 which was significantly lower than the value for days 12–180.			

Reference	n (number of samples)	Country	Maternal dietary intake (mg/day) mean ± SE (range)	Stage of lactation	Choline concentration (mg/L) mean ± SE	median	Analytical method	Comments
Fischer et al. (2010b)	51(51)	US American (89%), African-American (3%), Asian (6%), American Indian (1%), other (1%)	**Supplemented group (n = 48)** (Supplement: 750 mg choline/day) *Dietary choline: 338 ± 14 (124-622) *Total choline intake: 1 088 ± 14	45 days post partum	Free choline 11.0 ± 1.0 (Significantly higher than in placebo group). Total choline [(a)] 149.4		Liquid chromatography/ electrospray ionization isotope dilution mass spectrometry	103 participants: no breast milk data for 4 individuals and no dietary intakes for 9 individuals 3 days dietary records at 45 days postpartum. PC supplement or placebo from 18 weeks of gestation to 90 days postpartum Calculated duration of pregnancy (from duration of treatment) 34–42 weeks (for supplementation group) and 35–43 weeks (for placebo group) Maternal plasma choline concentration reported. Genetic polymorphism investigated. Correlation between breast milk concentration of choline or plasma concentration of choline and total choline intake.
	48(48)		**Placebo group (n = 46)** * Dietary choline: 364 ± 18 (139–671) *Total choline intake: 364 ± 18	45 days postpartum	Free choline 8.6 ± 0.8 Total choline [(a)] 124.8			

Reference	n (number of samples)	Country	Maternal dietary intake (mg/day) mean ± SE (range)	Stage of lactation	Choline concentration (mg/L) mean ± SE	Choline concentration (mg/L) median	Analytical method	Comments
Ozarda et al. (2014)	53	Turkey	Not reported	1–3 days post partum		Free choline 7.4 (2.2–13.6) (c) Total choline 42.4 (31.4–72.1) (b) (c)	HPLC - electrochemical detection (HPLC-EC) for water-soluble choline compounds after hydrolysis; phospholipid-bound choline by enzymatic colorimetric method.	Women who provided colostrum samples were not the same as the women who provided the mature milk samples.
	54			22–180 days post partum		Free choline 9.7 (7.0–13.9) (c) Total choline 159.6 (130.2–176.6) (b) (c)		Term infants. Expressed milk. Supplementation of the mothers not reported. Plasma CRP concentration reported (relationship between serum CRP and breast milk content investigated).
						Free and total choline median values at days 1-3 were significantly lower than at days 22-180. In colostrums positive correlation of water-soluble choline compounds with CRP in maternal serum and negative correlation with PC. No such correlation in mature milk.		
Davenport et al. (2015)	28	US	*Dietary choline: 380 (a) Supplement: 100 *Total choline intake: 480 **(n = 15)**	5 weeks post partum	mean ± SD (a) Free choline Baseline: 8.9 ± 4.2 Week 10: 16.5 ± 1.3 (a) Total choline (a) Baseline: 136.5 ± 26.0 Week 10: 104.2 ± 5.2		LC-MS/MS	No information about the term of the infants. Expressed milk. Maternal plasma choline concentration reported. Correlation between breast milk concentration of choline

Reference	n (number of samples)	Country	Maternal dietary intake (mg/day) mean ± SE (range)	Stage of lactation	Choline concentration (mg/L) mean ± SE	Choline concentration (mg/L) median	Analytical method	Comments
								and total choline intake.
			(b) Supplement: 550 *Total choline intake: 930 **(n = 13)**		(b) Free choline Baseline: 8.8 ± 5.7 Week 10: 15.4 ± 1.4 (b) Total choline [(a)] Baseline: 117.1 ± 22.8 Week 10: 125.0 ± 6.3 **All subjects** Free choline: Baseline: 8.8 ± 4.4 Total choline [(a)] Baseline: 127.5 ± 26.0			Increased circulating plasma choline during lactation The study also had a control group (nonpregnant, nonlactating women).

CRP, C-reactive protein; EC, electrochemical detection; GC-MS, gas chromatography-mass spectrometry; GPC, glycerophosphocholine; HPLC, high-performance liquid chromatography; LC-MS/MS, liquid chromatography–tandem mass spectrometry; PC, phosphatidylcholine; PChol, phosphocholine; SE, standard error; SPM, sphingomyelin; TLC, thin-layer chromatography.

(a): Total choline was the result of the sum of: free choline, phosphatidylcholine, phosphocholine, glycerophosphocholine, sphingomyelin.
(b): Total choline was the result of the sum of: free choline, phosphocholine, glycerophosphocholine, phospholipid-bound choline.
(c): Median (P25-P75).
The values of free choline and total choline concentration in breast milk reported in the articles were expressed in nmol/mL or mmol/L, those values were converted in mg/L using the following molecular mass (MM) (for free choline and total choline) = 104.17 g/mol.

Appendix B. Intervention and observational studies on the relationship between dietary choline and plasma homocysteine concentration

Author	Type of study	Subjects n, sex, age, country	*Intervention/design (trials) * Intake measurement (cross-sectional studies)	* Duration (trials) * Choline intake (mg/day) (cross-sectional studies)	tHcy in plasma (µmol/L)		Comment on tHcy in plasma	Other outcomes
					Plasma tHcy(fasting)	Post-methionine (0.1g/kg)		
(Olthof et al., 2005)	Double-blind cross-over RCT	26 (male), 50-71 years, NL	**2.6 g choline**/day as PC, n = 13	2 weeks	Mean ± SD Baseline 15.6 ± 4.0 Day 15 13.6 ± 2.5	Mean ± SD Baseline 27.0 ± 6.1 Day 15 22.3 ± 3.3	Choline 2.6 g/day for two weeks decreased significantly fasting plasma tHcy and 6-hour post-methionine plasma tHcy.	Choline supplement decreased serum folate and alkaline phosphatase, increased serum B6 and TAG; no change in cobalamin, ALT, AST, GGT, creatinine, total, LDL and HDL cholesterol.
			No supplement (wash-out period)	2 weeks	-			
			Placebo, n = 13	2 weeks	Baseline 16.5 ± 4.2 Day 15 16.6 ± 4.0	Baseline 31.8 ± 7.0 Day 15 31.6 ± 6.0		
(Wallace et al., 2012)	Double-blind RCT	42 (female, postmenopausal), 49–71 years, Ireland	**1 g choline**/day (as bitartrate), n = 19	12 weeks	Median Baseline 9.9 6 weeks 9.5 12 weeks 9.7		No significant difference of plasma tHcy at 6 and 12 weeks.	MTHFR genotype TT 10.5% in choline group. Plasma choline, betaine and DMG at six weeks significantly higher in choline group than placebo group
			Placebo (2.4 g tartaric acid)/day, n = 23		Baseline 9.7 6 weeks 10.1 12 weeks 10.0			MTHFR genotype TT 4.3% in placebo group.
(Atkinson et al., 2008)	Randomised, single-event, cross-over	8 (male), 19-40 years, New Zealand	**500 mg choline** as chloride	Once per week	Non-significant decrease.		Choline from a meal has a greater tHcy lowering effect than supplemental choline. Overall the effect is moderate.	Increase of plasma betaine except after the low-choline/low-betaine meal. Urinary betaine excretion did not change. Urinary DMG excretion increased after high-choline meal, not after choline supplement
			High-choline meal **(760 mg choline)**	Once per week	Significant decrease by 0.77 µmol after 4–6 h.			
			High-choline meal **(760 mg choline)** plus methionine load (100 mg/kg body weight)	Once per week		Significant lower rise at 4–6 h compared to low-choline meal by 6.9-7.6 µmol/L		
			Low-choline meal **(< 1 mg choline**)	Once per week				

Author	Type of study	Subjects n, sex, age, country	*Intervention/design (trials) * Intake measurement (cross-sectional studies)	* Duration (trials) * Choline intake (mg/day) (cross-sectional studies)	tHcy in plasma (µmol/L)		Comment on tHcy in plasma	Other outcomes
					Plasma tHcy(fasting)	Post-methionine (0.1g/kg)		
(Cho et al., 2006)	Cross-sectional study in a long-term cohort, offspring of Framingham cohort, start 1971, 5th examination 1991–1994	1 860, (1 040 females), 28-82 years, USA	FFQ	Energy-adjusted Intake Total choline (all forms): **313** ± 61 (mean ± SD) Quintiles (mean) Q1 **234** Q2 **283** Q3 **311** Q4 **339** Q5 **401**	Adjusted for age, sex, folate, B6, cobalamine intake, smoking, alcohol, caffeine, medication, serum creatinine Geometric mean (95% CI) 10.6 (10.2, 11.0) 10.4 (10.0, 10.8) 10.1 (9.7, 10.5) 9.7 (9.3, 10.1) 9.8 (9.5, 10.2)		Hcy lowering effect observed at choline intakes < 1 000 mg/day and stronger in men than in women.	
(Lee et al., 2010a) (follow-up from (Cho et al., 2006))	Cross-sectional study in long-term cohort study, Framingham Offspring study started 1971-1974	2 732 (1 325 male), 29–86 years, USA	FFQ, 6th examination 1995-1998	Energy-adjusted total intake Total choline (all forms): **308** ± 56 (mean ± SD) Quintiles (median ± SD) Q1 **234**±25 Q2 **278** ± 9 Q3 **305** ± 7 Q4 **334** ± 10 Q5 **379** ± 36	Adjusted for age, sex, folate, B6, cobalamin intake, smoking, alcohol, caffeine, total energy, serum creatinine Geometric mean (95% CI) 10.1 (9.8, 10.4) 10.1 (9.8, 10.4) 9.7 (9.5, 10.0) 9.7 (9.4, 9.9) 9.7 (9.4, 9.9) p for trend 0.001	Geometric mean (95% CI) 24.5 (23.8, 25.3) 25.6 (24.8, 26.4) 24.0 (23.3, 24.8) 24.4 (23.7, 25.2 24.3 (23.6, 25.0) N.S.	Inverse association between choline intake and either fasting or post-methionine plasma tHcy before folic acid fortification in the USA, not after. Association strongest for GPC and stronger for men than women.	Choline intake quintiles differ from 1 to 5 by 145 mg/day only.
(Chiuve et al., 2007)	Cross-sectional study within long-term cohort, Nurses' Health Study (NHS) and NHS 2; start 1976 and 1989, respectively	1 477 (healthy premenopausal females), 867 NHS (30–55 years at inclusion), 510 NHS2 (25-42 years at inclusion), USA	FFQ 1984, 1986, 1990 for NHS, and 1991, 1995, 1999 for NHS 2;	Energy-adjusted intake Total choline (all forms) Quintiles (median) Q1 **265** Q2 **297** Q3 **323** Q4 **345** Q5 **385**	Adjusted for age Median ± SEM 11.4 ± 0.3 10.7 ± 0.2 10.7 ± 0.2 10.2 ± 0.2 10.1 ± 0.3		Inverse relationship between plasma tHcy (age-adjusted, or further adjusted for diet and other lifestyle factors) and 1) total choline intake or 2) choline intake from PChol and GPC, particularly if folate intake is low; no relationship when further adjusting for riboflavin and folate intake	

ALT, alanine transaminase; AST, aspartate transaminase; DMG, dimethylglycine; CI, confidence interval; FFQ, food frequency questionnaire; GGT, γ-glutamyltransferase; GPC, glycerophosphocholine; HDL, high-density lipoproteins; LDL, low-density lipoproteins; MTHFR, methylene-tetrahydrofolate reductase; NHS, Nurses' Health Study; NL, the Netherlands; N.S., not significant; PC, phosphatidylcholine; Q, quintile; RCT, randomised controlled trial; SD, standard deviation; TAG, triacylgycerols; tHcy, total homocysteine.

2429 **Appendix C. SNPs of genes coding for enzymes involved in choline metabolism and their**
2430 **impact on choline requirement and/or risk to develop organ dysfunction while**
2431 **being fed a low-choline diet**

Enzyme gene	rs number	Base pair and change	Comments
Phosphatidylethanolamine methyltransferase *(PEMT)* (about 100 SNPs),	rs12325817 rs4646343 rs37601188	-744G → C C → A G→ A	Three SNPs that decrease the estrogen responsive PEMT induction (da Costa et al., 2014) associated with increased risk of choline deficiency on choline depletion. May increase the dietary requirement of choline. Eighteen of 23 female carriers of the variant rs12325817 allele developed organ dysfunction on choline depletion (OR of 25; 95% CI 2.0-256.0; p = 0.002), but men did not (da Costa et al., 2006b). Fischer et al. (2010a) found a gene dose-response relationship in 27 premenopausal women to develop signs of choline deficiency on choline depletion: 80%, 43 % and 13% with two, one and zero variant alleles, repectively developed liver dysfunction. Eleven of 22 postmenopausal women subjected to the standard choline depletion/repletion experiment who received oestrogen were four times less likely to develop choline-deficiency associated liver dysfunction than 11 women who received placebo. The rs12325817 CC genotype was associated with an increased risk for breast cancer mortality compared to the GG genotype (OR 1.30, 95% CI 1.01-1.67) (Xu et al., 2008). About 75% of the North Carolina population is carrier of at least one rs12325817 C allele and 18% are homozygous for the variant allele (Corbin and Zeisel, 2012). The rs12325817 allele was associated in 92% of 64 women with a rs4646343 allele (Kohlmeier et al., 2005; da Costa et al., 2006b; Resseguie et al., 2011).
	rs7946(3)	+5465 G→ A	Despite 30% loss of function, no increased susceptibility to choline deficiency (da Costa et al., 2006b). The PEMT rs79463 SNP is found more frequently in people with fatty liver consuming a low-choline diet (Ivanov et al., 2009) and in 67.9% of patients with NAFLD (healthy subjects 40.7%) (Song et al., 2005) Attenuates rise in plasma Hcy in men with the MTHFR 677TT genotype (Caudill et al., 2009). Gene frequency in 43 Mexican-American women: GG 3, GA 19, AA 21 (Ivanov et al., 2009). All effect alleles of *PEMT* occur frequently in American subjects of European origin (homozygosity 24-60%), followed by Mexican origin (9-12%) and least in subjects of Asian or African descent (Da Costa et al., 2014). Carrier status of offspring without effect on umbilical cord blood choline and its metabolites (Visentin et al., 2015).
Methylenetetrahydrofolate dehydrogenase1 (*MTHFD1)*	rs2236225	1958G → A -	Decreases the availability of methyl-THF for Hcy remethylation and increases reliance on choline-derived methyl groups. May increase the dietary requirement of choline and reduce the synthesis of PC (Ivanov et al., 2009): in a choline depletion/repletion study on 54 healthy adults (n = 26 men and n = 28 women), more than half of the participants developed organ dysfunction associated with choline deficiency. Signs of choline deficiency were significantly (> 15 times in premenopausal women) more likely to occur in subjects who were carriers of the A allele of the SNP rs2236225 of MTHFD1 (OR 7.0; 95% CI 2.0-25.0, p < 0.01) than in non-carriers during the low-choline diet, unless they were also treated with a folic acid supplement (Kohlmeier et al., 2005). Homozygous mothers for the SNP were found to have a 1.5–2 fold increased risk of carrying a child with an NTD (Brody et al., 2002). Carrier status of offspring without effect on umbilical cord blood choline and its metabolites (Visentin et al., 2015). 63% of subjects investigated in North Carolina possessed at least one allele of this SNP and 11% were homozygous carriers (da Costa et al., 2006b; Corbin and Zeisel, 2012).

Enzyme gene	rs number	Base pair and change	Comments
Choline dehydrogenase (*CHDH)*	rs9001	+114 A → C	Carriers may be protected against organ dysfunction upon choline depletion (OR 0.2; 95% CI 0.05–0.7, p = 0.03) (da Costa et al., 2006b).
	rs12676	+233 G → T	May increase the dietary requirement of choline in carriers of the variant associated with increased susceptibiliy to choline deficiency upon choline depletion in premenopausal women (OR 20.0; 95% CI 1.0-282.0; p = 0.04) (da Costa et al., 2006b). The T allele was associated with an increased risk (OR 1.19, 95% CI 1.00-1.41) for breast cancer compared to the major G allele (Xu et al., 2008). Forty and 75% lower ATP concentration in sperm of men with GT (n = 18) and TT (n = 5) genotypes compared to the GG (n = 17) genotype, respectively (Johnson et al., 2012). The TT genotype is present in 9% of the North Carolina population, the prevalence of the GT genotype is 45% (Johnson et al., 2012). Carrier status of offspring without effect on umbilical cord blood choline and its metabolites (Visentin et al., 2015).
Betainehomocysteine methyltransferase (*BHMT)*	rs3733890	+742G → A	Not associated with susceptibility to choline deficiency (da Costa et al., 2006b). This polymorphism was not associated with breast cancer risk (Xu et al., 2008), but with a reduced risk of breast cancer mortality (Xu et al., 2009). Carrier status of offspring without effect on umbilical cord blood choline and its metabolites (Visentin et al., 2015).
Choline kinase A (*CHKA)*	rs7928739 rs10791957 rs2512612	A → C A → C A → G	Three SNPs associated with a decreased risk for organ dysfunction on choline depletion in homozygotes (da Costa et al., 2014). Frequency is highest in subjects of African descent followed by Asian and European origin and least frequent in subjects of Mexican origin (da Costa et al., 2014). In a case control study on 103 cases of spina bifida and of 338 controls, the CHK SNP (rs7928739) genotype with at least one C allele was associated with a reduced risk of spina bifida (OR = 0.60, 95% CI = 0.38–0.94) (Enaw et al., 2006).
	Rs6591331	A → T	Associated with increased risk for organ dysfunction in postmenopausal women on choline depletion in homozygotes (da Costa et al., 2014).
Choline kinase B (*CHKB)*	rs1557502	G → A	Associated with an increased risk for muscle damage on choline depletion (da Costa et al., 2014). Most frequent in subjects of African descent, least frequent with European origin (da Costa et al., 2014). Nine of ten subjects who developed muscle damage were heterozygous or homozygous carriers of the effect alleles for *SLC44A1* rs2771040 (G) and *CHKB* rs1557502 (A).
CTP:phosphocholine cytidyltransferase (*CCT*)	rs939883	T → A	In a case control study on 103 cases of spina bifida and of 338 controls, the CCT rs939883 genotype AA was associated with an increased risk of spina bifida (OR = 1.89, 95% CI = 0.97–3.67) (Enaw et al., 2006).
Solute carrier 44A1 (choline transporter) *(SLC44A1)*	rs7873937 rs2771040 rs6479313 rs16924529 rs3199966	C → G A → G C → G G → A A → C	Associated with an increased risk for muscle damage on choline depletion with a low-choline diet (da Costa et al., 2014). Nine of ten subjects who developed muscle damage were heterozygous or homozygous carriers of the effect alleles for *SLC44A1* rs2771040 (G) and *CHKB* rs1557502 (A). Most frequent in subjects of African descent, least frequent with Asian origin (da Costa et al., 2014).
Methylenetetrahydrofolate reductase (*MTHFR*)	rs1801133	677C → T	Thermolabile enzyme, increases the reliance on choline-derived methyl groups for Hcy remethylation.when folate intake is insufficient (Yan et al., 2011). Significantly increased plasma Hcy, decreased plasma PC and SPM with low folate status/intake in both men and women with either CC (n = 28) or TT (n = 17) genotype, but no change in plasma choline and leukocyte global DNA methylation. Women with the TT genotype had a 10.3% increase in plasma PC while consuming adequate amounts of folate and choline. No changes in plasma PC in response to diet in subjects with the CC genotype (Abratte et al., 2009). In 60 healthy men, 29 with the TT genotype and 31 with the CC genotype, an intake of 300 mg choline/day for 12 weeks was sufficient to maintain liver and kidney function, but 438 µg DFE/day did not prevent a rise in plasma tHcy in subjects with the TT genotype. Under these conditions, choline supplementation (up to 1 900 mg/day) had no effect on plasma tHcy and serum folate concentrations. Choline intake decreased DNA methylation in subjects with the CC genotype but not in TT subjects (Solis et al., 2008; Veenema et al., 2008; Caudill et al., 2009). Carrier status of offspring without effect on umbilical cord blood choline and its metabolites (Visentin et al., 2015). TT genotype frequency varies between ethnic groups (2-35%).
	rs1801131	1298A → C	Reduced enzyme activity; no association with risk for choline deficiency in choline depletion/repletion studies (Kohlmeier et al., 2005).

ATP, adenosine triphosphate; BHMT, betaine-homocysteine methyltransferase; CCT, CTP:phosphocholine cytidyltransferase, CHDH, choline dehydrogenase; CHK, choline kinase; CI, confidence interval; DFE, dietary folate equivalent; DNA, deoxyribonucleic acid; Hcy, homocysteine; MTHFD1, 5,10-methylene-tetrahydrofolate dehydrogenase 1; MTHFR, methylene-tetrahydrofolate reductase; NTD, neural tube defect; OR, odds-ratio; PC, phosphatidylcholine; PEMT, phosphatidylethanolamine N-methyltransferase; SLC44A1, solute carrier family 44 (choline transporter); SNP, single-nucleotide polymorphism; SPM, sphingomyelin.

Appendix D. Depletion/repletion studies for choline

(choline intake per 70 kg body weight per day)

Author	Aim of investigation Duration	Outcome measurements	Participants	Design/duration	Results	Comment
(Zeisel et al., 1991) **1***	Experimental choline deficiency in humans	Choline, PC in plasma; PC in red blood cells; liver and kidney function, blood lipids, liver size and density by CT	Male, n = 15, healthy **A controls** n = 6**,** mean age 26.8 years; **B depleted** n = 8, mean age 29.1 years One recruited control subject was excluded (abnormal liver function tests on day 1)	Metabolic unit; **Week 1**: A and B: baseline diet (13 mg/70 kg body weight per day) + 500 mg/day choline **Week 2-4**: **A:** baseline diet + 500 mg/day choline **B:** baseline diet + placebo **Week 5** (i.e. 35 days): **A:** baseline diet + 500 mg/day choline **B:** baseline diet + 500 mg/day choline	**Week 1:** choline in plasma 9.6–10.9 µmol/L; plasma PC 1.3-2.0 mmol/L **Week 4**: **A**: no change in plasma choline/PC, increase by 14% in red blood cell PC, no change in ALT **B**: choline in plasma decreased by 30%, plasma PC (as % of day 7 value) decreased by 30%, decrease in red blood cell PC by 15%; significant **increase in ALT** by 50%; non-significant increase in liver size **Week 5:** **A**: no change **B**: plasma choline, plasma PC, ALT return to baseline	Plasma choline, plasma PC and serum ALT activity expressed as a change from day 7 to day 28. Three-week depletion of dietary choline (513 to 13 mg choline/day) significantly decreased plasma choline and PC and increases serum ALT activity in all subjects. No effects on other hepatic or kidney function parameters.
(Kohlmeier et al., 2005) **2**	Influence of genetic variants of folate metabolism on susceptibility to choline deficiency.	Liver by MRI, CK in serum, Plasma folate, plasma tHcy, SAM, SAH; tHcy response to methionine load before and after depletion; genotyping for *MTHFR*, *MTHFD1* and *RFC1* (reduced folate carrier1)	n = 54, female n = 28, mean age 38.7 years, healthy	Metabolic unit **Baseline**: 10 days, 550 mg choline/70 kg body weight per day+ 400 µg folic acid **Depletion** (up to 42 days): < 50 mg choline/70 kg body weight per day and 100 µg folate/day **A** plus 400 µg folic acid/day **B** placebo **Repletion** (increasing amount (137–550 mg/70 kg body weight per day) up to > 550 mg choline per day for ≥ 3 days)	**Organ dysfunction** 12/54 subjects 5-fold increase in CK 24/54 increase (at least by 28 %) in liver fat content, no effect of folate intake **Genotyping and % symptomatic choline deficiency:** *MTHFD1* 1958 GG n = 20: 40% *MTHFD1* 1958 GA n = 28: 82% *MTHFD1* 1958 AA n = 6: 83% GG versus GA/AA **OR 7.0 (95% CI 2.0–25) p = 0.007** *RFC1* 80 AG n = 20: 70% *RFC1* 80 GG n = 15: 73% AA versus AG/GG OR 1.82 (95% CI 0.56-5.9) N.S. Mean serum folate significantly lower in subjects with low folate intake (22.1 (B) versus 28.3 mmol/L (A)) without effect by genetic polymorphism.	More than 50% of the participants developed signs of organ dysfunction when consuming < 50 mg/70 kg body weight per day. Susceptibility to develop signs of choline deficiency on a 50 mg/70 kg body weight per day-choline diet greater in carriers of the *MTHFD1* G1958A polymorphism: OR 7.0 (95% CI 2.0–25; p < 0.01) unless they received additional folic acid. Susceptibility to develop signs of choline deficiency not influenced by polymorphism of *MTHFR* or *RFC1*.

Author	Aim of investigation Duration	Outcome measurements	Participants	Design/duration	Results	Comment
(da Costa et al., 2005) **3**	Choline deficiency and capacity to methylate tHcy	Total plasma tHcy, before and after Met load (100 mg/kg body weight) before and after choline depletion and repletion, plasma choline, betaine, PC, folate liver fat by MRI.	n = 8 males, age 20-46 years, healthy	Standardised depletion/repletion design **Baseline** diet (10 days): 550 mg choline/70 kg body weight per day + 400 DFE/day **Depletion** diet **(**up to 42 days): < 50 mg choline/70 kg body weight per day **Repletion** diet**:** 1) subjects not clinically choline deficient: 550 mg choline diet for three days 2) subjects clinically choline deficient: graded amounts of choline sequentially in 10 days periods (138, 275, 413, 550 mg/70 kg body weight per day until hepatic steatosis resolved).	**Organ dysfunction** 4/8 increase in liver **fat** **tHcy in plasma** **Depletion** fasting tHcy significantly increased by 1.3 µmol/L in clinically choline-deficient participants (no significant change in the non-deficient subjects) **Choline in plasma (mean)** Before depletion 10 µmol/L Clinically depleted 7 µmol/L Not clinically depleted 7 µmol/L **PC in plasma (mean)** Before depletion 1 818 µmol/L Clinically depleted 1 564 µmol/L Not clinically depleted 1 834 µmol/L **Betaine in plasma (mean)** Before depletion 66 µmol/L Clinically depleted 36 µmol/L Not clinically depleted 34 µmol/L	Half of the participants developed signs of liver dysfunction when consuming < 50 mg choline/70 kg body weight per day; no difference in change in plasma choline (or betaine) between those with and without organ dysfunction.
(da Costa et al., 2006b) **3**	Choline deficiency and lymphocyte apoptosis and DNA damage	CK, liver fat by MRI, 24 h-urine choline and betaine, plasma folate, peripheral lymphocytes at baseline, after depletion and repletion: DNA fragmentation (TUNEL) and strand breaks (COMET), activated caspase-3 (used as a marker for apoptosis).	n = 51, n = 31 female, age 18-70 years, healthy	Metabolic unit. Standardised depletion/repletion design **Baseline** diet (10 days): 550 mg choline/70 kg body weight per day + 400 DFE/day **Depletion** diet (up to 42 days): < 50 mg choline/70 kg body weight per day and 100 DFE/day **A** plus 400 µg folic acid/day, n = 26 **B** placebo, n = 25 **Repletion** diet 1) subjects not clinically choline deficient: 550 mg choline diet for three days 2) subjects clinically choline deficient: graded amounts of choline sequentially in 10 days periods (137.5, 275, 412.5 and 550 mg/70 kg body weight per day and > 550 mg for three days.	**Organ dysfunction** 33/51, including 26/51 liver dysfunction (18 females) 1/51 muscle dysfunction only 6/51 both liver and muscle dysfunction returning to normal after choline repletion **Plasma folate** Significant decrease during choline depletion without extra folic acid: 26.0 to 21.4 µmol/L (and p = 0.0003 without folate supplementation) **24-h urine choline and betaine** Decrease from about 25 to 10 and from 80 to about 30 µmol/g creatinine, respectively with choline depletion **Activated caspase-3 assay in lymphocytes** Higher amounts in cells from clinically choline deficient subjects, compared to non-deficient subjects (p < 0.05) **TUNEL assay** More TUNEL-positive lymphocyte cells during choline depletion with or without organ dysfunction, without folic acid supplement (p = 0.026). **COMET assay** COMET-Tail moment increase during choline depletion compared to baseline	Choline deficiency is associated with *in vitro* signs of DNA damage and of apoptosis in peripheral lymphocytes.

Author	Aim of investigation Duration	Outcome measurements	Participants	Design/duration	Results	Comment
(Fischer et al., 2007) **3**	Dietary requirement in healthy men and women and clinical sequelae of choline deficiency	Plasma choline, PC, SAM, SAH, Met, tHcy, methylglycine and DMG CK, Fat in liver by MRI	n = 57, n = 16 pre-menopausal women, n = 15 post-menopausal women, n = 26 men Age 18-70 years, healthy	Metabolic unit. Standardised depletion/repletion design **Baseline** diet (10 days): 550 mg choline/70 kg body weight per day + 400 DFE/day **Depletion** diet **(**up to 42 days): < 50 mg choline/70 kg body weight per day + 100 DFE/day **A** plus 400 µg folic acid/day **B** placebo **Repletion** diet**:** 1) subjects not clinically choline deficient: 550 mg choline diet for three days 2) subjects clinically choline deficient: graded amounts of choline sequentially in 10 days periods (137.5, 275, 412.5 and 550 mg/70 kg body weight per day, then > 550 mg for three days).	**Organ dysfunction** 39/57 as by changes in CK, AST, ALT, LDH or by hepatic steatosis, of which: 1) **6 while on 550 mg choline baseline diet (550 mg/70 kg body weight per day)**, all men 2) **33 while on low-choline diet (50 mg/70 kg body weight per day):** 14/20 men (70%), 7/16 (44%) premenopausal women 12/15 (80%) postmenopausal women; with **liver steatosis** alone: in 8/20 men, 12/15 postmenopausal women and 6/16 premenopausal women. . **Choline (metabolites) in plasma on depletion**: Choline decrease by 28–33%, betaine by ≈50%, PC only in subjects with organ dysfunction, Met decreased only in subjects with organ dysfunction, DMG and MG decreased, tHcy increased, SAM and SAH did not change. Serum uric acid increased in all subjects during depletion **Repletion of choline depleted subjects: see Table 2, Section 5.1.1.3**	Most men and postmenopausal women (68.4%) developed clinical choline deficiency when on < 50 mg choline/day independent on folate intake. 18/57 subjects did not develop signs of choline deficiency with < 50 mg choline/day;
(Niculescu et al., 2007) **3**	Organ dysfunction on low-choline diet and SNPs in genes involved in choline and folate metabolism/	Liver fat by MRI, CK in serum, Peripheral lymphocytes at 10 days and after depletion for genotyping *MTHFD1*, *PEMT*, *CHDH* and for change in expression with low-choline diet and DNA methylation	n = 33, age 20-67 years, 19 women, healthy	Metabolic unit. Standardised depletion/repletion design. **Baseline** diet (10 days): 550 mg choline/70 kg body weight/day + 400 DFE/day **Depletion** diet **(**up to 42 days): < 50 mg choline/70 kg body weight per day **A** plus 400 µg folic acid/day **B** placebo **Repletion** diet	No outcome measurements indicative of choline requirement	Previous studies showed that the *PEMT* (rs12325817) and *MTHFD1* (rs2236225) SNPs predispose subjects to develop organ dysfunction when they consume a low-choline diet (Kohlmeier et al., 2005; da Costa et al., 2006b). At baseline, subjects with the *PEMT* (rs12325817) and *MTHFD1* (rs2236225) SNPs, compared with subjects without the SNPs, had a different expression of genes involved in apoptosis, the DNA damage checkpoint, and cell proliferation control. This suggests that the presence of the *PEMT* and *MTHFD1* genotypes can lead to differences in the phenotypes at baseline (i.e. even before consuming a low-choline diet). Subjects may differ in their susceptibility to dietary choline deficiency. In women who are carriers of the *PEMT* allele, the risk of choline deficiency is higher.

Author	Aim of investigation Duration	Outcome measurements	Participants	Design/duration	Results	Comment
(Fischer et al., 2010a) **4**	Low-choline related organ dysfunction, in relation to number of alleles of rs12325817 in premenopausal women, and in relation to oestrogen in postmenopausal women	Liver fat by MRI , CK, AST, ALT Plasma choline (metabolites) Genotyping for PEMT rs12325817	**A**: n = 27 pre-menopausal women, age 18–49 years. **B:** n = 22 post-menopausal women, age 50–73 years, randomised to receive oestrogen (**B1**) or placebo (**B2**). Healthy.	Metabolic unit. Standardised depletion/repletion **Baseline** diet (10 days): 550 mg choline/70 kg body weight per day **Depletion** diet **(**up to 42 days): < 50 mg choline/70 kg body weight per day **Repletion** diet**:** 550–850 mg/70 kg body weight per day for up to 10 days. If signs of organ dysfunction did not resolve after 10 days of repletion diet: *ad libitum* diet for two weeks	**Among premenopausal women:** 11/27 developed choline deficiency/organ dysfunction. There was a dose-response effect of rs12325817 on the risk of choline related organ dysfunction: 80%, 43%, and 13% of women with 2, 1, or 0 alleles, respectively, developed organ dysfunction during the low-choline diet. **Among postmenopausal women:** only 2/11 (18%) who received oestrogen (B1) and 8/11 (73%) who received placebo (B2), developed organ dysfunction during the low-choline diet.	Dietary requirement for choline is higher in postmenopausal women (because of their lower oestrogen concentrations) than in premenopausal women. Choline requirements for both groups of women are further increased by rs12325817. 80% of homozygous women develop organ dysfunction on the depletion diet versus 43% of those with one copy and 13% of women homozygous for the wildtype. No oestrogen versus oestrogen increases four-fold the risk for organ dysfunction on the depletion diet. OEstrogen mitigates the effect of the *PEMT* SNP. OEstrogen may decrease choline requirement in postmenopausal women.
(Sha et al., 2010) **3**	Metabolomic profiling to predict organ dysfunction with deficient choline intake	Liver fat by MRI CK, AST, ALT Plasma choline (metabolites), Met, Hcy, sarcosine, DMG, cysteine, cystathionine, Metabolomic analysis of plasma	n = 53, n = 30 women, age 18–70 years, healthy	Metabolic unit. Standardised depletion/repletion design **Baseline** diet (10 days): 550 mg choline/70 kg body weight per day **Depletion** diet **(**up to 42 days): < 50 mg choline/70 kg body weight per day **Repletion** diet (≥ three days, (≥ 550 mg/70 kg body weight per day)	**Organ dysfunction** **Baseline diet:** 9 (17%) developed fatty liver (n = 4) or muscle dysfunction (n = 5), without special metabolome **Depletion** (n = 44): 23 fatty liver, 5 muscle dysfunction Higher plasma Hcy, cysteine, cystathionine, keto-acids at baseline in subjects who later develop fatty liver. Choline deficiency increased plasma carnitine and acyl-carnitine, decreased pyridoxate. Baseline plasma choline has no predictive value.	Metabolomic profiles of subjects at baseline could predict the development of liver dysfunction when deprived of dietary choline
(Spencer et al., 2011) **4**	Choline deficiency and hepatic steatosis and gut microbiome /2 months	Liver fat by MRI CK AST, ALT Sequencing of the 16S RNA bacterial genes in stool; genotyping of *PEMT* promoter SNP *rs12325817*	n = 15 females, age not reported, healthy	Standardised depletion/repletion design **Baseline** diet (10 days): 550 mg choline/70 kg body weight per day **Depletion** diet **(**up to 42 days): < 50 mg choline/70 kg body weight per day **Repletion** diet (10 days, (≥ 850 mg/70 kg body weight per day)	No statistically significant general microbial convergence with choline depletion	Host factors as well as gut bacteria respond to dietary choline deficiency, but individual microbiota persist although all subjects consumed the same diets.

Author	Aim of investigation Duration	Outcome measurements	Participants	Design/duration	Results	Comment

Da Costa et al., 2011
 	
3+4
	PC-DHA plasma concentration used as a non-invasive marker of liver PEMT activity	Plasma DHA, PC-DHA, ratio PC-DHA/total PC	n = 72, age 18-70 years;
			n = 20 men;
			n = 52 women of which n = 25 post-menopausal and n = 27 pre-menopausal
				Standardised depletion/repletion design.
				
				Baseline diet (10 days): 550 mg choline/70 kg body weight/day
				Depletion diet **(**up to 42 days): < 50 mg choline/70 kg body weight per day
				Repletion diet
					70% of the subjects possess at least one *PEMTrs12325817* allele.	Plasma ratio PC-DHA/total PC higher in pre-menopausal women than men or post-menopausal (at baseline and even when a low-choline diet).
						Plasma PC-DHA/total PC at baseline and PEMT activity in liver: lower in pre-menopausal women homozygous for the *rs12325817* polymorphism in the *PEMT* gene.

Da Costa et al., 2014 **3+4**	Identification of effect alleles of SNPs known to influence dietary requirement for choline	DNA concentration by spectrometry; genotyping of alleles	n = 79, 18-70 years old; n = 26 men n = 53 women of which n = 26 post- and n = 27 pre-menopausal	Standardised depletion/repletion design **Baseline** diet (10 days): 550 mg choline/70 kg body weight/day **Depletion** diet **(**up to 42 days): < 50 mg choline/70 kg body weight per day **Repletion** diet	Effect alleles identified of SNPs in genes for the choline transporter (SCC44A1) and choline kinase A and B (see Appendix C). Choline deficiency related organ dysfunction (liver or muscle: 50/79, including 20 of 26 postmenopausal women, 11 of 27 premenopausal women 19 of 26 men	29 of 79 healthy subjects did not develop organ dysfunction while consuming a low-choline diet for six weeks.

*Same numbers in the column "author" indicate references providing data from the same cohort.

ALT, alanine aminotransferase; AST, aspartate aminotransferas; CHDH, choline dehydrogenase; CK, creatine kinase; CT, computerised tomography; DHA, docosahexaenoic acid; DMG, dimethylglycine; CI, confidence interval; COMET, single-cell gel electrophoresis, DFE, dietary folate equivalent, DNA, deoxyribonucleic acid; LDH, lactate deshydrogenase; Met, methionine; MRI, magnetic resonance imaging; MG, methylglycine; MTHFD1, 5,10-methylene-tetrahydrofolate dehydrogenase 1; MTHFR, Methylene-tetrahydrofolate reductase; N.S., not significant; OR, odds-ratio; PC, phosphatidylcholine; PEMT, phosphatidylethanolamine N-methyltransferase; RFC1, reduced folate carrier 1; RNA, ribonucleic acid; SAH, S-adenosylhomocysteine; SAM, S-adenosyl-methionine; SNP, single-nucleotide polymorphism; tHcy, total homocysteine; TUNEL, terminal deoxynucleotidyl transferase mediated dUTP nick end labeling.

ABBREVIATIONS

Afssa	Agence française de sécurité sanitaire des aliments
AI	Adequate intake
ALT	Alanine aminotransferase
AR	Average requirement
ARA	Arachidonic acid
ARIC	Atherosclerosis Risk in Communities
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BADH	Betaine aldehyde dehydrogenase
BE	Barrett esophagus
BHMT	Betaine-homocysteine methyltransferase
BMI	Body mass index
bw	Body weight
CCT	Phosphocholine cytidyltransferase
CDP	Cytidine 5-diphosphate
CHK	Choline kinase
CHKA	Choline kinase A
CHKB	Choline kinase B
CHD	Coronary heart disease
CHDH	Choline dehydrogenase or choline oxidase
CI	Confidence Interval
CK	Creatine (phospho)kinase
COMA	Committee on Medical Aspects of Food Policy
COMET	Single-cell gel electrophoresis
CPT	Cytidine 5-diphosphate-choline
CRC	Colorectal cancer
CRP	C-reactive protein

CT	Computerised tomography
CTL1	Choline transporter-like protein 1
CTP	Cytidine triphosphate
CVA	Cerebrovascular accident
CVD	Cardiovascular disease
D-A-CH	Deutschland-Austria-Confoederatio Helvetica
DFE	Dietary folate equivalent
DMG	Dimethylglycine
DH	Department of Health
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DRV	Dietary Reference Values
EAC	Oesophageal adenocarcinoma
EAR	Estimated Average Requirement
EC	European Commission
ECG	Electrocardiogram
EFSA	European Food Safety Authority
EGJAC	Oesophagogastric junction adenocarcinoma
ESCC	Oesophageal squamous cell carcinoma
EU	European Union
FAO	Food and Agriculture Organization
FFQ	Food frequency questionnaire
FMO3	Flavin-containing monooxygenase isoform 3
GC-MS	Gas chromatographys-mass spectrometry
GGT	γ-glutamyltransferase
GPC	Glycerophosphocholine
Hcy	Homocysteine
HDL	High-density lipoprotein

HILIC LC-MS/MS	Hydrophilic interaction liquid chromatography-tandem mass spectrometry
HPLC	High-performance liquid chromatography
HR	Hazard ratio
IOM	U.S. Institute of Medicine of the National Academy of Sciences
K_m	Michaelis constant
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
LOAEL	Lowest Observed Adverse Effect Level
Met	Methionine
MG	Methylglycine
MI	Myocardial Infarction
MIDA	Multiple isotopomerdistribution analysis
MM	Molecular mass
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectrometry
MS	Methionone synthase
MTHFD1	5,10-methylenetetrahydrofolate dehydrogenase 1
MTHFR	Methylenetetrahydrofolate reductase
NAFLD	Non-alcoholic fatty liver disease
NHS	Nurses' Health Study
NHANES	National Health and Nutrition Examination Survey
NORCCAP	Norwegian Colorectal Cancer Prevention
N.S.	Not significant
NTD	Neural tube defect
OR	Odds ratio
PC	Phosphatidylcholine
PChol	Phosphocholine

PE	Phosphatidylethanolamine
PEMT	Phosphatidylethanolamine N-methyltransferase
PL	Phospholipase
Q	Quintile
RCT	Randomised controlled trial
RDA	Recommended Dietary Allowance
RFC1	Reduced folate carrier 1
RNA	Ribonucleic acid
RR	Relative risk
SAH	S-adenosylhomocysteine
SAH-H	S-adenosylhomocysteine hydrolase
SAM	S-adenosyl-methionine
SCF	Scientific Committee for Food
SD	Standard deviation
SEM	Standard error of the mean
SLC44A1	Solute carrier family 44 (choline transporter)
SNP	Single nucleotide polymorphism
SPM	Sphingomyelin
TAG	Triacylglycerol
tHcy	Total homocysteine
THF	Tetrahydrofolate
TMA	Trimethylamine
TMAO	Trimethylamine-N-oxide
TNF-α	Tumor necrosis factor-α
TTMA	Total trimethylamine
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UK	United Kingdom
UL	Tolerable upper intake level

UNU	United Nations University
US	United States
USDA	United States Department of Agriculture
VLDL	Very low density lipoproteins
WHO	World Health Organization

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