



Published in final edited form as:

World Rev Nutr Diet. 2010 ; 101: 73–83. doi:10.1159/000314512.

Choline: Clinical Nutrigenetic/Nutrigenomic Approaches for Identification of Functions and Dietary Requirements

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Abstract

Nutrigenetics/nutrigenomics (the study of the bidirectional interactions between genes and diet) is a rapidly developing field that is changing research and practice in human nutrition. Though eventually nutrition clinicians may be able to provide personalized nutrition recommendations, in the immediate future they are most likely to use this knowledge to improve dietary recommendations for populations. Currently, estimated average requirements are used to set dietary reference intakes because scientists cannot adequately identify subsets of the population that differ in requirement for a nutrient. Recommended intake levels must exceed the actual required intake for most of the population in order to assure that individuals with the highest requirement ingest adequate amounts of the nutrient. As a result, dietary reference intake levels often are set so high that diet guidelines suggest almost unattainable intakes of some foods. Once it is possible to identify common subgroups that differ in nutrient requirements using nutrigenetic/nutrigenomic profiling, targeted interventions and recommendations can be refined. In addition, when a large variance exists in response to a nutrient, statistical analyses often argue for a null effect. If responders could be differentiated from nonresponders based on nutrigenetic/nutrigenomic profiling, this statistical noise could be eliminated and the sensitivity of nutrition research greatly increased.

Challenges for Clinical Nutrigenetics/Nutrigenomics

The first challenge for developing clinical nutrigenetics/nutrigenomics is the growing misconception that only very large studies can develop evidence for associations between single nucleotide polymorphisms (SNPs) and phenotypes. The use of genome-wide profiling of common single nucleotide polymorphisms (SNPs) to identify such associations has become common. These genome-wide association studies (GWAS) often screen thousands to millions of genes and their variants in thousands of subjects. In order to avoid the issue of multiplicity, and because of expected modest effect sizes, the scientific community has adopted strict definitions of statistical significance (e.g. $p < 5 \times 10^{-7}$ [1]), which dictate the need for large sample sizes typically involving thousands of subjects. It is important to note that these definitions were adopted assuming an individual SNP has a small effect size and that large numbers of randomly selected SNPs are being screened for an association with a phenotype. Because of the enormous number of genotype-phenotype associations tested in a genome-wide study, spurious associations will substantially outnumber true ones unless rigorous statistical thresholds are applied; smaller p values generally provide greater support for a true association. However, standard Bonferroni correction is overly conservative because it assumes the independence of all tests performed, but in many association studies markers are not independent because they are in linkage disequilibrium.

Sadly, this growing consensus for requiring p values $< 5 \times 10^{-7}$ makes clinical nutrigenetics/nutrigenomics virtually impossible. Inherently, such studies involve tens to hundreds but not thousands of subjects and often measure phenotype parameters that are not practically measured in large populations. If the phenotype to be explained is not easily detected in thousands of people, a population GWAS approach is not possible. For example, we later discuss studies on fatty liver that require controlled diet conditions and mass resonance imaging. It might be possible to perform such studies on hundreds of people, but certainly not on tens of thousands. For clinical nutrigenetics/nutrigenomics approaches to be viable we need to use study designs that allow less stringent p values than are used for GWAS studies. The appropriate design elements have already been considered by the scientific panel that suggested the rigorous p values for GWAS studies [1].

Reasons that are appropriate for lowering the threshold for calling a finding of a particular SNP-phenotype association are the selection of targeted SNPs based on knowledge of the underlying processes causing the phenotype (e.g. selecting the gene for endogenous biosynthesis of a nutrient when studying the phenotype associated with deficiency of the nutrient), or selecting SNPs that are likely to result in defective protein products (such as non-synonymous coding SNPs) [1]. Selection of SNPs for which there is credible laboratory evidence or a validated *in silico* prediction *a priori* permits accepting a less rigorous p value; however, creating a credible biological hypothesis *post hoc* is not acceptable [1]. The lowering of the threshold for positively identifying a particular SNP-phenotype association must be declared before initiation of the analysis and not once the analysis has begun [1]. Additional information gathered from laboratory techniques, bioinformatic tools and *a priori* biological insight should be used to provide plausibility for interpreting genetic association findings [1]. It is important to limit the number of candidate SNPs considered as the number of multiple comparisons made in the analysis drives the possibility of false discovery. Inherently, small sample sizes can provide imprecise or incorrect estimates of the magnitude of the observed effects; thus, the effect size must be large enough to stand out from such noise. An estimated effect size that is large (that is, with an odds ratio greater than 2) in a small but well-powered study can lend credence to an association, because unknown confounding factors are less likely to produce large effects [1]. Finally, replication of the association between SNP and phenotype in an independent study is important.

Thus, though some geneticists initially expressed doubts about results generated in clinical nutrigenetics/nutrigenomics because they reflexively expect large population studies with very small p values, there are reasonable study designs under which clinical nutrigenetics/nutrigenomics is not only possible but practical (targeted and small number of SNPs studied based on biological insights with SNPs that have a large effect size and results that can be replicated).

Other Considerations before Undertaking Clinical Nutrigenetics/ Nutrigenomics

In GWAS or clinical nutrigenetics/nutrigenomic studies, a haplotype associated with the phenotype of interest can be identified. The identified polymorphism is rarely the actual phenotype-causing variant, but is more likely to be correlated, or in linkage disequilibrium with the functional SNP. Because SNP arrays do not assay every polymorphism in a genomic region, it is not possible to identify all the surrounding genetic variants that are correlated with the identified marker. However, we can define the boundaries within the gene where sequencing or subsequent fine-mapping experiments are appropriate [2].

Prototype Experiment in Nutrigenetics/Nutrigenomics: Studies on Choline Deficiency

The case study of the effects of genetic variation on dietary requirements for choline provides an excellent example of how clinical nutrigenetics/nutrigenomics can be used. In these studies, SNPs in the gene responsible for de novo biosynthesis of choline were associated with the risk for developing choline deficiency (phenotype). When young women were found to be resistant to developing choline deficiency, the role of estrogen in induction of choline biosynthesis was identified. In addition, the effects of choline on epigenetic regulation of gene expression were studied.

Choline Metabolism

Choline is involved in 3 major pathways: acetylcholine synthesis, methyl donation via betaine, and phosphatidylcholine synthesis [3]. Choline, via its irreversible oxidation to betaine [4], methylates homocysteine to form methionine. This is the precursor for synthesis of *S*-adenosylmethionine, the universal methyl donor needed for methylation of DNA, RNA and proteins. It is important to realize that choline, methionine and folate metabolism are inter-related at the step that homocysteine is methylated to form methionine [5]. Perturbing metabolism of one of the methyl-donors results in compensatory changes in the other methyl-donors due to the intermingling of these metabolic pathways [6–8]. Rats treated with the anti-folate, methotrexate, had diminished pools of choline metabolites in liver [7, 9]. Rats ingesting a choline-deficient diet had diminished tissue concentrations of methionine and *S*-adenosylmethionine [10] and doubled plasma homocysteine concentrations [11]. Humans who were choline deficient, even when fed adequate amounts of folic acid, had diminished capacity to methylate homocysteine [12].

Most of the foods we eat contain various amounts of choline, choline esters and betaine [13], and in 2004 the United States Department of Agriculture released a database on choline content in common foods (www.nal.usda.gov/fnic/foodcomp/Data/Choline/Choline.pdf). The foods with greatest abundance of choline are of animal origin, especially eggs and liver. Human breast milk also is a good source of free choline and choline esters [14], and the manufacturers of infant formulas have recently modified the content of choline compounds to levels similar with the ones in human breast milk [14, 15]. The only source of choline other than diet is de novo biosynthesis of phosphatidylcholine catalyzed by phosphatidylethanolamine-*N*-methyltransferase (PEMT) in liver. This enzyme uses *S*-adenosylmethionine as a methyl donor and forms a new choline moiety [16].

Studies in humans show that dietary choline is required (reviewed in [3] and discussed later). In 1998, the US Institute of Medicine (Food and Nutrition Board) established for the first time adequate intake (AI) and tolerable upper intake limit values for choline, based on limited human studies [17]. The AI is 550 mg/70 kg body weight, with upward adjustment in pregnant and lactating women; the upper intake limit ranges from 1,000 mg/day in children to 3,500 mg/day in adults [17]. For some age categories for which adequate data were missing, AI values have been set by extrapolating from adult values (for ages 1–18 years), and from infants (for ages 7–12 months) [17]. The 2005 NHANES survey reported that most people do not achieve the recommended AI for choline [18]. In participants from the Framingham Offspring Study the mean intake for total choline (energy adjusted) was below the AI values, with a mean intake of 313 mg/day; moreover, there was an inverse association between choline intake and plasma total homocysteine concentration in subjects with low folate intakes [19].

Consequences of Dietary Choline Deficiency in Humans

Using a clinical methodology for phenotyping individuals with respect to their susceptibility to developing organ dysfunction when fed a low choline diet [12, 20–22], adult men and women (pre- and postmenopausal) aged 18–70 years were admitted to the General Clinical Research Center, UNC Chapel Hill and fed a standard diet containing a known amount of choline (550 mg/70 kg/day; baseline). On day 11, subjects were placed on a diet containing <50 mg choline/day for up to 42 days. Blood and urine were collected to measure various experimental parameters of dietary choline status, and markers of organ dysfunction and liver fat were assessed. If at some point during the depletion period functional markers indicated organ dysfunction associated with choline deficiency, subjects were switched to a diet containing choline until replete. Most men and postmenopausal women fed low choline diets under controlled conditions developed reversible fatty liver (measure by mass resonance spectroscopy) as well as liver and muscle damage, while 56% of premenopausal women were resistant to developing choline deficiency [22]. This observation immediately suggested that estrogen moderated the dietary requirement for choline, and, indeed, estrogen induces the gene (*PEMT*) that makes endogenous synthesis of choline possible [23]. The classic actions of estrogen occur through its receptors ER α and ER β which bind as homodimers or heterodimers to estrogen response elements (EREs) in the promoters of many estrogen-responsive genes [24]. The consensus ERE (PuGGTCAnnnTGACCPy) [24] and some imperfect ERE half site motifs (ERE1/2) bind with ER α and ER β [25–27]. There are multiple EREs in the promoter region(s) of the *PEMT* gene [23] and estrogen caused a marked up-regulation in *PEMT* mRNA expression and enzyme activity in human hepatocytes [23]. Thus, premenopausal women have an enhanced capacity for de novo biosynthesis of choline moiety. During pregnancy, estradiol concentration rises from approximately 1 to 60 nM at term [28, 29], suggesting that capacity for endogenous synthesis of choline is highest during the period when females need to support fetal development.

Pregnancy and lactation are times when demand for choline is especially high. Large amounts of choline are delivered to the fetus across the placenta, where choline transport systems pump it against a concentration gradient [30, 31] and deplete maternal plasma choline in humans [32]. Plasma or serum choline concentrations are 6- to 7-fold higher in the fetus and newborn than they are in the adult [33, 34]. High levels of choline circulating in the neonate presumably ensure enhanced availability of choline to tissues. It is interesting that despite enhanced capacity to synthesize choline, the demand for this nutrient is so high that stores are depleted during pregnancy. Pregnant rats had diminished total liver choline compounds compared to non-mated controls and become as sensitive to choline-deficient diets as were male rats [35]. Because milk contains a great deal of choline, lactation further increases maternal demand for choline, resulting in further depletion of tissue stores [35, 36]. These observations suggest that women depend on high rates of *PEMT* activity, as well as on dietary intake of choline to sustain normal pregnancy. *Pemt*^{-/-} mice abort pregnancies at around 9–10 days of gestation unless fed supplemental choline (personal observation; [37]). As discussed later, choline nutrition during pregnancy is especially important because it influences brain development in the fetus [38–50].

Genetic Variation in Dietary Requirements for Choline

Though premenopausal women should be resistant to choline deficiency because of estrogen, a significant portion of them (44%) developed organ dysfunction when deprived of choline [22]. Genetic variation likely underlies these differences in dietary requirements. As noted earlier, *PEMT* encodes for a protein responsible for endogenous formation of choline, and 78% of female carriers of the variant (C) allele in a SNP in the promoter region of the

PEMT gene (rs12325817) developed organ dysfunction when fed a low choline diet (OR 25, $p < 0.00005$; p value based on 64 women studied) [51]. The frequency of this variant allele was 0.74 in North Carolina. The risk haplotype abrogated the induction of *PEMT* by estrogen, while the wild-type haplotype did not [Resseguie et al., manuscript submitted]. The SNP rs12325817 is not located in an estrogen response element but probably is in linkage disequilibrium with a functional SNP within such a response element.

Other SNPs in choline metabolism genes may have some influence on the dietary requirements for choline, though the p values for these associations are not as robust as for rs12325817. The first of 2 SNPs in the coding region of the choline dehydro-genase gene (*CHDH*; rs9001) had a protective effect on susceptibility to choline deficiency, while a second *CHDH* variant (rs12676) was associated with increased susceptibility [51]. We did not have the power in this study to identify any association of a SNP in the betaine:homocysteine methyltransferase gene (*BHMT*; rs3733890) with susceptibility to choline deficiency [51].

Genetic variants of genes in folate metabolism also modified the susceptibility of these subjects to choline deficiency [52]. Premenopausal women who were carriers of the very common 5,10-methylenetetrahydrofolate dehydrogenase-G1958A (*MTHFD1*; rs2236225) gene allele were more than 15 times as likely as non-carriers to develop signs of choline deficiency ($p < 0.0001$) on the low choline diet. Sixty-three percent of our study population had at least 1 allele for this SNP. The rs2236225 polymorphism alters the delicately balanced flux between 5,10-methylene tetrahydrofo-late and 10-formyl tetrahydrofolate and thereby influences the availability of 5-methyl THF for homocysteine remethylation [53]. This increases demand for choline as a methyl-group donor. It is of interest that the risk of having a child with a neural tube defect increases in mothers with the rs2236225 SNP [54]. We did not have sufficient power in the study to detect any effects of other folate metabolism SNPs (C677T and A1298C polymorphisms of the 5,10-methylene tetrahydrofolate reductase gene and the A80C polymorphism of the reduced folate carrier 1 gene) [52].

Choline and Neural Development

Rats and mice fed a low choline diet in late pregnancy (gestational days 12 to 17 in mice, days 12 to 18 or 20 in rats) had reduced neural progenitor cell proliferation and increased apoptosis in fetal hippocampus and cortex [38, 44, 55]. Similar outcomes were reported when pregnant mice are fed a low-folate diet [56], suggesting, again, potential synergistic mechanisms of action between folate and choline.

The mechanisms associating choline deficiency with decreased cell proliferation are, in part, related to the over-expression of cyclin-dependent kinase inhibitors (Cdkn) like p27Kip1 [40], p15Ink4b [40, 45] and Cdkn3 [45, 57], suggesting that choline deficiency inhibits cell proliferation by inducing G_1 arrest due to the inhibition of the interaction between cyclin-dependent kinases and cyclins. Using mouse hippocampal and cortical progenitor cells exposed to choline deficiency for 48 h, oligonucleotide-array analysis of gene expression showed expression changes in more than a thousand genes, of which 331 were related to cell division, apoptosis, neuronal and glial differentiation, methyl metabolism, and calcium-binding protein ontology classes [58], consistent with the phenotype of reduced cell proliferation, increased apoptosis, and increased differentiation.

Choline Deficiency Alters Gene Expression via Epigenetic Mechanisms

Neural development is influenced by DNA methylation. Overall levels of methylation decrease as neuronal differentiation proceeds [59] and the treatment of neural progenitor

cells with demethylating agents induces them to differentiate into cholinergic and adrenergic neurons [60]. Although the relationship between nutrition and epigenetics has been firmly established in the last few years [61], less is known about the role nutrition has in the epigenetic regulation of fetal brain development. Because dietary choline is an important player in the maintenance of the *S*-adenosylmethionine pool (the methyl donor for DNA methylation), along with folate and methionine, it is reasonable to expect that choline influences the epigenetic status of the developing brain. Global DNA methylation is decreased in the neuroepithelial layer of the hippocampus in choline deficient mouse fetal brains. Along with decreased global methylation, changes in gene-specific methylation were reported, where a cyclin-dependent kinase (*Cdkn3*) was hypo-methylated in its promoter by choline deficiency [45, 57] in the progenitor layer of the hippocampus. These alterations were associated with increased protein expression of this cyclin-dependent kinase inhibitor [45], and this model is consistent with previous findings showing that there is epigenetic regulation of cyclin-dependent kinase inhibitors that regulate cell proliferation [62].

Long-Lasting Consequences of Prenatal Choline Availability

The changes induced by dietary choline in fetal brain have long-lasting effects that alter brain function throughout life. Maternal dietary choline availability during late pregnancy was associated with long-lasting changes in the hippocampal function of the adult offspring. Choline supplementation during this period enhanced visuo-spatial and auditory memory in the adult rats throughout their life-span [63–67]. It also enhanced a property of the hippocampus, long-term potentiation [46, 68, 69]. The offspring from mothers fed a choline-deficient diet manifested opposite outcomes [64, 68].

Implications for Human Brain Development

It is always difficult to extrapolate findings reported using animal models to humans. However, limited data are available to support the hypothesis that similar mechanisms are involved in humans. Due to ethical constraints, no studies are available in children or pregnant mothers to validate the rodent model. Because the 2005 National Health and Nutrition Examination Survey (NHANES) data suggests that pregnant women do not consume adequate amounts of choline [18], and case-control studies in California suggest that women eating lower choline diets are at increased risk for giving birth to babies with neural tube defects [70] and cleft palate [71], the recommendation that pregnant women should attempt to consume diets adequate in choline seems reasonable. In addition, because half of the population has gene polymorphisms that affect choline and folate metabolism [52, 72], it is likely that different individuals may have different dietary requirements for choline and may need to pay special attention to choline intake during pregnancy.

Acknowledgments

This work was funded by grants from the National Institutes of Health (DK55865, AG09525). Support for this work was also provided by grants from the NIH to the UNC Nutrition & Obesity Research Center (DK56350).

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