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Lymphocyte gene expression in subjects fed a low-choline diet differs between those who develop organ dysfunction and those who do not[2]

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Abstract

Background—Some humans fed a low-choline diet develop hepatosteatosis, liver and muscle damage, and lymphocyte apoptosis. The risk of developing such organ dysfunction is increased by the presence of single-nucleotide polymorphisms (SNPs) in genes involved in folate and choline metabolism.

Objective—We investigated whether these changes that occur in the expression of many genes when humans are fed a low-choline diet differ between subjects who develop organ dysfunction and those who do not. We also investigated whether expression changes were dependent on the presence of the SNPs of interest.

Design—Thirty-three subjects aged 20−67 y were fed for 10 d a baseline diet containing the recommended adequate intake of choline. They then were fed a low-choline diet for up to 42 d or until they developed organ dysfunction. Blood was collected at the end of each phase, and peripheral lymphocytes were isolated and used for genotyping and for gene expression profiling with the use of microarray hybridization.

Results—Feeding a low-choline diet changed the expression of 259 genes, and the profiles of subjects who developed and those who did not develop signs of organ dysfunction differed. Group clustering and gene ontology analyses found that the diet-induced changes in gene expression profiles were significantly influenced by the SNPs of interest and that the gene expression phenotype of the variant gene carriers differed significantly even with the baseline diet.

Conclusion—These findings support our hypothesis that a person's susceptibility to organ dysfunction when fed a low-choline diet is modulated by specific SNPs in genes involved in folate and choline metabolism.

Keywords

Diet; choline; gene expression; lymphocytes; single-nucleotide polymorphism; methylenetetrahydrofolate dehydrogenase; MTHFD; phosphatidylethanolamine methyltransferase; PEMT; choline dehydrogenase; CHDH; choline deficiency

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INTRODUCTION

Choline is an essential dietary nutrient involved in a multitude of metabolic roles (1). It is a major source of methyl groups for methionine synthesis and is needed for the structural integrity of cell membranes, the transport of lipids from the liver, and cholinergic neurotransmission (1). Adequate intake (AI) recommendations for choline have been established (2), and, in population studies, diets low in choline were associated with a greater risk of birth defects (3,4) and with high homocysteine concentrations in blood (5). We previously reported that some humans fed a diet low in choline developed hepatosteatosis, experienced liver and muscle damage, and had greater lymphocyte apoptosis (6–10). All of these changes were reversed after a period of choline repletion. The susceptibility to these outcomes was not the same in all subjects; it varied with the presence of single-nucleotide polymorphisms (SNPs) within the genes involved in folate and choline metabolism (7,8). With the notable exception of the carriers of the choline dehydrogenase (CHDH) variant allele (318 A→C; rs9001), who were protected against developing organ dysfunction (8), carriers of the methylenetetrahydrofolate dehydrogenase (MTHFD1) synthase variant (1958 G→A; rs2236225), the CHDH variant (432 G→T; rs12676), and the phosphatidylethanolamine methyltransferase (PEMT) variant (−744 G→C; rs12325817) alleles were at greater risk of developing organ dysfunction when fed a low-choline diet than were the carriers of the corresponding wild-type alleles (7,8).

In this study, we fed humans a diet containing recommended amounts of choline, then fed the same subjects diets low in choline content, and then assessed gene expression in lymphocytes at the end of each feeding period. We determined whether ingesting a low-choline diet was associated with changes in gene expression, whether those subjects who developed organ dysfunction differed in gene expression from those who did not, and whether changes in gene expression were related to the presence of SNPs.

SUBJECTS AND METHODS

Subjects

Healthy men ($n = 31$) and women ($n = 35$) were recruited for the study. Inclusion was contingent on an age-typical good state of health as determined by physical examination and standard clinical laboratory tests, such as complete blood count, blood chemistries, and fasting lipids and liver function tests and on the absence of known chronic diseases. Twenty-two subjects admitted to the study had minor elevations in blood lipids that were not deemed of clinical significance by the study physician. Of the originally recruited 66 subjects, 61 completed at least the initial and depletion phases. Of those 61 subjects, 1 was excluded because of a 9-kg weight loss during the study, 3 were excluded because they did not comply with diet restrictions, and 6 were excluded because they did not have baseline measurements; thus, 51 subjects were included in analyses. Of those subjects, 18 were excluded from the gene array analyses (*see* Microarray data analysis). The remaining 14 men and 19 women ranged in age from 20 to 67 y and had a body mass index (BMI; in kg/m^2) between 19 and 31, which they maintained throughout the study. The ethnic distribution of these participants was 61% white, 30 African American, and 9% Asian, which reflects the local population characteristics of the Raleigh-Durham-Chapel Hill area.

Written informed consent was obtained from all participants. The study protocol was approved by the Institutional Review Board at the University of North Carolina at Chapel Hill (UNC-CH).

Study design

The participants were admitted to the UNC-CH General Clinical Research Center, where they remained under the supervision of study staff for the duration of the study. Research diets administered to the subjects, which were composed of 0.8 g high-biologic-value protein/kg body wt, with 30% of energy from fat and 70% of energy from carbohydrate, were prepared in-house to protocol specifications and have been described in detail elsewhere (11). Total food intake was adjusted to be isocaloric and to provide adequate intakes of macronutrients and micronutrients. Initially, all participants received a diet of commonly eaten foods containing 550 mg choline · 70 kg body wt^{-1} · d^{-1}—the presumed AI (2)—and 400 dietary folate equivalents (DFE)/d. The dietary choline content was confirmed as described previously (11), and the folate content was calculated by using the US Department of Agriculture SR16 database and PRONUTRA software (version 3.1.0.13; Viocare, Princeton, NJ). After 10 d of this baseline diet, liver fat was measured, and 48 mL blood was collected by venipuncture and processed for peripheral lymphocytes as described below. The subjects were randomly assigned to 2 groups—diet folate only or diet folate supplemented with 400 μg folic acid/d—and then were fed a diet in which the choline content was reduced to <50 mg/d, as confirmed by analysis of duplicate food portions. For the rest of the study, all diets offered to the diet-folate-only group contained 100 DFE/d, whereas the folic acid–supplemented group received an additional 668 DFE/d. Periodic measurements of urinary choline and betaine concentrations (12) were used to confirm compliance with the dietary restrictions. Subjects followed this depletion diet until they developed organ dysfunction associated with choline deficiency or for 42 d if they did not develop organ dysfunction. Blood (48 mL) was again collected at the end of the depletion phase. Humans were deemed to have organ dysfunction associated with choline deficiency if they had a >5-fold increase in serum creatine phosphokinase (CPK) activity (measurements were taken every 3−4 d) or if they had a >28% increase in liver fat content while following the choline-depletion diet (measurements were taken on days 21 and 42 of the choline-depletion diet) and if this increase in CPK activity or liver fat content was resolved when choline was returned to the diet. The change from baseline in liver fat content was estimated by using magnetic resonance imaging (MRI) in a clinical magnetic resonance system (Vision 41.5T; Siemens Medical Solutions, Malvern, PA) with a modified “In and Out of Phase” procedure that was described previously (13). Fat content was derived from measurements across 3−5 liver slices per subject and standardized to similarly measured slices of spleen.

Isolation of lymphocyte RNA and quality assessment

At the end of the baseline and choline-depletion diet phases, peripheral lymphocytes were isolated from blood within 2 h of collection by using Ficoll-Hypaque gradient in evacuated tubes with sodium citrate (Vacuatainer CPT tubes; Becton Dickinson, Franklin Lakes, NJ), and suspended in TRIzol Reagent (Invitrogen, Carlsbad, CA). Total RNA was isolated according to the manufacturer's protocol, purified with the use of an RNeasy kit (Qiagen, Valencia, CA), and diluted to a standard concentration of 100 μg/mL. The subsequent procedures were performed at the UNC-CH Genomics Core Facility. Each sample (0.5 μg), including the reference RNA, was first tested on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) to ensure high-quality RNA before being diluted for hybridization.

Genotyping

Genomic DNA was prepared from peripheral blood with a commercial extraction kit (PureGene; Gentra Systems, Minneapolis, MN) according to the manufacturer's instructions and diluted to a standard concentration of 1 μg/mL. The cytoplasmic MTHFD synthase (MTHFD1-G1958A) was amplified by multiplex polymerase chain reaction (PCR), purified,

and then analyzed with matrix-assisted laser desorption ionization time-of-flight mass spectrometry (7,14). For the *PEMT* and *CHDH* genes, DNA sequencing was performed on double-stranded DNA templates obtained from genomic DNA by using PCR amplification (8).

Microarray hybridization and data retrieval

We mixed 12 μg of sample and an equal amount of universal human reference RNA (Stratagene, La Jolla, CA) with anchored oligo-dT Primer mixture, and amplified the mixture by using a thermal cycler (70 °C for 5 min to anneal; 42 °C for 1.5 h). The cDNA was then labeled in a reverse transcriptase reaction with dUTP CyDye–labeled nucleotides (Cy3 for reference and Cy5 for experiment) by using a CyScribe First-Strand cDNA Labeling Kit [GE Healthcare (formerly Amersham Biosciences), Piscataway, NJ]. The labeled cDNA was then purified by degrading the mRNA with NaOH at 37 °C for 15 min and washing the samples with Tris-EDTA buffer by using a Microcon PCR filter (Millipore, Bedford, MA). The combined Cy3+Cy5 sample was then applied to a glass slide array, sealed in a hybridization chamber, and incubated in a 65 °C water bath overnight. The human oligo-arrays with 16 000 spots were produced at the Genomics Core Facility in the same batch by using 60-mer oligonucleotides (Compugen USA, Jamesburg, NJ). After hybridization, the array slides were washed and spun dry before being scanned to collect the fluorescent images (GenePix 4000B fluorescent scanner; Axon Instruments, Union City, CA). Images were gridded, and data were collected with the use of GENEPIX PRO microarray acquisition and analysis software (version 5.0; Axon Instruments). Detailed protocols are available at http://cancer.med.unc.edu/genomicscore/. Images obtained were analyzed by superimposing a grid for each array with the use of the GENEPIX PRO software. All spots of poor quality (as determined by visual inspection) were flagged as bad and removed from further analysis.

Microarray data analysis

All of the collected raw data files were further processed by uploading them into the UNC Microarray Database (https://genome.unc.edu/), and the data were filtered and retrieved according to the following criteria: *1*) data were retrieved by the immutable Stanford University Identification (SUID) reference number to average the replicate spots by gene name and present the result as one (ie, to collapse them); *2*) spots were selected only if they had both Channel 1 and Channel 2 Lowess-normalized means ≥30% above background; and *3*) genes were selected only if they had >70% good data. No cutoffs were selected. Final data were expressed as log (base 2) of lymphocyte RNA-reference RNA Lowess normalized ratio (mean). The final number of arrays (and hence the number of subjects included in further analyses) was influenced by various criteria: not all RNA samples were of good quality, some blood samples did not contain enough RNA, and not all arrays passed the quality test. Therefore, of the 51 subjects initially included in the study, only 33 had arrays that were suitable for analysis. Of these 33, not all had 2 arrays (1 for baseline and 1 for depletion), so the number of arrays available at baseline was 30, and the number of arrays available at depletion was 25 (Table 1).

Statistical analysis of gene expression

Subjects were grouped for 12 comparisons (Table 1) according to the following criteria: presence or absence of organ dysfunction when fed a low-choline diet (hepatosteatosis and liver and muscle damage); timepoint (end of the baseline phase or end of the depletion phase); and the presence of a specific SNP for *PEMT*, *MTHFD*, and *CHDH*. Significance analysis of microar-rays (SAM) (15) was applied to the final data by using TIGR MEV software [version 3; Dana-Farber Cancer Institute, Boston, MA (16)]. We used the one-class response type and 100 permutations or the maximum allowed permutations (if <100) to select the genes that are significantly changed across each of the groups. An arbitrary false discovery rate (FDR) of

maximum 5% was chosen, and the closest threshold value (delta, or Δ) was selected for each group. The FDR is the expected percentage of false predictions; therefore, at 5% FDR, 95% of the observations are reproducible and not due to chance. The software generated a list of significantly overexpressed and underexpressed genes (*d* score assessment), and the *q* value (the lowest FDR at which the gene is called significant) was computed for each gene. The data generated by the use of SAM were converted accordingly for subsequent gene ontology (GO) classification: −1 for underexpression, 0 for no change, and 1 for overexpression. The defined groups (Table 1) were clustered according to their changes in gene expression (16). Each group was considered as an experiment, and gene changes were expressed as an average of all the arrays within the group.

Cluster analysis

The TIGR MEV software was used for cluster analysis, in which groups defined in Table 1 were clustered according to changes in gene expression (post-SAM analysis). Hierarchical clustering was assessed by using the average Euclidean distance between groups.

Gene ontology classification

GOMINER software (version 1.22; Georgia Tech University, Atlanta, GA; Internet: http://discover.nci.nih.gov/gominer/) was used to construct a GO list of the significantly changed genes (17), by using gene symbols as identifiers. Data from the SAM output file were converted to text files and GOMINER generated a list of genes classified by their various GO classes, according to the default database (com.mysql.jdbc.Driver at jdbc:mysql://discover.nci.nih.gov/GEEVS). Fisher's exact tests were performed to determine the significance of changes within the total number of genes in each GO class, and significance was separately assessed for the number of genes that were overexpressed, underexpressed, or both ($P < 0.05$).

Gene expression validation

A small subset of genes was selected to determine the validity of the array-generated data. Real time reverse transcriptase (RT)–PCR was used to assess the expression of 4 of the most overexpressed genes and 4 of the most underexpressed genes. Primers for these 8 genes—*FOXA1*, *PRAME*, *TERT*, *CDCA8*, *CHEK1*, *IL2RB*, *TNFAIP3*, and *NFKBIA*—were purchased from SuperArray (Frederick, MD) as was 18S rRNA, which was used as the normalization gene. Equal amounts of RNA were pooled from all subjects at baseline and depletion, respectively. For all genes but one, 100 ng pooled template RNA was used in quintuplicate reactions (one-step RT-PCR) in a QuantiTect SYBR Green RT-PCR kit (Qiagen); for 18S RNA, 10 pg template RNA was used. All reactions were performed on an iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) under the following cycling conditions: reverse transcription for 30 min at 50 °C; initial activation for 15 min at 95 °C; and 45 cycles of denaturation for 15 s at 94 °C, annealing for 30 s at 55 °C, and extension for 30 s at 72 °C, which were followed by data acquisition for the melting curves. The differences and statistical assessment in gene expression were computed by using the comparative threshold ($\Delta\Delta C_T$) method with the REST384 add-in for EXCEL (Internet: http://www.gene-quantification.de/) (18), and gene expression changes were expressed as 18S-normalized ratios between depletion and baseline. Statistical significance of change was assessed by using both the Student *t* test and the nonparametric pairwise fixed reallocation randomization test provided by REST (18).

Regulation of gene expression by DNA methylation

We used the DNA Methylation Database (Internet: http://www.methdb.de/; last accessed 12/05/2006) to compare genes found to be changed by dietary choline with those reported to have their expression regulated by DNA methylation (19).

RESULTS

Changes in gene expression between baseline and depletion

Of the 12 034 unigenes present within the arrays, 152 were underexpressed, and 107 were overexpressed in subjects after the low-choline diet phase of the study. A list of selected genes (the 20 most overexpressed and the 20 most underexpressed genes) is shown in Table 2. When subjects were further subclassified according to the absence or presence of signs of organ dysfunction (fatty liver, increased plasma CPK, or both) after being fed a low-choline diet, the gene expression profiles of the 2 groups could be differentiated (Table 2). From the ALL SUBJECTS group, we chose a limited number of genes to validate the array data by real-time RT-PCR (Table 3). With the exception of *CHEK1*, changes in gene expression, given as the relative ratio between depletion and baseline, were confirmed as significant by one or both of the statistical tests (*t* test and the pairwise fixed reallocation randomization test).

Hierarchical clustering between defined groups

Using the data obtained from SAM analysis, we clustered the groups defined in Table 1 to determine whether specific changes in gene expression profiles were associated with the presence of signs of organ dysfunction when subjects were following a low-choline diet, with the presence of PEMT, CHDH, and MTHFD1 SNPs, or both. In addition, we investigated whether gene expression profiles at baseline were different between these groups (which would suggest a different phenotype at baseline). The tree node of the groups, each one being considered an experiment, is shown in Figure 1. According to the node distribution, 2 main branches were generated for all the experiments. One branch included the *PEMT* and *MTHFD1* groups surrounding the group with all subjects at the end of the depletion phase [ALL(D)]. The other main branch includes both *CHDH* polymorphisms, and the groups based on the presence (SIGNS) or the absence (NO SIGNS) of signs of organ dysfunction, all centered around the main ALL SUBJECTS group. The ALL SUBJECTS, SIGNS, and NO SIGNS groups were used to compare gene expression at the end of the depletion phase and the end of the baseline phase. ALL(D) compared the SIGNS group with the NO SIGNS group at the end of the depletion period. Four clusters were identified with the smallest distance: *1*) subjects with the PEMT SNP fed a low-choline diet [PEMT(D)] associated with women with the PEMT SNP fed a low-choline diet [PEMT(W)]; *2*) ALL(D) associated with subjects with the MTHFD1 SNP fed a low-choline diet [MTHFD1(D)]; *3*) ALL SUBJECTS associated with subjects who developed organ dysfunction when fed a low-choline diet (SIGNS); and *4*) one of the CHDH SNPs [CHDH(G→T)] associated with the FOLATE group.

Changes in gene expression across all groups

When the SAM analysis was performed for all groups defined in Table 1, changes for 1054 unigenes were significant. A complete list of these genes is available at http://www.unc.edu/zeisel_lab/. On the basis of our GO analysis, we present data for a limited number of genes (Figure 2) that are included in the following GO classes of special interest for our human study: folate-thymidine metabolism (GO 19860, 42083, and 46104), apoptosis (GO 6915, 6916, 6917, 8632, 30693, 43027, 43065, and 43154), DNA damage-repair (GO 77, 724, 725, 31570, 42770, and 51908), cell cycle regulation-proliferation (GO 4861 and 8283), immune-inflammatory response and lymphocyte differentiation-activation (GO 1772, 6952,

6954, 6955, 19731, 19732, 19864, 30098, 42113, 42742, and 50832), epigenetic regulation (GO 8327, 35035, and 45815), and telomere maintenance (GO 3720, 3721, 5697, and 42162).

Gene ontology analysis

GO analysis was performed for all genes deemed significantly changed within any of the groups defined in Table 1. Fisher's exact test indicated either unidirectional (up or down) or bidirectional (both up and down) changes for 1216 GO classes (a complete list of changes within GO classes is available at http://www.unc.edu/zeisel_lab/). Changes across selected GO classes listed above are shown in Table 4, where the vast majority of changes are in one direction, which suggests that, within each GO class, changes in gene expression are functionally convergent.

Genes regulated by DNA methylation

Previous reports in cell culture and animal model systems show that dietary choline modulates gene expression by altering DNA methylation (20,21), but no data are yet available in humans fed a low choline diet. Using the only available public database on DNA methylation (*see* Subjects and Methods), we identified 13 of the 1024 genes changed in our study that were reported to be regulated by DNA methylation in various studies using human tissues or human cells in culture (Table 5).

DISCUSSION

We found that a low-choline diet induced changes in lymphocyte gene expression in humans. These changes included (but were not limited to) changes in expression of genes functionally involved in folate metabolism, apoptosis, DNA damage-repair, cell cycle regulation, immune response, epigenetic regulation, and telomere maintenance, which suggested that dietary choline deficiency can alter the functionality of many pathways. Subjects who developed organ dysfunction while following a low-choline diet differed from those who did not develop organ dysfunction in their expression of many genes, including some of those related to any or all of apoptosis, the DNA integrity checkpoint, and genes of cell cycle regulation. Subjects with the PEMT (rs12325817) and MTHFD1 (rs2236225) SNPs, previously shown to predispose a person to developing organ dysfunction when fed a low-choline diet (7,8), differed at baseline from those subjects without the SNP in their expression of apoptosis, the DNA damage checkpoint, and cell proliferation control genes, which suggests that they are phenotypically different even before a low-choline diet is administered.

In different comparison groups, choline deficiency induced different patterns of change (Figure 1). Moreover, many of the results reported within all subjects (baseline compared with depletion, ALL SUBJECTS) could be misleading because these changes were not homogenous when subjects were classified on the basis of signs of organ dysfunction (Table 2 and Figure 2). For instance, 3 of the genes with the largest change in expression (*CHEK1*, *GBE1*, and *KIF20A*) were differently expressed in the NO SIGNS group than in the SIGNS group (Table 2). *CHEK1* is involved in DNA repair in human T lymphocytes (22). *GBE1* is required for sufficient glycogen accumulation and is normally underexpressed in whole human blood (23). *KIF20A* regulates the transport of Golgi membranes and associated vesicles along microtubules (24).

By clustering the groups according to genotype (Figure 1), we found that different patterns in gene expression do indeed support this classification. For example, the previously reported (8) protective *CHDH* (318 A→C) genotype grouped close to the NO SIGNS group—those who had no clinical symptoms while following the low-choline diet—whereas the *CHDH* (432 G→T) genotype, which was reported to increase susceptibility to choline deficiency (8),

grouped with the SIGNS group. Moreover, the NO SIGNS and SIGNS groups were intercalated by the ALL SUBJECTS group, which supports the heterogeneity of the previously reported responses to dietary choline deficiency. Included in the other arm from the clustering analysis were the *PEMT* and *MTHFD1* genotypes, which grouped around the ALL(D) group. This cluster supported our hypothesis that genetic differences account for the presence or absence of organ dysfunction in humans depleted of choline (7,8). This analysis also found that the presence of the *PEMT* and *MTHFD1* genotypes could confer differences in the phenotypes at baseline, which suggests that different persons may have different susceptibility to dietary choline deficiency and that the risk of choline deficiency is greater in women who are carriers of the *PEMT* allele [this group was closer to the ALL(D) group than to the PEMT(D) group]. Harder to interpret is the unexpected clustering of the FOLATE group (those receiving folate supplements versus those who did not) close to the *CHDH* (432 G→T) genotype.

To construct a better picture of the potential functional significance of these gene expression patterns, we used GO analysis to group genes according to their functional roles. The heterogeneity of the response to a low choline diet was also shown at this level of analysis. Genes involved in folate metabolism were most affected in the carriers of the *MTHFD1* and *CHDH* (432 G→T) alleles (Table 4). A different pattern was observed for genes involved in apoptosis (Table 4), in which the *MTHFD1* polymorphic allele carriers were the most affected (increases in apoptosis, induction of apoptosis, caspase activity, positive regulation of apoptosis, and decreased expression of genes involved in negative regulation of caspase activity). This result is consistent with our previous data, which showed that humans who developed organ dysfunction when fed a choline-deficient diet were more likely to have the *MTHFD1* variant allele (7) and to have increased lymphocyte DNA damage and apoptosis, as measured by caspase activity, than were those who did not develop organ dysfunction (9). It is interesting that the same trends were present in these subjects at baseline, which suggests that their higher susceptibility to choline deficiency may be due to effects present even when subjects are consuming a normal diet. For carriers of the *PEMT* polymorphic allele, the most affected genes were grouped within GO classes involved in telomere maintenance; notably, these alterations were more extensive in female *PEMT* allele carriers. Telomeres are the protein-DNA structures that protect chromosome ends from being recognized as double-stranded DNA breaks, and their maintenance is important for cell longevity, normal cell cycling, and prevention of cancer (25).

Our findings suggest that dietary choline deficiency may affect the homeostatic mechanisms responsible for telomere maintenance, perhaps by epigenetic changes in gene expression. The folate status of subjects had little effect on the gene expression changes seen in GO analyses.

Some genes that are regulated by DNA methylation were also identified as being changed by a low-choline diet (Table 5). In cultured human neuroblastoma cells and in rodent models, choline deficiency alters both global and gene-specific DNA methylation and the expression of these genes (20,21). Therefore, we suggest that the observed choline deficiency–induced changes in gene expression occurred because of altered methylation in promoter regions of the genes involved. Among the genes changed in choline deficiency that are known to be regulated by gene methylation, the insulin-like growth factor 2 (*IGF2*) is an imprinted gene; loss of imprinting is associated with cancer in various experimental models (as reviewed in reference 26). Another gene identified as being changed by dietary choline and known to be regulated by methylation is telomerase reverse transcriptase (*TERT*), the product of which is the protein component of a ribonucleoprotein polymerase that maintains telomere ends by addition of the telomere repeat TTAGGG, and its deregulation is involved in both cellular senescence (telomere shortening) and carcinogenesis in leukemic cells (27).

In conclusion, dietary choline deficiency induced changes in gene expression profiles in human lymphocytes, and these patterns correlated with the occurrence of organ dysfunction and apoptosis in humans fed a low-choline diet. These outcomes also correlated with polymorphisms in genes that regulate folate and choline metabolism. Further studies are required to determine whether these changes are regulated by epigenetic mechanisms and to identify other populations at risk for dietary choline deficiency.

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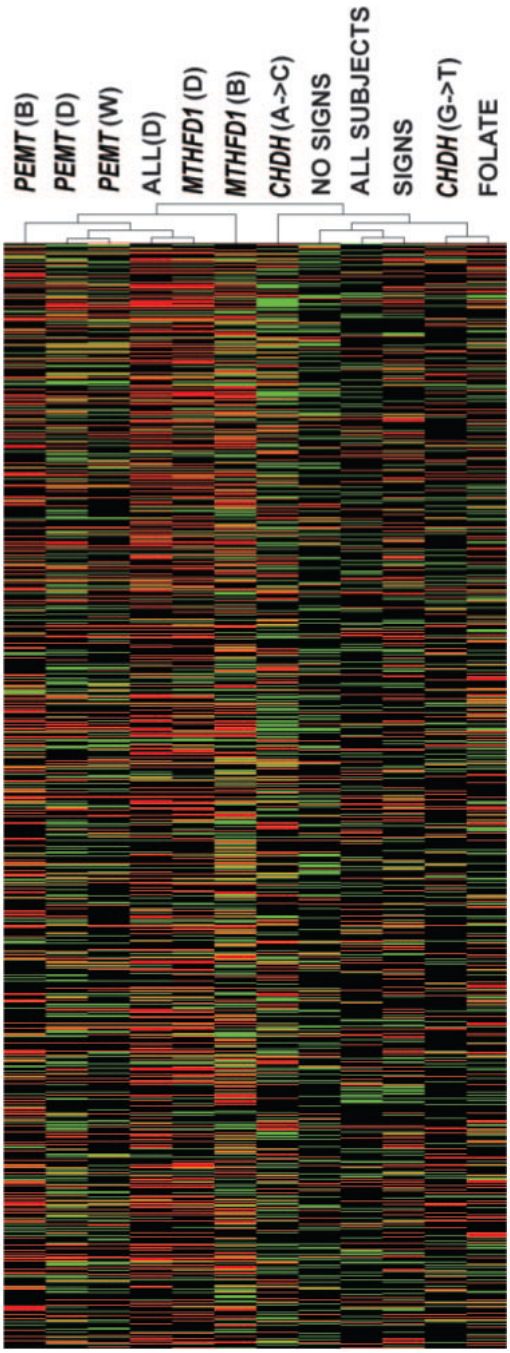

FIGURE 1.

Cluster analysis. All comparison groups defined in Table 1 were subjected to cluster analysis (*see* Materials and Methods) to determine the distance of change between groups, as defined by the node structure indicated. ALL SUBJECTS, comparison of all subjects (the end of the depletion phase compared with the end of the baseline phase); SIGNS, the subjects who developed organ dysfunction when fed the low-choline diet; NO SIGNS, the subjects who did not develop organ dysfunction (comparing the end of the depletion phase with the end of the baseline phase). For each polymorphism, the gene symbol is indicated. The choline dehydrogenase single-nucleotide polymorphisms are indicated by the nucleotide replacement, and the sample is at the end of depletion phase. The notation "(B)" after the gene symbol

indicates the sample after the baseline (adequate choline) phase; “(D)” after the gene symbol indicates the sample after the low-choline diet phase; “(W)” after the gene symbol indicates women only after the depletion phase. FOLATE compares subjects with and without folate supplementation at the end of the depletion phase. The number of arrays for each comparison is provided in Table 1. Red indicates overexpression, green indicates underexpression, and black indicates no change in gene expression.

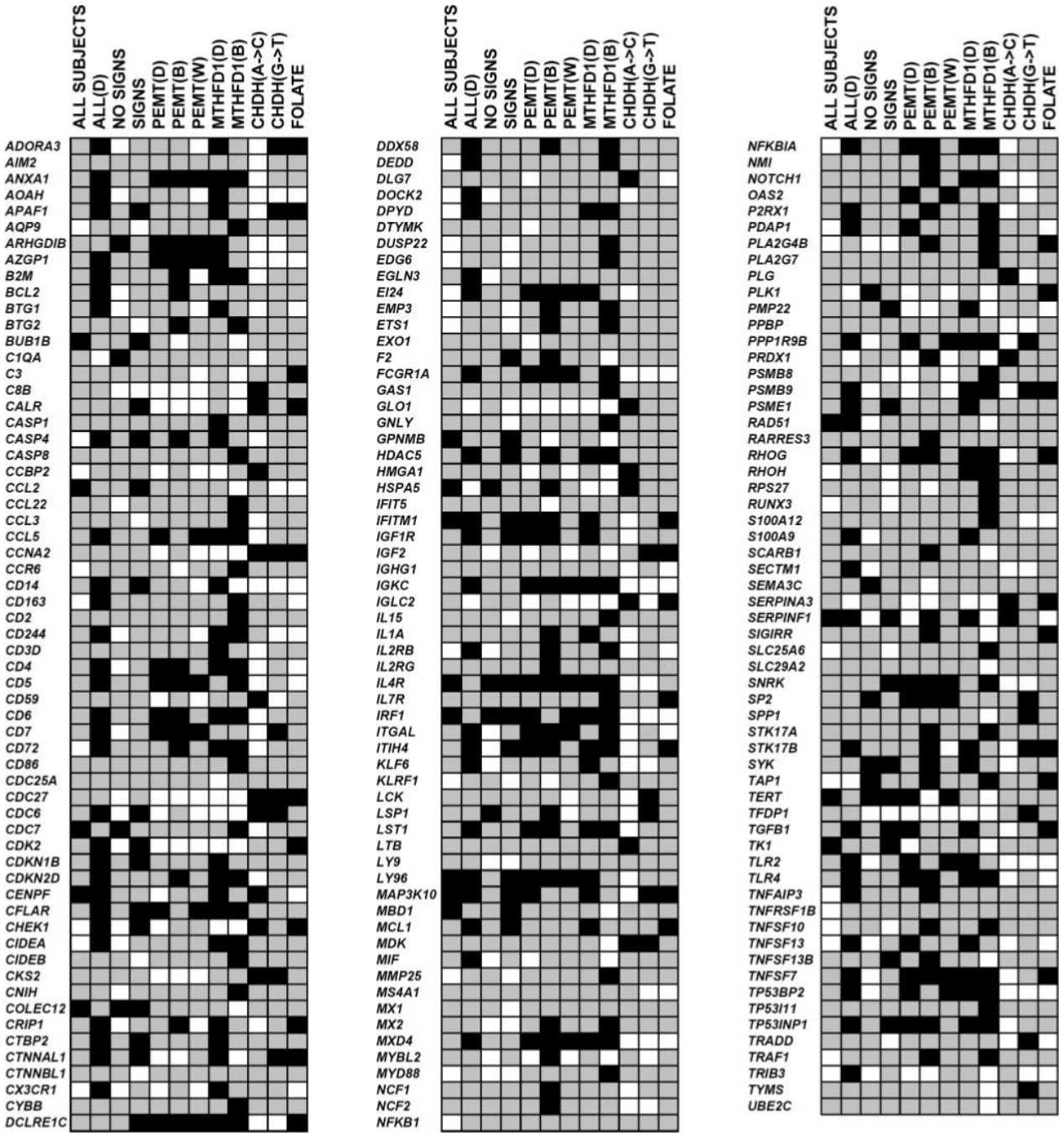

FIGURE 2.

Gene expression profiles across all groups. The computed change in expression is shown for each gene included in gene ontology (GO) classes pertaining to folate-thymidine metabolism, apoptosis, DNA damage-repair, cell cycle regulation-proliferation, immune-inflammatory response and lymphocyte differentiation-activation, epigenetic regulation, and telomere maintenance. ■, overexpression; ▫, no change; □, underexpression within each comparison group (by significance analysis by microarray; the false discovery rate was <5%) as indicated in Table 1. ALL SUBJECTS, comparison of all subjects (the end of the depletion phrase compared with the end of the baseline phase); SIGNS, the subjects who developed organ dysfunction when fed the low-choline diet; NO SIGNS, the subjects who did not develop organ

dysfunction (comparing the end of the depletion phase with the end of the baseline phase). For each polymorphism, the gene symbol is indicated. The choline dehydrogenase single-nucleotide polymorphisms are indicated by the nucleotide replacement, and the sample is at the end of depletion phase. The notation "(B)" after the gene symbol indicates the sample after the baseline (adequate choline) phase; "(D)" after the gene symbol indicates the sample after the low-choline diet phase; "(W)" after the gene symbol indicates women only after the depletion phase. FOLATE compares subjects with and without folate supplementation at the end of the depletion phase. The number of arrays for each comparison is provided in Table 1.

Table 1

Groups for analyses

Analysis group	Symbol	Analysis group	Arrays	Genes changed
			n	*n*
All subjects	ALL SUBJECTS	Depletion vs Baseline	25vs30	259
Subjects with no organ dysfunction on low-choline diet	NO SIGNS	Depletion vs Baseline	12vs14	293
Subjects with organ dysfunction on low-choline diet	SIGNS	Depletion vs Baseline	13vs16	339
All subjects at depletion	ALL(D)	Signs vs No Signs	13vs12	416
PEMT(−744 G→C) group at baseline	PEMT(B)	SNP vs WT	22vs 8	357
PEMT(−744 G→C) group at depletion	PEMT(D)	SNP vs WT	19vs 6	441
PEMT(−744 G→C) group at depletion (women)	PEMT(W)	Women with SNP vs All WT	13vs 6	325
MTHFD1(−1958 G→A) group at baseline	MTHFD1 (B)	SNP vs WT at baseline	19vs11	558
MTHFD1(−1958 G→A) group at depletion	MTHFD1 (D)	SNP vs WT at depletion	17vs 8	409
CHDH(318 A→C) group at depletion	CHDH(A→C)	SNP vs WT at depletion	9vs16	450
CHDH(+432 G→T) group at depletion	CHDH(G→T)	SNP vs WT at depletion	9vs16	233
FOLATE group at depletion	FOLATE	Folate supplementation vs no supplementation at depletion	13vs12	360

[1] ALL SUBJECTS, comparison of all subjects (depletion phase versus end of baseline); SIGNS, subjects who developed organ dysfunction when fed the low-choline diet; NO SIGNS subjects who did not develop organ dysfunction (end of depletion phase versus end of baseline); ALL(D), comparison of all subjects at the end of the low-choline diet (clinical signs versus absence of signs). For each polymorphism, single-nucleotide polymorphism (SNP) denotes the presence of the respective mutation, and WT is the wild-type genotype (*see* Introduction for genotype descriptions). The SNPs are indicated by the nucleotide position relative to the transcription starting site and by the nucleotide replacement. “(B)” after a gene symbol indicates the sample after the baseline (adequate choline) phase; “(D)” after a gene symbol indicates the sample after low-choline diet phase; “(W)” after a gene symbol indicates women only after the low-choline diet; FOLATE compares to subjects with and without folate supplementation at the end of the low-choline diet.

Table 2

Selected genes with expression changes between baseline and depletion[1]

Gene symbol	Gene name	*d* Score		
		ALL SUBJECTS (*n* = 25–30)	NO SIGNS group (*n* = 13–16)	SIGNS group (*n* = 12–14)
ALDH1A2	Aldehyde dehydrogenase 1 family, member A2	3.9	4.0	3.6
ARHGAP9	Rho GTPase—activating protein 9	−5.0	−4.5	=[2]
BUB1B	Budding uninhibited by benzimidazoles 1 homolog β	4.7	=	4.9
CCL2	Chemokine (C-C motif) ligand 2	4.0	=	3.9
CD163	CD163 molecule	−4.0	=	=
CD36	CD36 molecule (thrombospondin receptor)	−7.3	−8.5	=
CDCA8	Cell division cycle—associated 8	4.1	4.2	3.9
CHEK1	CHK1 checkpoint homolog	−5.0	−7.2	3.1
COLEC12	Collectin subfamily member 12	4.7	4.6	4.6
CST3	Cystatin C (amyloid angiopathy and cerebral hemorrhage)	−4.2	−3.7	=
CTSL	Cathepsin L	4.0	4.7	=
CX3CR1	Chemokine (C-X3-C motif) receptor 1	−8.5	−7.9	=
DOCK2	Dedicator of cytokinesis 2	−4.1	−4.2	=
FOXA1	Forkhead box A1	6.0	7.3	4.9
GBE1	Glucan (1,4-α), branching enzyme 1	4.2	−1.4	4.0
GZMK	Granzyme K (granzyme 3; tryptase II)	−4.5	=	=
HSPA5	Heat shock 70-kDa protein 5 (glucose-regulated protein, 78 kDa)	4.0	4.9	=
HSPCB	Heat shock protein 90-kDa α (cytosolic), class B member 1	−4.4	=	−3.8
IL2RB	Interleukin 2 receptor β	−8.2	−7.8	=
KIF20A	Kinesin family member 20A	5.4	−1.1	4.2
KIF23	Kinesin family member 23	3.9	4.8	3.5
KLRF1	Killer cell lectin—like receptor subfamily F, member 1	−4.3	=	−3.8
MAL2	Mal, T cell differentiation protein 2	5.8	6.1	5.4
MARCH1	Membrane-associated ring finger (C3HC4) 1	−4.3	=	−3.8
MGP	Matrix Gla protein	6.4	6.5	=
MMP2	Matrix metallopeptidase 2	4.7	4.8	=
MYOM2	Myomesin (M protein) 2, 165 kDa	−4.0	=	−4.0
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B cell inhibitor α	−4.1	=	=
PDAP1	PDGFA-associated protein 1	−4.4	=	=
PLAUR	Plasminogen activator, urokinase receptor	−4.3	=	=
PRAME	Preferentially expressed antigen in melanoma	5.9	6.9	=
PTPRCAP	Protein tyrosine phosphatase, receptor type, C-associated protein	4.4	=	4.5
RGS19	Regulator of G protein signaling 19	−4.6	−4.1	=
RNPEPL1	Arginyl aminopeptidase (aminopeptidase B)—like 1	−4.5	−3.7	=
SFRP1	Secreted frizzled-related protein 1	5.1	5.8	4.5
SLC7A7	Solute carrier family 7 (cationic amino acid transporter, y + system), member 7	−5.9	−5.6	−5.8
SMARCA1	*SWI/SNF*-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 1	5.9	=	4.5
TERT	Telomerase reverse transcriptase	4.7	3.8	5.3
TNFAIP3	Tumor necrosis factor-α—induced protein 3	−4.3	=	=
UCK1	Uridine-cytidine kinase 1	5.1	=	5.6

[1] Listed are the 20 most overexpressed and the 20 most underexpressed genes. Changes in the genes are presented as *d* scores. All *d* scores were calculated by using a delta value corresponding to a maximum false discovery rate of 5% for each comparison group. A positive *d* score indicates overexpression, and a negative *d* score indicates underexpression. ALL SUBJECTS, comparison of all subjects (depletion phase versus end of baseline); SIGNS, subjects who developed organ dysfunction when fed the low-choline diet; NO SIGNS, subjects who did not develop organ dysfunction (end of the depletion phase versus end of baseline).

[2] No change according to significance analysis of microarrays (all such).

Table 3

Real-time reverse transcriptase polymerase chain reaction (RT-PCR) validation of the gene array data[1]

	FOXA1	*PRAME*	*TERT*	*CDCA8*	*CHEK1*	*IL2RB*	*TNFAIP3*	*NFKBIA*
RT-PCR ratio	28.53	11.03	2.15	4.84	0.81	0.67	0.61	0.81
± SE	± 5.77	± 2.25	± 0.36	± 0.81	± 0.10	± 0.14	± 0.07	± 0.15
t Test	*[2]	*	*	*		*	*	*
Randomization	*	*		*		*	*	*
SAM *d* score[3]	6.0	5.9	4.7	4.1	−5.0	−8.2	−4.3	−4.1

[1] SAM, significance analysis of microarrays. Gene expression data from the microarray study was compared with real-time RT-PCR for 8 of the genes indicated in Table 1 (ALL SUBJECTS analysis).

[2] Significant value, $P < 0.05$.

[3] A positive score indicates overexpression, and a negative score indicates underexpression per SAM analysis.

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Table 4

Gene expression changes in selected gene ontology (GO) groups[1]

GO Identifier	GO class	ALL SUBJECTS	ALL (D)	NO SIGNS	SIGNS	PEMT (D)	PEMT (B)	PEMT (W)	MTHFD1 (D)	MTHFD1 (B)	CHDH (A→C)	CHDH (G→T)	FOLATE
Folate-thymidine metabolism													
19860	Uracil metabolism	↓	↑	=	=	=	=	=	↑	↑	=	=	=
42083	5,10-Methylenetetrahydrofolate—dependent methyltransferase activity	=	=	=	=	=	=	=	=	↓	=	↑	=
46104	Thymidine metabolism	↓	↑	=	=	=	=	=	↑	↑	=	=	=
Apoptosis													
6915	Apoptosis	↓	↑	=	↑	=	↑	=	↑	↑	↓	=	=
6916	Antiapoptosis	=	=	=	=	=	↑	=	=	=	=	=	=
6917	Induction of apoptosis	↓	↑	=	=	=	=	=	↑	↑	↓	=	=
8632	Apoptotic program	=	↑	=	↑	=	=	=	=	=	=	↑	=
30693	Caspase activity	=	↑	=	↑	=	=	=	↑	↑	=	=	=
43027	Caspase inhibitor activity	=	↓	↑	=	=	=	=	=	=	=	=	=
43065	Positive regulation of apoptosis	↓	↑	=	=	=	=	=	↑	↑	↓	=	=
43154	Negative regulation of caspase activity	↑	↓	↑	=	=	=	=	↓	=	↑	=	=
DNA damage-repair													
77	DNA damage checkpoint	=	=	=	=	↓	=	=	↓	↓	↑	=	↑
724	Double-strand break repair via homologous recombination	↑	=	=	=	=	=	=	=	=	=	=	=
725	Recombinational repair	↑	=	=	=	=	=	=	=	=	=	=	=
31570	DNA integrity checkpoint	=	=	↓	↑	↓	=	↓	↓	↓	↑	↑	↑
42770	DNA damage response, signal transduction	↓	=	=	=	=	=	=	↓	=	=	=	↑
51908	Double-stranded DNA—specific 5′−3′ exodeoxyribonuclease activity	=	=	=	=	↓	=	=	=	=	=	=	=
Cell cycle regulation-proliferation													
4861	Cyclin-dependent protein kinase inhibitor activity	=	↑	=	=	=	=	=	↑	=	=	=	=
8283	Cell proliferation	=	↑	↓	↑	=	↑	=	↑	↑↓	↓	=	=
Immune-inflamatory response and lymphocyte differentiation-activation													
1772	Immunologic synapse	=	↑	=	=	↑	↑	=	↑	=	↓	=	↓
6952	Defense response	↓	↑	↑↓	↑↓	↑	↑	↑↓	↑	↑	↓	↓	↑↓
6954	Inflammatory response	=	↑	↓	=	↑	↑	=	↑	↑	↓	↓	↑↓
6955	Immune response	↓	↑	↑↓	↓	↑	↑	↑↓	↑	↑	↓	↓	↑↓
19731	Antibacterial humoral response	↑	=	↑	↑	=	=	=	=	=	=	=	=
19732	Antifungal humoral response	↑	=	↑	↑	=	=	=	=	↓	=	=	=
19864	Immunoglobulin G binding	=	↑	=	=	↑	↑	↑	=	↑	↓	↓	↓
30098	Lymphocyte differentiation	=	=	=	=	=	=	=	↑	↑	↓	=	=
42113	B cell activation	=	↑	=	=	=	=	=	↑	=	↓	↓	=
42742	Defense response to bacteria	↑	=	=	=	=	=	=	=	=	↓	↓	=
50832	Defense response to fungi	=	=	=	=	=	=	=	=	↑	=	=	=
Epigenetic regulation of gene expression													
8327	Methyl-CpG binding	↑	=	=	↑	=	=	=	=	↓	=	=	=
35035	Histone acetyltransferase binding	=	↓	=	=	=	=	=	=	=	=	=	=
45815	Positive regulation of gene expression, epigenetic	=	=	=	=	=	=	↓	=	=	↑	=	=
Telomere maintenance													
3720	Telomerase activity	↑	=	↑	=	=	↓	↑	=	=	=	=	=
3721	Telomeric template RNA reverse transcriptase activity	↑	=	↑	↑	↑	↓	↑	=	↓	=	=	=
5697	Telomerase holoenzyme complex	↑	=	↑	=	=	↓	↑	=	=	=	=	=
42162	Telomeric DNA binding	=	=	↑	=	=	=	=	=	=	=	=	=

[1] GO analysis was used to assess the significance of change for genes according to their GO class (*see* Materials and Methods). All analysis groups are as defined in Table 1. ALL SUBJECTS, comparison of all subjects (depletion phase versus end of baseline); SIGNS, subjects who developed organ dysfunction when fed the low-choline diet; NO SIGNS, subjects who did not develop organ dysfunction (end of depletion phase versus end of baseline). For each polymorphism, the gene symbol is given. The choline dehydrogenase (*CHDH*)

single-nucleotide polymorphisms are indicated by the nucleotide replacement, and the sample is at the end of the depletion phase. The notation “(B)” after the gene symbol indicates sample after the baseline (adequate choline) phase; “(D)” after the gene symbol indicates sample after the low-choline diet phase; “(W)” after the gene symbol indicates women only after the depletion phase. FOLATE compares subjects with and without folate supplementation at the end of the depletion phase. The number of arrays for each comparison is specified in Table 1. Fisher's exact test was used to assess significance of change within each GO class within genes that are overexpressed, underexpressed, or both. Up arrows denote significance of the number of genes that were overexpressed; down arrows denote significance for the number of the underexpressed genes, whereas both arrows indicate significance for the total number of both overexpressed and underexpressed genes within each GO class ($P < 0.05$). An equals sign denotes no change.

Table 5

Genes of which the expression is changed by a low-choline diet and that are known to be regulated by DNA methylation[1]

Gene	ALL SUBJECTS	ALL (D)	NO SIGNS	SIGNS	PEMT (D)	PEMT (B)	PEMT (W)	MTHFD1(D)	MTHFD1(B)	CHDH (A→C)	CHDH (G→T)	FOLATE
CASP8									Up			
COL6A1					Down		Down	Down		Up		
CSPG2		Up			Up	Up	Up	Up	Up	Down		
DSCR5			Up		Up	Up	Up		Up	Down	Down	Down
GAGE2									Down			
IFNA8		Up	Down		Down		Down	Down	Down			Up
IGF2			Down		Down		Down		Down		Up	Up
PFKL		Down		Down	Down			Down	Down			Down
RASSF1		Up			Up	Up		Up	Up	Down		Down
RCN1						Up			Down	Up	Up	
TERT	Up		Up	Up	Up	Down	Up		Down			
TFF1			Down			Down						
TIMP3											Down	

[1]"Up" and "Down" indicate the direction of change in gene expression. Within all genes changed in the comparison groups defined in Table 1, 13 genes were found to be regulated by DNA methylation according to a search with the DNA Methylation Database (*see* Materials and Methods). ALL SUBJECTS, comparison of all subjects (depletion phase versus end of baseline); SIGNS, subjects who developed organ dysfunction when fed the low-choline diet; NO SIGNS, subjects who did not develop liver dysfunction (end of depletion phase versus end of baseline). For each polymorphism, the gene symbol is given. The choline dehydrogenase (*CHDH*) single-nucleotide polymorphisms are indicated by the nucleotide replacement, and the sample is at the end of depletion phase. The notation "(B)" after a gene symbol indicates the sample after the baseline (adequate choline) phase; "(D)" after a gene symbol indicates the sample after the low-choline diet phase; "(W)" after a gene symbol indicates women only after the depletion phase. FOLATE compares subjects with and without folate supplementation at the end of the depletion phase. The number of arrays for each comparison is specified in Table 1.