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## Polymorphism of the *PEMT* gene and susceptibility to nonalcoholic fatty liver disease (NAFLD).

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### Abstract

Phosphatidylethanolamine *N*-methyltransferase (*PEMT*) catalyzes phosphatidylcholine synthesis. *PEMT* knockout mice have fatty livers, and it is possible that, in humans, nonalcoholic fatty liver disease (NAFLD) might be associated with *PEMT* gene polymorphisms. DNA samples from 59 humans without fatty liver and from 28 humans with NAFLD were genotyped for a single nucleotide polymorphism in exon 8 of *PEMT* which leads to a V175M substitution. V175M is a loss of function mutation, as determined by transiently transfecting McArdle-RH7777 cells with constructs of wildtype *PEMT* open reading frame or the V175M mutant. Met/Met at residue 175 (loss of function SNP) occurred in 67.9% of the NAFLD subjects and in only 40.7% of control subjects ( $p < 0.03$ ). For the first time we report that a polymorphism of the human *PEMT* gene (V175M) is associated with diminished activity and may confer susceptibility to NAFLD.

### Keywords

phosphatidylethanolamine *N*-methyltransferase (*PEMT*); choline; liver function; single nucleotide polymorphism (SNP); Nonalcoholic Fatty Liver Disease (NAFLD) pregnancy; neural tube defect (NTD); transfection; site-directed mutagenesis

### Introduction

Nonalcoholic Fatty Liver Disease (NAFLD) is the most common reason for abnormal liver function, and may occur in as much as 25% of the population (1). NAFLD can progress to liver cell necrosis, fibrosis and cirrhosis of the liver (1). The mechanisms underlying NAFLD are not well understood. Obesity, diabetes and hypertriglyceridemia are predictive risk factors, but it can appear in humans who are otherwise normal (2,3). Humans ingesting diets deficient in the nutrient choline develop fatty liver (4,5) because phosphatidylcholine is required for hepatic secretion of triacylglycerol in very low density lipoproteins (VLDL) (6–8).

Phosphatidylethanolamine *N*-methyltransferase (*PEMT*; EC 2.1.1.17) catalyzes *de novo* synthesis of phosphatidylcholine in liver (9,10) and is responsible for about 30% of phosphatidylcholine formed in liver; the remainder being formed from preexisting choline moiety via an alternative pathway (11). *PEMT* knockout mice do not express any *PEMT* activity in liver and completely depend on dietary choline intake to meet daily choline

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requirements (12,13). When fed a diet deficient in choline they develop severe fatty liver; a choline supplemented diet prevents this (14) and can reverse hepatic damage if begun early enough (15). The *PEMT* gene is highly polymorphic; 98 single-nucleotide polymorphisms (SNPs) in *PEMT* were found in 48 Japanese individuals (16). It is possible that some of these SNPs have functional significance, and if so, could make humans susceptible to fatty liver when dietary intake of choline is low. We identified a variant that resulted in an amino acid substitution (V175M) and report for the first time that this SNP results in partial loss of activity of encoded *PEMT* and that this SNP occurs 1.7x as frequently in humans with NAFLD as in normal controls.

## MATERIALS AND METHODS

### Cell culture and transient transfection

McArdle RH-7777 rat hepatoma cells (American Type Culture Collection (ATCC), Manassus, VA) were maintained in Dulbecco's modified Eagle's medium/F12 (DMEM/F12; Gibco-BRL Rockville, MD), 10% horse serum, 10% fetal bovine serum, 100 units/ml penicillin (Gibco-BRL) and 100 µg/ml streptomycin sulfate (Gibco-BRL) at 37 °C under 5% CO<sub>2</sub>. On day 0, cells were plated at a density of  $1.5 \times 10^6$  cells/60-mm dish. When cells reached 70% confluence they were transfected with test plasmids or mock transfected with empty expression vector using Lipofectamine (Invitrogen, Carlsbad, CA) as per the manufacturer's protocol. 24 hours post transfection, the expression of enhanced green fluorescent protein (EGFP) was observed under a green fluorescent microscope and the transfection efficiency was calculated based on fluorescence intensity. 48 hours later, cells were harvested and total cellular homogenate and total particulate fraction were prepared for protein assay and enzyme activity analysis.

### PEMT activity assay

Liver homogenate or total particulate fraction from McArdle-RH7777 cells were assayed for *PEMT* activity using a modified method of Ridgway and Vance (17,18). Briefly, *PEMT* activity was assayed using 50 µg of protein in 125 mM Tris-HCl (pH 9.2; Mallinckrodt, Paris, KY) and 5mM DTT buffer (Sigma, St. Louis, MO) in the presence of 200 µM *S*-adenosyl-L-methionine containing 0.5 µCi of *S*-adenosyl-L-methionine (55.70 Ci/mmol; Amersham Biosciences, Piscataway, NJ) and 0.4 mM exogenous phosphatidylmethylethanolamine (P2, Avanti Polar-lipids, Inc., Alabaster, AL). The reaction was carried out for 30 minutes at 37°C and stopped by addition of chloroform/methanol/hydrochloric acid mixture (100:50:1, v/v). An aliquot of the chloroform phase was applied to a silica gel thin layer chromatography (TLC) plate (Si250-PA (19C)-Silica Gel, Baker, Inc., Phillipsburg, NJ) and was developed in chloroform: methanol: acetic acid: water (50:30:5:2, v/v). Disintegrations per minute from [<sup>3</sup>H]-PtdCho were determined in bands that co-migrated with authentic standards using liquid scintillation spectrophotometry (Wallac 1410, Pharmacia LKB Nuclear Inc, Gaithersburg, MD).

### PEMT protein expression assay

Proteins (25 µg) were separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Amersham Biosciences) which was probed with anti-FLAG M2 antibody (Sigma), washed extensively with 1X PBS (Gibco) containing 0.1% Tween 20 (Sigma), and then probed with horseradish peroxidase-conjugated goat anti-mouse IgG (Pierce, Rockford, IL). *PEMT*-FLAG protein was visualized by a reaction with Supersignal chemiluminescent substrate (Pierce) and exposed to x-ray film (Denville Scientific, Metuchen, NJ). The film was scanned and the integrated optical densities of the bands were measured using the ScionImage software (Scion Corporation, Frederick, MD, USA).

## Recombinant plasmid construction /site-directed mutagenesis

Total RNA was extracted from an adult white male human's liver using Trizol (Life Technologies Inc., Rockville, MD) according to the manufacturer's instructions. 3µg of total RNA, previously treated with RNase-free DNase I (Life Technologies Inc.), was reverse transcribed using an 18mer oligo(dT) primer and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. The oligonucleotide (5'*Mlu*I *P*EMT) TAACACGCGTAGTTATGACCCGGCTGCTGGGCTA was used as the 5' primer, and the oligonucleotide ATCATCATCCTTGTAGTCGAGCACGACGAAGCTGAGAATGTA (3' *P*EMT -WT), which adds 19 nucleotides encoding the FLAG epitope (DYKDDDDK), was used as the 3' primer. The PCR product was subcloned into *Nhe*I and *Mlu*I multicloning sites of mammalian expression vector pBI-EGFP-tet (Clontech, Palo Alto, CA), a bidirectional response plasmid that allows simultaneous expression of both EGFP and PEMT-FLAG under the control of a single tetracycline (doxycycline)-responsive element. With PEMT-WT as the template, site-directed mutagenesis was conducted using GeneTailor™ Site-Directed Mutagenesis System per manufacturer's instructions (Invitrogen, Carlsbad, CA). Oligonucleotides 5' TGGTGGCCCTCACCTACATAGTGGCTCTCCTATA3' and 5' TATGTAGGTGAGGGCCACCAGCACCGTCAG3' were the forward and reverse primers to introduce the M175V mutation (subsequently we realized that V175M is the SNP and V at 175 is the WT and data analysis was performed accordingly; see discussion).

## Human liver specimens

40 human liver samples were obtained from the Liver Procurement and Distribution System (LTPADS) (University of Minnesota, Minneapolis MN 55455, USA; funded by NIH contract N01 DK92310). Of these 40 subjects, 28 had fatty liver and 12 had normal livers. Synopses of tissue donors' medical histories, including pathologist's impression diagnosis on liver fat content, were also obtained. The specimens were snap-frozen once removed from the organ donors, delivered on dry ice and stored at -80°C until analysis.

## Normal human blood collection

47 healthy volunteers were recruited for a protocol approved by the Institutional Review Board at the University of North Carolina at Chapel Hill. These individuals consumed a diet adequate in choline content and had no liver disease by review of medical history, serum liver function tests (bilirubin, alanine aminotransferase, aspartate aminotransferase, creatine phosphokinase, γ-glutamyl transpeptidase, lactic dehydrogenase, alkaline phosphatase, prothrombin time, partial thromboplastin time, and albumin) and did not have fatty liver as assessed by magnetic resonance imaging of liver (see below). Blood samples were obtained by venipuncture and peripheral lymphocytes isolated by Ficoll-Hypaque gradient using Vacutainer® CPT™ tubes with sodium citrate (Becton Dickinson, Franklin Lakes, NJ) (19,20) and prepared for SNP analyses as described below.

## Magnetic Resonance Imaging of liver

Changes in relative hepatic fat levels were determined utilizing a modified "In and Out of Phase" magnetic resonance imaging (MRI) technique of Dixon (21–23) using a Siemens Vision 1.5T clinical MR system. Briefly, quantification of fat within the liver using MRI is possible because of the resonant frequency differences between fat and water. The resonant frequency differences in fat and water is reflected in the transverse magnetization changes and signal intensity changes at particular time intervals. Utilizing a "breath-hold" fast field echo sequence (FLASH; TE=2.2 msec and 4.5 msec, with a flip angle of 80°, and TR=140 msec) at an echo time of 2.2 msec (TE) MRI signals from water and fat will be 180° out-of-phase with each other while at a TE=4.5 msec the signals from fat and water will be in-phase with each other.

MR images obtained from the in-phase and the out-of phase can then be processed to derive the fat fraction from the differences in the MR image pixel intensity values. Serial FLASH MRI studies utilizing a TE= 2.2 and 4.5 msec were performed on subjects. The MR image sets were processed to determine the fat fraction in the liver utilizing software provided by Siemens Medical Solutions (Malvern, PA). Five liver slices in each subject were analyzed and compared to the fat fraction found in the spleen. Additionally, the relative changes in the levels of fat and water were monitored utilizing a localized single volume proton magnetic resonance spectroscopy technique (PRESS; TE=135, TR=1500 msec ) (24).

### Genomic DNA extraction

Genomic DNA was extracted from liver tissues using TriZol (Life Technologies Inc.) and from peripheral lymphocytes using PureGene (Gentra Systems, Minneapolis, MN) according to the manufacturer's instructions.

### SNPs detection

DNA sequencing was performed on double stranded DNA templates obtained from genomic DNA by PCR amplification. Exon 8 of *PEMT* was amplified with the oligonucleotides 5' GGAGCACTTTGCCCCAGAATC3' and 5'GACTTGGAGCCTTCAGAGCG3' as forward and reverse primers, respectively. PCR products were purified with QIAquick® PCR Purification Kit 250 (QIAGEN Inc, Valencia CA) according to the manufacturer's instructions. Sequencing reactions were performed by the University of North Carolina at Chapel Hill Genome Analysis Facility, using a capillary sequencing machine (model 3100, Applied Biosystems, Foster City, CA). The sequences obtained were compared with ones stored in the NCBI database (<http://www.ncbi.nlm.nih.gov/entrez/>, accession number AF294467) using ClustalW multiple sequence alignment software (<http://www.ebi.ac.uk/Tools/sequence.html>). Sequence homology identity was determined in accordance with criteria as previously described (25).

### Statistics

All data are presented as mean  $\pm$  standard error of the mean. Differences in the prevalences of V175M polymorphic genotypes in normal controls and patients with NAFLD were tested with Fisher's Exact Test (26). *PEMT* activity data were analyzed using one way analysis of variance (27).

## RESULTS

### In Vitro study

Wildtype and V175M *PEMT* plasmids were transiently transfected into McArdle-RH7777 cells, which does not show endogenous *PEMT* activity (Figure 1). Transfection efficiency as assessed by the expression of EGFP was not different in the three groups (vector, wildtype or V175M; data not shown). The amount of *PEMT* protein expressed was assessed by measuring the FLAG epitope expressed. Whether *PEMT* activity was expressed per mg protein or per expressed FLAG, the V175M form of *PEMT* had significantly diminished activity (Figure 1). Thus, V175M is a loss of function polymorphism. We analyzed enzyme activity using saturating concentrations of substrates; it is possible that activity differences between the polymorphic proteins might be different using other incubation conditions.

### Subject demographics

In patients with NAFLD (n=28; all confirmed as NAFLD from liver biopsy specimens with appropriate history), there were 12 males and 16 females, age range was 5–77 years (mean 51  $\pm$  3 years), with 23 Caucasians, 2 African Americans, 1 Hispanic and 1 Asian (ethnicity not

known for 1 subject) and with body mass indices ranging from 18.4 to 48.5 (mean  $30 \pm 1.4$  units; BMI unknown for 2 subjects). In control subjects (n=59; 12 confirmed normal from liver biopsy specimens and 47 confirmed as having normal liver by MRI), there were 30 males and 29 females, age range was 5–72 years (mean  $37.1 \pm 2.1$  years), with 37 Caucasians and 14 African Americans, 3 Hispanics, 3 Asians, 1 Native American, and 1 Trinidadian. Body mass indices ranged from 15.3 to 33 (mean  $24.7 \pm 0.5$  units) in the controls.

### SNP detection

The V175M (G to A substitution) polymorphism in *PEMT* was differentially distributed in controls and NAFLD patients. The allele frequency for G and A were 0.19 and 0.81 respectively in 28 NAFLD patients. Among 59 normal controls, they are 0.39 and 0.61 respectively. The frequencies of the three genotypes among patients were GG (Val/Val) 7.1%, GA (Val/Met) 25%, and AA (Met/Met) 67.9%. The frequencies of the three genotypes among normal controls were GG, 18.6%, GA, 40.7%, and AA, 40.7%. (Table 1). Almost twice as many controls had the GG and GA genotype than did the NAFLD patients, while the AA genotype was over represented in the NAFLD patients compared with the normal controls ( $p=0.02$  by two tailed Fischer's Exact Test). We observed no significant sexual dimorphism in this distribution. The observed distribution of the V175 and M175 alleles is similar to that previously reported in the public SNP databases.

### Discussion

Nonalcoholic fatty liver disease is the most common reason for liver test abnormalities in the general population (1). While it is usually identified in adults with obesity, it can also occur in people with normal body weight without underlying diabetes and hyperlipidemia and has been described in children as well (2,3). We identified a loss of function SNP in *PEMT* (G→A in exon 8 of the *PEMT* gene in humans; results in a V175M substitution in the encoded protein) that occurs more frequently in patients with NAFLD. This SNP was previously detected in humans in a Japanese population but no functional significance was attributed to it (16). Currently, there are no noninvasive tests to diagnose and stage NAFLD (1). Liver biopsy remains the most sensitive diagnostic test but cannot distinguish NAFLD from other causes of fatty liver disease, such as alcohol abuse (28). Identification of genetic predisposition factors to this disease would help to identify individuals who are at risk for NAFLD and who warrant evaluation for pre-symptomatic prediction and prevention.

The mechanism whereby a loss of function SNP in *PEMT* might be associated with NAFLD involves the role of this enzyme in lipoprotein secretion from liver. Triacylglycerol is formed in the liver and then secreted in very low density lipoprotein (VLDL). Synthesis of new phosphatidylcholine molecules is required for VLDL formation, and when they are not available fat droplets accumulate in the cytosol of liver cells (6,7,29). When diet is deficient in choline (4,8,30) or when *PEMT* activity is inhibited or deleted (12,13,15,29), fatty liver ensues.

In the literature, the sequence for human *PEMT* was originally reported to contain a methionine at residue 175 (GenBank™ accession number AAK19172); however in later reports on human *PEMT* and in the reported sequence for mouse, rat and cow (GenBank™ accession numbers are NP\_009100, AAH26796, Q08388, and AAQ01191, respectively) *PEMT* contains a valine at this residue (Table 2). Therefore, we argue that in evolution the earliest sequence for *PEMT* contains a valine at this position, and that the mutation to encode a methionine is the genetic polymorphism.

We recognize that our observation is derived from a small study and that the group of NAFLD patients was drawn from a national pool, while many of our control subjects were recruited in

North Carolina. The demographics of the two groups were similar, though average BMI was higher in the NAFLD group. Since high BMI can increase incidence of fatty liver, it is possible that the PEMT SNP we report is somehow associated with factors that increase BMI. However, it is unlikely that this is the sole reason that this SNP is associated with risk for NAFLD.

The relatively common occurrence of this loss of function SNP (81% of controls and 93% of NAFLD have at least one allele) suggests that it provides some evolutionary advantage to humans. We previously reported (31) that mice in which this gene is deleted have excess *S*-adenosylmethionine available for methylation reactions because PEMT activity uses an appreciable portion of available methyl groups for formation of choline. Perhaps, when humans eat enough choline in their diet, the V175M SNP is beneficial because it reduces waste of methyl-groups for making choline moiety. Sometimes a SNP is selected for because it protects against disease (32). Human erythrocytes infected with Plasmodium develop new pathways for accumulating choline from plasma (33). Up to 45% of the malaria parasite is composed of phosphatidylcholine and the malaria parasite actively accumulates choline from its host (34). Finally, there is a clear correlation between antimalarial activity of some drugs and their ability to inhibit choline uptake into the parasite (35). Perhaps the V175M SNP that we describe diminishes the availability of choline in the human host and thereby impairs the replication of the malaria parasite. Whatever the reason, it is interesting that the V175M SNP is so prevalent in humans.

It is interesting that the incidence of NAFLD is lower in premenopausal women than in men or postmenopausal women (36). This would be consistent with our hypothesis that low PEMT activity is a risk factor for developing NAFLD. Female rats are less likely to develop fatty liver when fed choline deficient diets than are male rats (37), because females have greater capacity to form the choline moiety *de novo* via PEMT pathway in liver. It is estimated that female rats have 10–50% more PEMT activity than do males (38,39). A woman's capacity to form the choline moiety *de novo* may be highest before menopause because estrogens increase PEMT activity in humans (40) and in castrated-rats (41). Thus, premenopausal women may be less sensitive to a loss of function SNP in PEMT because they have excess PEMT activity as compared to men or postmenopausal women.

The requirement for choline (from diet or from PEMT synthesis) is spared, in part, by the availability of methyl groups from 1-carbon metabolism (via methyltetrahydrofolate) (42). It is possible that the PEMT SNP we describe will interact with other commonly known SNPs in humans. For example, the thermolabile variant (677C→T) of 5,10-methylenetetrahydrofolate reductase (MTHFR, E.C. 1.5.1.20) occurs in 15–30% of humans (43). We found that mice in which MTHFR was deleted develop fatty liver that resolves when mice are fed the choline metabolite betaine (43). These mice require more choline or betaine because homocysteine remethylation to methionine, in the absence of 1-carbon units via the folate pathway shifts to a pathway that uses choline as a precursor. Homocysteine can be remethylated to methionine by methionine synthase, using 5-methylfolate which is supplied by MTHFR (43). Alternatively, betaine:homocysteine methyltransferase (BHMT, EC 2.1.1.5) catalyzes a methyl transfer from betaine to homocysteine (43). When 5-methylfolate is not available, more betaine is required. Thus, humans who have diminished capacity to synthesize choline moiety via PEMT activity, and who have diminished capacity to form 5-methylfolate will have difficulty producing increased betaine from choline when it is needed for homocysteine methylation.

We studied a relatively small number of subjects (28 with NAFLD and 59 controls); it would be valuable to characterize this SNP in larger populations. It would also be useful to determine whether diets high in choline reduce hepatic steatosis in humans with this SNP. We recently published data on choline content of foods (44), and the US Department of Agriculture

maintains an updated food composition table online (<http://www.nal.usda.gov/fnic/foodcomp/Data/Choline/Choline.html>). We are currently examining whether there are other loss of function SNPs in the *PEMT* gene.

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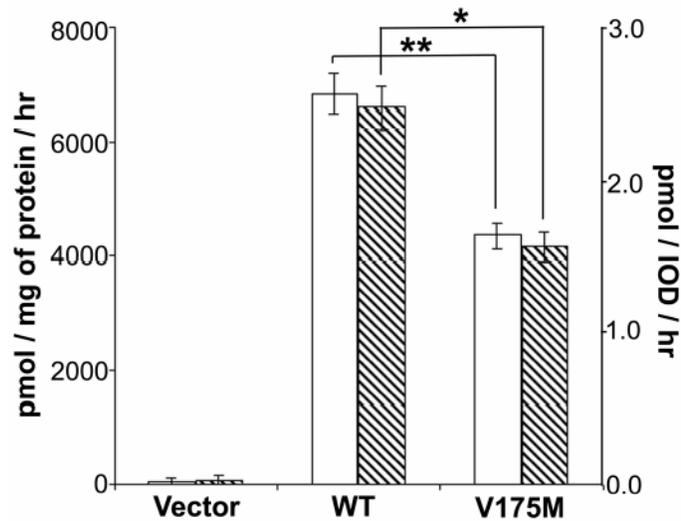
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**Figure 1.**

Val to Met substitution at residue 175 of PEMT leads to expression of PEMT with reduced activity.

McArdle-RH7777 cells were transiently transfected with plasmids containing sequence for wild type PEMT (WT) or V175M mutated form (V175M) or vector only (Vector). 50  $\mu$ g of total particulate protein from cells harvested 48 hr after transfection was used for PEMT activity assay in the presence of 200  $\mu$ M AdoMet. Results are expressed as mean  $\pm$  standard error for n=3/group. Open bars: Activity expressed as pmol phosphatidylcholine formed per mg protein per hour. Hatched bars: Activity expressed as pmol phosphatidylcholine formed per integrated optical density of FLAG epitope per hour. The experiment was duplicated with similar results. \* =  $p < 0.01$  by one way ANOVA; \*\* =  $p < 0.005$  by one way ANOVA.

**Table 1***PEMT* Exon 8 SNP distribution in human subjects.

	GG Val/Val	GA Val/Met	Percent of Population AA Met/Met
NAFLD (n=28)	7.1	25.0	67.9*
Controls (n=59)	18.6	40.7	40.7

DNA was isolated from subjects with nonalcoholic fatty liver disease (NAFLD) and from controls with normal liver fat and was analyzed for the V175M SNP. Data are presented as percent of group (n/group indicated in parentheses).

\* =  $p < 0.03$  that AA genotype (loss of function SNP) occurred more frequently in NAFLD patients than in control subjects by two-tailed Fisher's Exact Test.

**Table 2**  
Amino acid sequence alignment of mammalian PEMT orthologs.

RAT	LDNPMYWGSTANYLGWALMHASPTGLLLLTVLVALVYVVALLFE	180
MOUSE	LDNPMYWGSTANYLGWALMHASPTGLLLLTVLVAIVYVVALLYE	180
HUMAN	LDNPMYWGSTANYLGWAIMHASPTGLLLLTVLVALTYIVALLYE	180
COW	LDNPMYWGSTAIYLGWAIIVHASPTGLLLLTALVALIYMVAIVYE	180
	*****:*****:*****:***: * *: : *	

175V is conserved in various mammalian PEMT sequences. GenBank<sup>TM</sup> accession numbers for rat, mouse, human and cow are Q08388, AAH26796, NP\_009100 and AAQ01191, respectively.

\* = completely conserved; : = human same as rat.