ARIC Manuscript Proposal #763

PC Reviewed: 01/16/01	**Status: A**	**Priority: 1**
SC Reviewed: 01/30/01	**Status: A**	**Priority: 1**

Title: Thermolabile variant of methylene tetrahydrofolate reductase (MTHFR), homocysteine, and venous thromboembolism: A nested case-control study of the Longitudinal Investigation of Thromboembolism Etiology (LITE)

(CHS/ARIC Manuscript Proposal: Ancillary Study Manuscript
CHS Ancillary Study C5, ARIC Ancillary Study, "Epidemiology of Venous Thrombosis and Pulmonary Embolism in the ARIC and CHS Cohorts")

Short Title: MTHFR polymorphism, homocysteine and venous thromboembolism

Timeline: Analysis: November 2000; Draft MS: January 2001; Submission: February 2001

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Key Words: deep vein thrombosis / pulmonary embolism / venous thromboembolism / methylene tetrahydrofolate reductase / homocysteine / epidemiology

NOTE: The analysis in this study is part of the LITE ancillary study, and will be conducted by Albert Tsai, funded by the LITE study.

Background

A number of risk factors for VTE have been established, including immobilization, surgery, cancer, and exogenous female hormones. Pathophysiologically, three major contributing components may be important in the development and propagation of a thrombus: a hypercoagulable state, endothelial vessel injury, and stasis of the blood. Any factor associated with these three components is likely to affect the risk of thrombosis.

Of particular interest for this study is a point mutation (C677T) in the MTHFR gene, which results in elevated levels of plasma homocysteine in homozygotes.[1] MTHFR catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is the major

carbon donor in the remethylation of homocysteine to methionine. The mutation in this gene is a C to T transition at nucleotide 677 which results in an alanine-to-valine substitution at position 222. The variant resultant enzyme is thermolabile and has reduced activity**,** thus causing elevated plasma levels of homocysteine in homozygotes or heterozygotes. This thermolabile variant is an inherited autosomal recessive trait. Estimates of general population prevalence of homozygous mutants range 5%[2] to up to 17%[3].

Hyperhomocysteinemia

Jacques showed that homozygotes for the MTHFR thermolabile mutation had elevated fasting homocysteine concentrations when plasma folate concentration was low, but not when folate was high.[4] Hyperhomocysteinemia itself appears to increase risk of VTE approximately three-fold;[5-9] a recent meta-analysis[10] reported a pooled odds ratio of 2.5 (95% CI 1.8-3.5) for fasting plasma homocysteine concentration above the 95th percentile or mean plus two standard deviations. Much of the evidence supports increased risk of venous thrombosis with hyperhomocysteinemia, but there is less known about the risks for recurrent events, idiopathic events, or the risk in the presence of known predictors of VTE such as the Factor V Leiden mutation.[7]

MTHFR

While the inherited thermolabile variant of MTHFR is associated with higher plasma homocysteine levels,[4] not all studies have shown a corresponding increased risk of venous thrombosis. In a study of 577 patients with objectively diagnosed VTE and 500 healthy controls, Brown et al.[11] found no significant associations between homozygosity and risk of VTE. Other small case control studies of 65,[12] 194[13] and 216[14] patients did not find any evidence of increased risk. Arruda et al.[15] showed that homozygotes with the MTHFR mutation were at increased risk for venous thrombosis (OR = 2.93, 95% CI 1..23-7.01), but the sample included only 127 patients. Cattaneo et al. observed no association between homozygous MTHFR and VTE in 77 cases, but slight additional risk with the presence of Factor V Leiden mutation [16]. This study was also limited by a small sample size. However, Grandone et al.[17] found an odds ratio of 2.1 (95% CI 1.0-4.5) for the homozygote MTHFR genotype in 12/42 case women with a previous DVT during pregnancy or in the postpartum period, versus 34/213 control subjects. The effect, whether independent or by a gene-by-environment interaction, of the MTHFR gene mutation, has yet to be elucidated in a population based study. Thus, the relationship between the homozygous genotype and the level of homocysteine with risk of VTE is still uncertain.

5. Research questions/hypothesis:

1. Is presence of the MTHFR C677T polymorphism associated with increased risk of VTE?
2. Is there an association between high levels of serum homocysteine and increased risk of VTE?
3. Does the association of the MTHFR polymorphism with VTE vary in subgroups with incident or recurrent VTE, or with idiopathic vs. secondary VTE? Similarly, does the association of serum homocysteine level with VTE vary in subgroups with incident or recurrent VTE, or with idiopathic vs. secondary VTE?

6. Methods

Subjects
The parent study, LITE, is a prospective cohort study which combines the CHS and ARIC cohorts. The current analysis is based on the LITE nested case-control population. Cases of possible VTE were identified primarily by hospital discharge codes. Details of case ascertainment and validation are found elsewhere.[18]

Control selection
Potential controls were first assigned follow-up times between 0 days and the maximum number of follow-up days that subjects could have participated in the study. Potential controls were then retained if their actual lengths of follow-up were greater than their assigned follow-up times. This selection process was performed so that the set of potential controls included individuals who could have been diagnosed with a VTE (had one occurred) at the assigned follow-up time. This yielded a random selection of control subjects who were alive and under observation at the assigned times. Follow-up times were then binned into two-year intervals for a total of four intervals. Frequency matching was performed by race (black, non-black), gender (female, male), age group (five groups), and follow-up time in two-year blocks (four groups) for a total of 80 strata.

Laboratory Methods
We use digestion by *Hinf*I to detect the thermolabile $C_{677}T$ mutation of the MTHFR gene. Genomic DNA was amplified by polymerase chain reaction (PCR) using a sense primer from exon 4 (5'TGAAGGAGAAGGTGTCTGCGGGA3') and an antisense primer from intron 4 (5'AGGACGGTGCGGTGAGAGTG3'). The 198 bp PCR product was then digested with *HinfI* at 37° C for 3.5 hours, applied to a 10% polyacrylamide gel and electrophoresed for 1.5 hours at 150 volts. The gel was stained with ethidium bromide and visualized under UV light. A mutation at nucleotide 677 creates a *HinfI* recognition sequence which digests the normal 198 bp fragment into 175 and 23 bp fragments.

Total homocysteine was measured by a reverse-phase HPLC method which uses a thio-specific fluorogenic reagent, as described by Ubbink et al.[19] Serum thiols are derivatized with ammonium 7-fluorobenzo-2-oxa-1, 3-diazole-4-sulphonate (SBD-F), a thiol-specific fluorogenic probe and subjected to HPLC using a mobile phase of pH 2.1. Quantitation is achieved by measuring the peak height of the thiol containing derivative relative to the internal standard peak height and comparing to a standard line of known concentration.

Analysis
Dependent variable: venous thromboembolism (VTE) status.

Independent variables: MTHFR genotype; serum homocysteine level

Univariate statistics (means and proportions) for potential covariates amongst cases and controls will be calculated. Logistic regression models will be used to estimate odds ratios of VTE. Tests for interaction will precede tests for confounding and independence. Potential interaction terms include age, race, sex and presence of the Factor V Leiden mutation. We will adjust for

potential confounders which include, but are not restricted to, the following variables: age, sex, race, body mass index, and diabetes mellitus.

Expected Results
We hypothesize that (1) presence of the MTHFR mutation will be associated with an increased risk of VTE; (2) increased homocysteine levels will be associated with an increased risk of VTE; (3) The joint presence of high homocysteine and factor V Leiden will confer supra-additive risk.

Conclusions
This study will add to the body of evidence concerning the possible association of the MTHFR gene mutation and hyperhomocysteinemia with risk of venous thrombosis in the general population.

7.a. Will the data be used for non-CVD analysis in this manuscript? ____ Yes _X___ No

b. If Yes, is the author aware that the file ICTDER01 must be used to exclude persons with a value RES_OTH = "CVD Research" for non-DNA analysis, and for DNA analysis RES_DNA = "CVD Research" would be used? ____ Yes ____ No
(This file ICTDER01 has been distributed to ARIC PIs, and contains the responses to consent updates related to stored sample use for research.)

8.a. Will the DNA data be used in this manuscript? __X__ Yes ____ No

8.b. If yes, is the author aware that either DNA data distributed by the Coordinating Center must be used, or the file ICTDER01 must be used to exclude those with value RES_DNA = "No use/storage DNA"? ___X_ Yes ____ No

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