

## PDF hosted at the Radboud Repository of the Radboud University Nijmegen

The following full text is a publisher's version.

For additional information about this publication click this link.

<http://hdl.handle.net/2066/25463>

Please be advised that this information was generated on 2019-09-21 and may be subject to change.

## A Common 844INS68 Insertion Variant in the Cystathionine $\beta$ -Synthase Gene

Leo A. J. Kluijtmans,\* Godfried H. J. Boers,† Frans J. M. Trijbels,\* Henriëtte M. A. van Lith-Zanders,\* Lambert P. W. J. van den Heuvel,\* and Henk J. Blom\*

\*Department of Pediatrics and †Department of Internal Medicine, University Hospital Nijmegen, 6500 HB Nijmegen, The Netherlands

Received April 29, 1997

**Mildly elevated plasma homocysteine has been shown to be associated with an elevated risk for cardiovascular disease. In this study, we analyzed the frequency of a common 844ins68 insertion variant in the cystathionine  $\beta$ -synthase gene (CBS) in patients with arterial occlusive disease and in controls and assessed the association between the insertion variant and plasma homocysteine concentrations. The insertion variant was equally distributed between both study groups. Furthermore, the presence of this insertion variant, either in the heterozygous or the homozygous state, is not associated with hyperhomocysteinemia. We therefore conclude that this common 844ins68 variant is a neutral insertion variant.** © 1997 Academic Press

**Key Words:** cystathionine  $\beta$ -synthase; insertion variant; homocysteine; vascular disease; alternative splicing; genetic marker.

Mild hyperhomocysteinemia has been recognized as an independent and graded risk factor for arteriosclerosis (1) and thrombosis (2) and may result from environmental and genetic factors. The main regulating enzymes in homocysteine metabolism are cystathionine  $\beta$ -synthase (CBS; EC 4.2.1.22) and methylenetetrahydrofolate reductase (MTHFR). CBS, which is a homotetrameric enzyme, catalyzes the first reaction in the transsulfuration pathway, in which homocysteine and serine are condensed to cystathionine. Since the cloning and characterization of the human CBS cDNA (3), many efforts have been made to elucidate mutations causing severe hyperhomocysteinemia due to CBS deficiency, which resulted in the identification of >20 mutations in the CBS cDNA (4–8). The interest in the molecular basis of CBS is enhanced due to its

potential involvement in mild hyperhomocysteinemia and premature vascular disease as suggested by Boers *et al.* (9) and Clarke *et al.* (10), who reported decreased CBS activities in fibroblasts of hyperhomocysteinemic vascular disease patients.

In our study on the involvement of heterozygosity for CBS deficiency in cardiovascular disease (11), we also encountered the 844ins68 mutation in the coding region of exon 8 of the CBS gene, an insertion variant which exactly duplicates the splice junction site of exon 8 and which was previously described by Sebastio *et al.* (7) as a possible pathogenic mutation in an Italian homocystinuria patient. Recently, the same group (12) and another one (13) provided evidence that this 68-bp insertion is a common insertion variant in the CBS gene and is most likely skipped by alternative splicing of the nuclear CBS mRNA. Unlike Tsai *et al.* (13), Sperandio *et al.* (12) did not measure plasma homocysteine, so the effect of this insertion variant with data on plasma homocysteine could not be studied. Furthermore, they speculated that this variant, in combination with homozygosity for the 677C  $\rightarrow$  T mutation in the MTHFR gene, might further hamper homocysteine metabolism. In this report, we present our results on the prevalence of this common insertion variant in patients with premature arterial occlusive disease and controls and provide data on its effect on homocysteine metabolism.

### MATERIALS AND METHODS

#### *Subjects*

We analyzed 60 patients with different forms of arterial occlusive disease and 107 controls recruited

**TABLE 1**  
**Genotype Distribution of the 844 ins68 Mutation**  
**in Vascular Disease Patients and in Controls**

Genotype	Vascular disease patients (n = 60)	Controls (n = 107)
NN	50 (83.3%)	89 (83.2%)
NI	10 (16.7%)	15 (14.0%)
II	0 (0%)	3 (2.8%)

Note.  $\chi^2 = 1.86$ ;  $P = \text{NS}$ ; N, normal sequence; I, insertion.

from a general practice; both groups have been described extensively before (11,14). All individuals agreed to participate in this study.

#### DNA Amplification and Detection of Insertion

DNA was extracted from peripheral blood lymphocytes by a standard method (15). Approximately 100 ng of DNA was amplified by PCR, using 50 ng forward primer (5'-CTGCCTTGAGCCCTGAAGCC-3'; intronic), 50 ng reverse primer (5'-CGTCCTGTC-CAGCACCGT-3'; cDNA 937-954), 200  $\mu\text{M}$  each dNTP, and 1 unit *Taq* polymerase (Life Technologies, Breda, the Netherlands) in a standard PCR buffer. After an initial denaturation at 95°C for 3 min, amplification was performed in 35 cycles (92°C/1 min, 52°C/1 min, 72°C/1 min), followed by a final extension at 72°C for 7 min. The PCR products were subjected to agarose gel electrophoresis, ethidium bromide staining, and UV illumination for detection of the 844ins68 insertion variant. The wild-type allele yielded a fragment of 159 bp, whereas amplification of the inserted allele generated a fragment of 227 bp.

#### Statistics

Differences in allele frequencies and genotype distribution between vascular disease patients and controls were assessed by  $\chi^2$  analysis. A  $P$  value less than 0.05 was considered statistically significant.

## RESULTS AND DISCUSSION

The allele frequency of this insertion was 0.083 among vascular disease patients versus 0.098 among controls ( $\chi^2 = 0.063$ ;  $P = \text{NS}$ ), which is in line with the observations by Tsai *et al.* (13), who found an allele frequency of 0.085 versus 0.058 in coronary artery disease patients and controls, respectively. The genotype distribution of this insertion among patients and controls (Table 1) is in agreement with

the Hardy-Weinberg equation. In our study, there was no substantial difference in carrier status of the insertion between patients and controls ( $\chi^2 = 1.86$ ,  $P = \text{NS}$ ).

Mild aberrations in the transsulfuration pathway are often found to be associated only with elevated plasma homocysteine concentrations after a standardized methionine loading. We therefore compared not only fasting but also post-load plasma homocysteine concentrations and the increase in plasma homocysteine upon methionine loading within the three different genotypes of combined groups of patients and controls (Table 2). The individuals with the insertion on both alleles did not have increased homocysteine concentrations. Their plasma homocysteine levels were even lower, although not significant, compared with the other genotypes, both in fasting state and after a methionine loading test. Heterozygosity for the insertion variant also did not influence plasma homocysteine concentrations. Because homozygosity for the 677C  $\rightarrow$  T transition in the MTHFR gene, which has been shown to be associated with elevated plasma homocysteine concentrations (11), could mask a possible effect of the 844ins68 variant on plasma homocysteine levels, we also assessed the homocysteine concentrations in individuals after excluding those subjects homozygous for the 677C  $\rightarrow$  T polymorphism in the MTHFR gene. Again, we observed that the insertion variant does not affect plasma homocysteine concentrations (data not shown).

The 844ins68 insertion variant was previously described as a possible pathogenic mutation in an Italian homocystinuria patient and was postulated to introduce a premature translation termination codon, which would result in a truncated CBS mono-

**TABLE 2**  
**Relationship between Insertion and Plasma**  
**Homocysteine Concentrations**

	Genotype		
	NN	NI	II
Fasting HCy ( $\mu\text{mol/L}$ )	13.2 $\pm$ 4.8 (n = 137)	12.7 $\pm$ 2.8 (n = 25)	10.2 $\pm$ 2.8 (n = 3)
Post-load HCy ( $\mu\text{mol/L}$ )	41.7 $\pm$ 16.2 (n = 137)	36.4 $\pm$ 10.1 (n = 24)	34.0 $\pm$ 12.4 (n = 3)
Increase ( $\mu\text{mol/L}$ )	28.4 $\pm$ 13.5 (n = 136)	23.7 $\pm$ 9.1 (n = 24)	23.9 $\pm$ 11.3 (n = 3)

Note. Results are expressed as mean  $\pm$  SD. Increase is post-load homocysteine minus fasting homocysteine concentration. N, normal sequence; I, insertion.

mer (7). However, RT-PCR-amplified cDNA of individuals carrying the insertion variant only yielded normal size mRNA (12,13) (Kluijtmans *et al.*, unpublished results). Although we have no data on CBS enzyme activities in fibroblasts of individuals carrying this insertion in either the homozygous or the heterozygous state, a significantly deleterious effect of this 844ins68 mutation on CBS expression or activity should be reflected in elevated homocysteine concentrations, especially after a methionine loading. Because of the lack of hyperhomocysteinemia in heterozygotes and in homozygotes for this insertion, even after exclusion of those individuals homozygous for the 677C → T mutation in MTHFR, our data favor the hypothesis that this mutation is a neutral insertion variant which is skipped from the mRNA through preferential usage of the second acceptor splice site. Therefore, this 844ins68 polymorphism is likely a neutral insertion variant, which might serve as an intragenic marker in epidemiologic studies on the involvement of CBS in hyperhomocysteinemia and cardiovascular disease.

### ACKNOWLEDGMENT

This study was financially supported by Grant 93.176 from the Netherlands Heart Foundation.

### REFERENCES

1. Boushey CJ, Beresford SAA, Omenn GS, Motulsky AG. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease: Probable benefits of increasing folic acid intakes. *J Am Med Assoc* **274**:1049–1057, 1995.
2. Den Heijer M, Koster T, Blom HJ, Bos GMJ, Briët E, Reitsma PH, Vandenbroucke JP, Rosendaal FR. Hyperhomocysteinemia as a risk factor for deep-vein thrombosis. *N Engl J Med* **334**:759–762, 1996.
3. Kraus JP, Le K, Swaroop M, Ohura T, Tahara T, Rosenberg LE, Roper MD, Kozich V. Human cystathionine  $\beta$ -synthase cDNA: Sequence, alternative splicing and expression in cultured cells. *Hum Mol Genet* **2**:1633–1638, 1993.
4. Kraus JP. Molecular basis of phenotype expression in homocystinuria. *J Inher Metab Dis* **17**:383–390, 1994.
5. Kluijtmans LAJ, Boers GHJ, Stevens EMB, Renier WO, Kraus JP, Trijbels JMF, Van den Heuvel LPWJ, Blom HJ. Defective cystathionine  $\beta$ -synthase regulation by S-adenosylmethionine in a partially pyridoxine responsive homocystinuria patient. *J Clin Invest* **98**:285–289, 1996.
6. Kluijtmans LAJ, Blom HJ, Boers GHJ, Van Oost BA, Trijbels JMF, Van den Heuvel LPWJ. Two novel missense mutations in the cystathionine  $\beta$ -synthase gene in homocystinuric patients. *Hum Genet* **96**:249–250, 1995.
7. Sebastio G, Sperandio MP, Panico M, De Franchis R, Kraus JP, Andria G. The molecular basis of homocystinuria due to cystathionine  $\beta$ -synthase deficiency in Italian families and report of four novel mutations. *Am J Hum Genet* **56**:1324–1333, 1995.
8. Sperandio MP, Panico M, Pepe A, Candito M, De Franchis R, Kraus JP, Andria G, Sebastio G. Molecular analysis of patients affected by homocystinuria due to cystathionine  $\beta$ -synthase deficiency: Report of a new mutation in exon 8 and a deletion in intron 11. *J Inher Metab Dis* **18**:211, 1995.
9. Boers GHJ, Smals AGH, Trijbels JMF, Fowler B, Bakkeren JAJM, Schoonderwaldt HC, Kleijer WJ, Kloppenborg PWC. Heterozygosity for homocystinuria in premature peripheral and cerebral occlusive arterial disease. *N Engl J Med* **313**:709–715, 1985.
10. Clarke R, Daly L, Robinson K, Naughten E, Cahalane S, Fowler B, Graham I. Hyperhomocysteinemia: An independent risk factor for vascular disease. *N Engl J Med* **324**:1149–1155, 1991.
11. Kluijtmans LAJ, Van den Heuvel LPWJ, Boers GHJ, Stevens EMB, Frosst P, Van Oost BA, Trijbels JMF, Rozen R, Blom HJ. Molecular genetic analysis in mild hyperhomocysteinemia: A common mutation in the methylenetetrahydrofolate reductase gene is a genetic risk factor for cardiovascular disease. *Am J Hum Genet* **58**:35–41, 1996.
12. Sperandio MP, De Franchis R, Andria G, Sebastio G. A 68-bp insertion found in a homocystinuric patient is a common variant and is skipped by alternative splicing of the cystathionine  $\beta$ -synthase mRNA. *Am J Hum Genet* **59**:1391–1393, 1996.
13. Tsai MY, Bignell M, Schwichtenberg K, Hanson NQ. High prevalence of a mutation in the cystathionine  $\beta$ -synthase gene. *Am J Hum Genet* **59**:1262–1267, 1996.
14. Den Heijer M, Blom HJ, Gerrits WBJ, Rosendaal FR, Haak HL, Wijermans PW, Bos GMJ. Is hyperhomocysteinemia a risk factor for recurrent venous thrombosis? *Lancet* **345**:882–885, 1995.
15. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* **16**:1215, 1988.