(Table). Longitudinal analysis by the generalized estimating equation (GEE) model[2,3] revealed no evidence of interaction between genotype and time: the coefficients were −0.41 (P=0.19) for total cholesterol, −0.04 (P=0.71) for HDL-C, and −0.38 (P=0.22) for nonHDL-C. Based on the GEE model, the TT genotype had average higher levels of total cholesterol, LDL-C, and nonHDL-C than the (GG+GT) group, by 11.48 (P=0.04), 9.98 (P=0.049), and 12.40 (P=0.03), respectively, during the 15-year follow-up. HDL-C levels were not significantly different among the three genotypes in all 6 examinations.

However, the studies of the −493 G/T MTP polymorphism have yielded conflicting results. The Framingham Offspring Study,[4] in which the participants were primarily middle-aged (the mean age of 52 years) whites, reported no significant association, although they found that the TT genotype was associated with higher cholesterol levels. In contrast, studies of the Northern European men reported an opposite result to our finding, with the T allele associated with lower levels of total cholesterol, LDL-C, and triglycerides.[5,6] A functional study has shown that the T allele has 2-fold higher transcriptional activity than the G allele,[5] which is hard to reconcile with the opposite effects of the TT genotype observed in Northern European men. It is possible that the MTP gene interacts with other genes or environment leading to a complex situation. However, more studies are necessary to address these intriguing findings.

In summary, analysis of the 15-year data reveals a significant genetic effect of the MTP promoter polymorphism on lipids. The genotypic effect did not change over time. More studies are needed to address the conflicting observations between blacks and whites, as well as between Americans and Northern Europeans.

The Differences of Total Cholesterol (TC), LDL-C, and nonHDL-C Data (mg/dL) Between the TT and GT+GG Groups After Adjusting for the Center Effect, Body Mass Index, and Baseline Age

	ΔMean (SE)	*P* Value
TC		
1st measure	10.0 (5.8)	0.08
2nd measure	14.0 (6.2)	0.03
3rd measure	19.1 (6.2)	0.002
4th measure	14.4 (6.8)	0.03
5th measure	1.8 (6.6)	0.79
6th measure	13.9 (8.1)	0.09
GEE analysis for TC	11.48 (5.63)	0.04
LDL-C		
1st measure	8.6 (5.4)	0.11
2nd measure	11.2 (6.1)	0.06
3rd measure	17.0 (5.6)	0.003
4th measure	9.7 (6.6)	0.14
5th measure	0.0 (6.0)	0.97
6th measure	14.5 (7.0)	0.04
GEE analysis for LDL-C	9.98 (5.06)	0.049
nonHDL-C		
1st measure	10.5 (5.8)	0.07
2nd measure	14.4 (6.3)	0.02
3rd measure	18.7 (6.3)	0.003
4th measure	14.5 (7.0)	0.04
5th measure	2.1 (6.8)	0.76
6th measure	15.7 (8.3)	0.06
GEE analysis for nonHDL-C	12.40 (5.67)	0.03

The GG and GT genotypes were pooled.

GEE model is adjusted for center, baseline age, and time-dependent body mass index.

Suh-Hang Hank Juo
Laura Colangelo
Zhihua Han
Jonathan D. Smith
Kiang Liu

Genome Center (S.-H.H.J.) and Department of Epidemiology (S.-H.H.J.), Columbia University, New York, NY; Laboratory of Biochemical Genetics and Metabolism (Z.H., J.D.S.), Rockefeller University, New York, NY; and Department of Preventive Medicine (L.C., K.L.), Northwestern University, Chicago, Ill

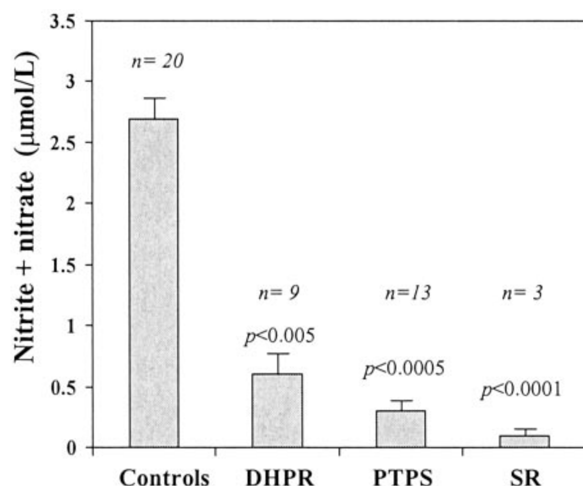
1. Juo SH, Han Z, Smith JD, Colangelo L, Liu K. Common polymorphism in promoter of microsomal triglyceride transfer protein gene influences cholesterol, ApoB, and triglyceride levels in young African American men: results from the coronary artery risk development in young adults (CARDIA) study. *Arterioscler Thromb Vasc Biol*. 2000;20:1316–1322.
2. Liang KY, Zeger SL. Longitudinal data analysis using generalized linear models. *Biometrika*. 1986;73:13–22.
3. Zeger SL, Liang KY. Longitudinal data analysis for discrete and continuous outcomes. *Biometrics*. 1986;42:121–130.
4. Couture P, Otvos JD, Cupples LA, Wilson PW, Schaefer EJ, Ordovas JM. Absence of association between genetic variation in the promoter of the microsomal triglyceride transfer protein gene and plasma lipoproteins in the Framingham Offspring Study. *Atherosclerosis*. 2000;148:337–343.
5. Karpe F, Lundahl B, Ehrenborg E, Eriksson P, Hamsten A. A common functional polymorphism in the promoter region of the microsomal triglyceride transfer protein gene influences plasma LDL levels. *Arterioscler Thromb Vasc Biol*. 1998;18:756–761.
6. Ledmyr H, Karpe F, Lundahl B, McKinnon M, Skoglund-Andersson C, Ehrenborg E. Variants of the microsomal triglyceride transfer protein gene are associated with plasma cholesterol levels and body mass index. *J Lipid Res*. 2002:43:51–58.

Possible Impact of Tetrahydrobiopterin and Sepiapterin on Endothelial Dysfunction

To the Editor:

Vasquez-Vivar et al[1] reported on diminished tetrahydrobiopterin (BH_4) concentrations in vessels from hypercholesterolemic rabbits. This is an interesting finding because both in serum and urine from patients with coronary artery diseases and hypercholesterolemia, total plasma biopterin concentrations were found to be unchanged[2] (unpublished data, 2002). However, total biopterin represents the sum of BH_4, 7,8-dihydrobiopterin (BH_2), and fully oxidized biopterin, and one cannot exclude possible the effect of either reduced BH_4 or increased BH_2 concentrations on the endothelial dysfunction in these patients. In normal plasma, almost all biopterin (>95%) is present as BH_4, and measurement of biologically active tetrahydro-derivative seems to be essential. Differential oxidation with iodine and subsequent high-pressure liquid chromatography (HPLC), according to Fukushima and Nixon,[3] is a simple method to measure different oxidation forms of biopterin. In this method, under acidic conditions, BH_4 and BH_2 are oxidized to biopterin, while under basic conditions, only BH_2 is oxidized to biopterin, and BH_4 undergoes a side-chain cleavage to form the blue fluorescing pterin. The difference in biopterin content between the two oxidations represents the actual BH_4 levels. HPLC separation of biopterin from pterin and isoxanthopterin is essential for the correct interpretation.

Another important finding of Vasquez-Vivar et al[1] is the observation that supplementation with sepiapterin, an intermediate in the salvage pathway of BH_4, worsens responses to endothelium-dependent agonists Ach and A23187 and that sepiapterin in high concentrations uncouples purified endothelial NO synthase (eNOS) and leads to generation of superoxide O_2^-. Recently, it has been shown that BH_2 and sepiapterin inhibit NOS in vitro by displacing the prebound BH_4 with >80% efficiency.[4] BH_2 and sepiapterin are metabolites that accumulate in patients with variants of BH_4 deficiency, and it has been suggested that they may potentate the superoxide formation during the uncoupled reaction of NOS.[5,6] NO



Mean concentrations of nitrite+nitrate in cerebrospinal fluid from patients with different forms of BH_4 deficiency. DHPR indicates dihydropteridine reductase deficiency; PTPS, 6-pyruvoyl-tetrahydropterin synthase deficiency; SR, sepiapterin reductase deficiency.

production was found to be significantly reduced in the brain in these patients.[7] Particularly, patients with sepiapterin reductase deficiency, in which both BH_2 and sepiapterin accumulate in the brain, have extremely low levels of nitrate and nitrite (end products of NO) in cerebrospinal fluid (Figure) and low-dose treatment with BH_4 (2.5 to 5.0 mg/kg) was not efficient to restore the brain's NO production. Thus, both the oxidation of BH_4 to BH_2 and sepiapterin formation promote uncoupling of the NOS reaction and stimulate superoxide and peroxynitrite production. Although patients with BH_4 deficiency show no cardiovascular problems, one can speculate that, in patients with hypercholesterolemia and other diseases presenting with endothelial dysfunction, a similar situation occurs. Sepiapterin is first reduced in endothelial vessels to BH_2 by sepiapterin reductase, but the capacity of dihydrofolate reductase to reduce BH_2 to BH_4 may be to low, resulting in formation of toxic metabolites. Incubating the vessels with methotrexate, an inhibitor of dihydrofolate reductase, may bring additional information on how sepiapterin and BH_2 promote the superoxide formation.

A number of studies documented that pro-inflammatory cytokines increase BH_4 formation in cultured vascular endothelial cells.[8] Another factor which may influence vascular BH_4 levels is vitamin C. It has been shown recently that long-term treatment with vitamin C decreases BH_2 levels and increase BH_4 levels in aortas of apolipoprotein-deficient mice.[9] Simultaneous application of vitamin C + E, as suggested in patients with sepiapterin reductase deficiency in order to improve the efficiency of the BH_4 therapy, needs further investigation.

Nenad Blau
Beat Thöny
Division of Clinical Chemistry and Biochemistry, University Children's Hospital, Zurich, Switzerland

1. Vasquez-Vivar J, Duquaine D, Whitsett J, Kalyanaraman B, Rajagopalan S. Altered tetrahydrobiopterin metabolism in atherosclerosis: implications for use of oxidized tetrahydrobiopterin analogues and thiol antioxidants. *Arterioscler Thromb Vasc Biol*. 2002;22:1655–1661.
2. Walter R, Blau N, Kierat L, Schoedon G, Reinhart WH. Systemic tetrahydrobiopterin (BH_4) levels and coronary artery disease. *Cardiology*. 2000;94:265–266.
3. Fukushima T, Nixon JC. Analysis of reduced forms of biopterin in biological tissues and fluids. *Anal Biochem*. 1980;102:176–188.
4. Jones CL, Vasquez-Vivar J, Kalyanaraman B, Griscavage-Ennis M, Gross SS. Tetrahydropterins but not dihydropterins attenuate the reduction of superoxide from eNOS: a novel role for tetrahydrobiopterin. *Pteridines*. 2001;12:52–53.
5. Bonafé L, Thöny B, Penzien JM, Czarnecki B, Blau N. Mutations in the sepiapterin reductase gene cause a novel tetrahydrobiopterin-dependent monoamine neurotransmitter deficiency without hyperphenylalaninemia. *Am J Hum Genet*. 2001;69:269–277.
6. Zorzi G, Redweik U, Trippe H, Penzien JM, Thöny B, Blau N. Detection of sepiapterin in CSF of patients with sepiapterin reductase deficiency. *Mol Genet Metab*. 2002;75:174–177.
7. Zorzi G, Thöny B, Blau N. Reduced nitric oxide metabolites in CSF of patients with tetrahydrobiopterin deficiency. *J Neurochem*. 2002;80: 362-364.
8. Rosenkranz-Weiss P, Sessa WC, Milstien S, Kaufman S, Watson CA, Pober JS. Regulation of nitric oxide synthesis by proinflammatory cytokines in human umbilical vein endothelial cells: elevations in tetrahydrobiopterin levels enhance endothelial nitric oxide synthase specific activity. 1994;93:1875–1876.
9. D'Uscio LV, Milstien S, Richardson D, Smith L, Katusic ZS. Long-term vitamin C treatment increases vascular tetrahydrobiopterin levels and nitric oxide synthase activity. *Circ Res*. 2003;92:88–95.

In Response:

Inadequate tetrahydrobiopterin (BH_4) concentration has been linked to impaired endothelial NO synthase (eNOS) activity and to loss of endothelial function in vascular conditions such as hypercholesterolemia. Blau and Thöny correctly contend that despite the critical importance of BH_4 in NO formation from eNOS, patients with BH_4 deficiency typically show no evidence of vascular disease. Studies that have evaluated the effects of BH_4 supplementation have further observed divergent effects in animal models as well as in humans, with some studies reporting beneficial or no effects and a few reporting actual worsening of vascular function.[1] We have recently presented an alternative hypothesis that may reconcile these apparently contradictory findings. This is that the relative concentrations of fully reduced (BH_4) and oxidized forms of tetrahydrobiopterin (BH_2), rather than the absolute concentration of BH_4, are what governs the nature of products generated from eNOS.[2] At low BH_4 or high BH_2 (7,8-dihydrobiopterin or sepiapterin) levels, eNOS generates superoxide that is not inhibited by saturating L-arginine or ascorbate.[2] In addition, experimental BH_4 deficiency (such as that induced by treatment with 2,4-diamino-6-hydroxy-pyrimidine and *N*-acetylserotonin, a GTP cyclohydrolase-I and sepiapterin reductase inhibitor, respectively) shows that supplementation with sepiapterin in the presence of *N*-acetylserotonin causes further increases in superoxide production by endothelial cells. This indicates that the BH_4/BH_2 ratio controls superoxide formation in the endothelium and may represent a physiologically relevant mechanism to regulate eNOS products.

Based on our study, it appears that BH_4 homeostasis in atherosclerotic vascular tissue is complex. While the tissue retains the ability to generate BH_4 from sepiapterin, this activity does not translate to improvements in vascular relaxation. Cotreatment with *N*-acetylcysteine augments BH_4 concentrations beyond normal levels and yet did not ameliorate vascular function. This result indicates that BH_4 metabolism is altered in experimental atherosclerosis, and that mere supplementation with sepiapterin, an oxidized precursor of BH_4, alone or with antioxidants does not translate to therapeutic benefits. The mechanisms involved in the regulation of BH_4 levels in the vascular wall are not known. Likewise, it is unclear how ascorbate may influence the BH_4/BH_2 ratio in the endothelium. It has been shown that chemical reduction of 7,8-BH_2 to generate BH_4 is not a mechanism by which ascorbate alters BH_4/BH_2 ratio.[3] Also, ascorbate does not improve de novo BH_4 synthesis, nor is it a first-line defense against superoxide and peroxynitrite.[4] Thus a thorough understanding of the mechanisms involved in regulating the balance between the different redox forms of BH_4, and the role of anti-oxidants in maintaining this balance, appears to be critical in designing rational therapeutic strategies to ameliorate endothelial dysfunction. This objective, however, will not be accomplished with the available analytical methods for BH_4 analysis. Indirect methodology such as that based on BH_4 oxidation to secondary products is complicated by fact that BH_4 is oxidized to generate several products. Clearly, this methodology prevents full identification and

quantification of the analogs involved in the process. The development of more direct methods for quantifying BH_4 and its metabolites in cells and tissues is warranted.

Jeannette Vásquez Vivar
Sanjay Rajagopalan
Medical College of Wisconsin (J.V.V.), Biophysics Research Institute and Free Radical Research Center, Milwaukee, Wis; and University of Michigan (S.R.), Department of Internal Medicine, Ann Arbor, Mich

1. Huraux C, Makita T, Kurz S, Yamaguchi K, Szlam F, Tarpey MM, Wilcox JN, Harrison DG, Levy JH. Superoxide production, risk factors, and endothelium-dependent relaxations in human internal mammary arteries. *Circulation*. 1999;99:53–59.
2. Vásquez-Vivar J, Martásek P, Whitsett J, Joseph J, Kalyanaraman B. The ratio between tetrahydrobiopterin and oxidized tetrahydrobiopterin analogues controls superoxide release from endothelial nitric oxide synthase: an EPR spin trapping study. *Biochem J*. 2002;362:733–739.
3. Vásquez-Vivar JM, Martásek P, Kalyanaraman B. BH4/BH2 ratio but not ascorbate controls superoxide and nitric oxide generation by eNOS. *Circulation*. 2000;102(Suppl II):63. Abstract
4. Vásquez-Vivar J, Santos AM, Augusto O. Peroxynitrite-mediated formation of free radicals in human plasma: EPR-detection of ascorbyl, albumin-thiyl and uric-acid-derived free radicals. *Biochem J*. 1995;314:869–876.