

The association of the Glu298Asp polymorphism of endothelial nitric oxide synthase (eNOS) with outcome after subarachnoid hemorrhage

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A. Introduction

Subarachnoid hemorrhage (SAH) accounts for 25% of all cerebrovascular deaths. Mortality rates after SAH have been reported to be as high as 50%¹; among the remaining survivors, 50% are left severely disabled. Morbidity and mortality are largely due to rebleeding and vasospasm.² Currently, prediction of outcome relies on demographic, clinical, and radiological factors. Yet, accurate prediction of outcome after SAH remains imprecise.^{3,4} Prior studies examining prognostic factors have found that the amount of hemorrhage, clinical grade (Hunt & Hess or WFNS), age, preexisting hypertension, aneurysm size and location, and vasospasm all contribute to poor outcome⁵.

Delayed ischemic neurological deficit (DIND) due to the reduction in cerebral blood flow from vessel narrowing in cerebral vasospasm is one of the most serious consequences of SAH. The incidence of angiographic vasospasm can be in excess of 50%, with symptomatic vasospasm occurring in 30% of patients.⁶ Predicting the neurological decline of a patient with vasospasm can be difficult, as not every patient with vasospasm becomes clinically symptomatic. However, early detection of vasospasm is essential to ensure rapid treatment before ischemic damage occurs whether patients are symptomatic or not. Considerable research effort has been directed toward demonstrating both the mechanisms and potential predictors of delayed cerebral vasospasm following SAH.⁷

The mechanism of vasospasm has not been completely elucidated and multiple factors are likely to play a role. Free radical reactions triggered by oxyhemoglobin released from the subarachnoid clot are considered to be important in the development of cerebral vasospasm. Oxyhemoglobin scavenges nitric oxide after SAH creating a deficit of NO and an imbalance between vasodilatory and vasoconstrictive factors. Prior studies have helped to demonstrate the important role of NO in vasospasm. Animal studies have shown that NO replacement reverses cerebral vasospasm.^{8,9} Further, an intracellular NO donor, hydroxylamine, attenuated post-SAH neurological deficit in a rat model of SAH.¹⁰

¹ Hop JW, Rinkel GJ, Algra A, van Gijn J. Case-fatality rates and functional outcome after subarachnoid hemorrhage: a systematic review. *Stroke*. 1997. 28: 660–664.

² Kassell NF, Torner JC, Haley EC Jr, Jane JA, Adams HP, Kongable GL. The International Cooperative Study on the Timing of Aneurysm Surgery, part 1: overall management results. *J Neurosurg*. 1990;73:18–36.

³ Oshiro EM, Walter KA, Piantadosi S, Witham TF, Tamargo RJ. A new subarachnoid hemorrhage grading system based on the Glasgow Coma Scale: a comparison with the Hunt and Hess and World Federation of Neurological Surgeons scales in a clinical series. *Neurosurgery*. 1997;41: 140–148.

⁴ Chiang VLS, Claus EB, Awad IA. Toward more rational prediction of outcome in patients with high-grade subarachnoid hemorrhage. *Neurosurgery*. 2000;46:28–36.

⁵ Ohman, J., et al (1991). Risk factors for cerebral infarction in good-grade patients after aneurysmal subarachnoid hemorrhage: A prospective study. *J. Neurosurg*. 74, 14-20

⁶ Kassell NF, Torner JC, Haley EC Jr, et al: The International Cooperative Study on the Timing of Aneurysm Surgery. Part 1: Overall management results. **J Neurosurg** 73:18–36, 1990

⁷ Yanaka K, Kamezaki T, Fujita K, et al: Prevention of delayed cerebral vasospasm after aneurysmal subarachnoid hemorrhage. **Drugs Today** 37:573–578, 2001

⁸ Wolf, EW., Banerjee, A., et al. Reversal of cerebral vasospasm using an intrathecally administered nitric oxide donor. *J Neurosurg*. 1998; 89: 279-288.

Nitric oxide is synthesized by three different isoforms of nitric oxide synthase (NOS) and NO is known to play an important role in vascular smooth muscle relaxation and proliferation, decreased platelet and leukocyte adhesion and endothelial permeability. Endothelial nitric oxide synthase (eNOS or NOS-3) is the main contributor of circulating NO and for this reason will be the main focus of this study.

In previous studies, the Glu298Asp polymorphism of eNOS has been associated with myocardial infarction^{11,12} Alzheimer's Disease¹³, and stroke¹⁴. This study will attempt to determine what effect the Glu298Asp polymorphism of eNOS has on vasomotor function and the predictive ability of this polymorphism on vasospasm and long-term neurological outcome. Further, this study will analyze the nitric oxide pathway from a novel vantage point and will help to further define nitric oxide's role in vasospasm and long-term neurological outcome.

a. Hypothesis

We hypothesize that carriers of the Glu298Asp eNOS polymorphism (genotype G/T or T/T) will be more likely to develop vasospasm and delayed ischemic neurological deficit and will have a worse outcome after SAH. eNOS is considered to have a protective effect in SAH models primarily by its ability to increase NO levels in the endothelium. Therefore, a genetic variant known to diminish eNOS production of NO will likely have a negative effect on prevention of vasospasm and recovery after SAH.

B. Methods

a. Conceptual and Operational Definitions

The primary outcome of this study will be defined as the presence or absence of vasospasm. To diagnose vasospasm, daily transcranial doppler (TCD) examinations, and routine clinical assessments will be performed. TCDs will be acquired at the bedside and will be done daily by trained personnel for the entire course of the patient's hospital stay. The diagnosis of vasospasm will be further confirmed by angiography.

Long-term Patient Outcomes will be assessed at 6 months and one year according to the Glasgow Outcome Scale (GOS), modified Rankin scale, modified Lawton scale, and SIP depression scale. A good or moderately disabled score on these tests generally indicates that the patient is able to live independently, caring for all their needs, and are able to participate in a normal social life and will be classified as a good outcome. Patients who die or are not capable of living independently will be classified as having an unfavorable outcome.

b. Study Design

This will be a prospective, observational case-control study. Two populations will be considered, those who carry the Glu298Asp allele of the eNOS gene (genotype G/T, T/T) and those who do not carry this allele (genotype G/G). The presence or absence of vasospasm in the two populations as determined by TCD, clinical and angiographic data will be considered. As vasospasm is one of the top contributors to morbidity and mortality after SAH by causing delayed ischemic neurological deficit, it is a key predictor of outcome. Other assessments of outcome after SAH will be used and will include the GOS, modified Rankin, modified Lawton and SIP depression scores.

⁹ Afshar JKB, Pluta RM, Boock RJ, Thompson BG, Oldfield EH. Effect of intracarotid nitric oxide on primate cerebral vasospasm after subarachnoid hemorrhage. *J Neurosurg.* 1995;83:118-122

¹⁰ Ryba, MS. Et al. Hydroxylamine attenuates the effects of simulated subarachnoid hemorrhage in the rat brain and improves neurological outcome. *Brain Research.* 850(1-2):225-33, 1999 Dec 11.

¹¹ Hibi, K. Ishigami, T, et al. Endothelial nitric oxide synthase gene polymorphism and acute myocardial infarction. *Hypertension.* 1998; 32:521-526.

¹² Shimasaki, Y, Yasue, H, et al. Association of the Missense Glu298Asp Variant of the Endothelial Nitric Oxide Synthase Gene with Myocardial Infarction. *JACC.* Vol. 31, No.7 June 1998: 1506-10.

¹³ Dahiyat, M., et al. Association between Alzheimer's disease and the NOS3 gene. *Ann Neurol.* 1999; 46: 664-667

¹⁴ Elbaz, A, Poirier, O, et al. Association between the Glu298Asp polymorphism in the endothelial constitutive nitric oxide synthase gene and brain infarction. *Stroke.* 2000; 31: 1634-1639.

C. Patient population

All patients (18 years of age or older) presenting to the Columbia Presbyterian Hospital emergency room or transferred from an outside hospital to Columbia Presbyterian with subarachnoid hemorrhage independent of etiology will be considered for genetic testing irrespective of their clinical status. It is expected that approximately ninety SAH patients will be eligible for recruitment each year based on prior census history. Population-based studies have demonstrated that 97% of patients suffering from SAH are hospitalized; thus, minimal bias in patient selection is likely.¹⁵

Exclusion criteria will include patients less than 18 years of age, pregnant women, employees, students or other affiliates of Columbia University, and subjects who are unable to give informed consent either directly or through a health proxy.

Patients will not be given compensation and will receive no direct benefit from this study.

Informed Consent: Written or verbal, informed consent will be obtained on every patient enrolled in this study. In those patients who are unable to give informed consent (Grade IV and V patients), surrogate consent will be obtained from a legally authorized representative or if such a representative is not available the patient's next of kin. High-grade patients often do improve with treatment and may be able to provide informed consent themselves during their recovery.

If Columbia University determines that surrogate consent is not acceptable for high-grade SAH patients, a waiver of consent will be requested as the study involves minimal risk to the patient. The only foreseeable risks are those associated with venipuncture and breach of confidentiality. Venipuncture is a minimally invasive procedure as the primary associated risks are pain or local bruising at the insertion site and a minimal risk for infection. We will further minimize this risk by making every effort to combine the blood sample collection with a venipuncture ordered by another physician for treatment purposes or by eliminating the venipuncture altogether if a catheter (central venous or arterial line) suitable for blood drawing is already present.

D. Protection of patient confidentiality

A potential breach of confidentiality will be minimized by storing all study information in locked research files and password protected databases that will be accessible only by study personnel. No record of the patient's participation in this study will be placed in his/her medical record. Any genetic material stored for future use will be stripped of identifying information and replaced with a randomly assigned code. The key linking this code to the identity of the subject will be electronically maintained in a password-protected database at Columbia University and will be accessible only to study personnel

Patient Variables to be recorded and analyzed will include but will not be limited to the following:

- *Patient characteristics:* age, sex, ethnicity, history of smoking, hypertension, preexisting cardiac disease or vascular disease
- *Admission status:* Hunt & Hess grade, systolic blood pressure, admission CT findings (i.e. Fisher grade), admission angiogram findings (i.e. aneurysm size & location, presence of vasospasm)
- *Pre-op and post-op clinical course:* rebleed, ventriculostomy, ICP, technique of aneurysm obliteration, timing of surgery, seizures, surgical complications, medical complications

These variables have been chosen as the most likely sources of potential confounders when analyzing the data and are the most important variables to consider.

¹⁵ Longstreth, W. et al: Clinical course of spontaneous subarachnoid hemorrhage: a population-based study in King County, Washington. **Neurology** 43: 712-718, 1993.

a. Management Protocol

All patients will be managed according to standard of care including early surgery, aggressive prevention and treatment of intracranial hypertension and vasospasm, and any further comprehensive intensive care when needed. Patients who develop symptomatic vasospasm will be treated with induced hypertension (as part of Triple H therapy). Angioplasty or other similar procedures will be used to ameliorate vasospasm when other therapies are ineffective.

b. Genetic Analysis

DNA sampling will take place either during the inpatient stay or during an outpatient follow-up appointment. We will also contact individuals that previously participated in the SAH Outcomes Project (IRB# 7718) who indicated willingness to be contacted for future studies

Once the potential subjects are identified and enrolled, DNA samples will be collected by buccal (cheek) swab for genetic analysis. Only if subjects are intubated, approximately 20cc of their blood will be collected for DNA extraction. All patient identifiers will be removed before the DNA samples are submitted for genetic analysis.

Glu298Asp eNOS polymorphism will be identified with PCR followed by RFLP analysis with the restriction enzymes DpnII and BanII to digest mutant and wild type alleles, respectively. The restriction products will be analyzed through electrophoresis on a 2% agarose gel.

c. Statistical Analysis

TCD velocity data, GOS, modified Rankin, modified Lawton, and SIP depression scores will be summarized as a mean ± standard deviation, or as the median if the data deviates from a normal distribution. An unpaired Student t- test will be used to analyze differences between group means for continuous variables with a normal distribution. Otherwise, a Mann-Whitney test will be used to evaluate differences between groups whose distributions fail to meet the assumptions of a t-test. Where appropriate, Chi-square analyses will be performed on all categorical patient variables and Student t-test will be performed on all continuous patient variables to determine differences between the control (G/G genotype) and experimental populations (G/T, T/T genotype). Multivariate analyses will be performed using logistic regression to determine the effect of presence of the Glu298Asp eNOS polymorphism in relation to other clinical and demographic variables. Significance for all tests will be set at a 0.05 significance level.

d. Sample Size

The proposed number of subjects to be enrolled (450) is justified based on a conservative power calculation. With the significance level set at 0.05 and a given power of 80%, approximately 200 subjects will be needed for each group as determined by a Chi Square Test. This calculation was based on the known frequency of the Glu298Asp polymorphism of endothelial nitric oxide synthase (eNOS). The effect size of this variant was based on literature in the acute myocardial infarction population. This population provides an ischemia-reperfusion model that is similar to the ischemia-reperfusion that is associated with SAH. The frequency of the allele in the acute myocardial infarction population was found to be 0.211 and the frequency of the allele in the control group was found to be 0.134.¹⁶

e. Measurement of NO metabolites

A genetic contribution of eNOS to plasma NO metabolite levels has been demonstrated.^{13,14} Several polymorphisms have been identified in the eNOS gene (NOS3), among which is a polymorphism located in exon 7 (G894T) that modifies its coding sequence (Glu298Asp)

Multivariate analysis will need to be performed for: 1 **Fisher grading** -has been used for evaluating the amount of the subarachnoid clot and for predicting the occurrence of vasospasm.¹⁷ 2 **Age-**

¹⁶ Shimasaki et al. Association of the Missense Glu298Asp Variant of the Endothelial Nitric Oxide Synthase Gene with Myocardial Infarction. JACC Vol 31, No. 7 June 1998; 1506-10.

¹⁷ Fisher CM, Kistler JP, Davis JM: Relation of cerebral vasospasm to subarachnoid hemorrhage visualized by computerized tomographic scanning. **Neurosurgery** 6:1-9, 1980

there seems to be a significant increased risk of poor outcome after the age of 60 years.¹⁸ 3. **Preexisting medical conditions** (arterial hypertension, diabetes, lung disease, cerebrovascular and cardiac diseases).

¹⁸ Lanzino, G. et al. Age and outcome after aneurysmal subarachnoid hemorrhage: why do older patients fare worse? *Journal of Neurosurgery*. 85(3):410-8, 1996 Sep