A *NOS3* Polymorphism Determines Endothelial Response to Folate in Children with Type 1 Diabetes or Obesity

Esko J. Wiltshire, MD, FRACP[1,2], Alexia S. Peña, PhD, FRACP[2,3], Karen MacKenzie, PhD, FRACP[2,3], Tulika Bose-Sundernathan, MSc[4], Roger Gent, DMU[5], and Jennifer J. Couper, MD, FRACP[2,3]

Objective To determine the effect of polymorphisms in *NOS3* and folate pathway enzymes on vascular function and folate status and endothelial response to folate in children with diabetes or obesity.

Study design A total of 244 subjects (age 13.8 ± 2.8 years, 125 males) were studied for *NOS3* and/or folate pathway polymorphisms using polymerase chain reaction/restriction fragment length polymorphism, including at baseline: 139 with type 1 diabetes; 58 with obesity; and 47 controls. The effect of *NOS3* genotype on endothelial response to folate (5 mg) was assessed in 85 subjects with diabetes and 28 obese subjects who received active treatment during intervention trials. Vascular function (flow-mediated dilatation [FMD] and glyceryl trinitrate-mediated dilatation), clinical, and biochemical measurements were assessed at baseline and 8 weeks in folate intervention studies.

Results Folate pathway enzyme and *NOS3* polymorphisms did not significantly affect baseline vascular function. The polymorphism in intron 4 of endothelial nitric oxide synthase altered endothelial response to folate significantly: in subjects with diabetes FMD improved by 6.4 ± 5% (insertion carriers) vs 2.3 ± 6.6% (deletion carriers), $P = .01$; in obese subjects FMD improved by 1.8 ± 5.4% (insertion carriers) and deteriorated by −3.2 ± 7.2% (deletion carriers), $P = .05$. More subjects carrying the insertion normalized FMD after folate supplementation (insertion 64% vs deletion 28%, $\chi^2 = 10.14$, $P = .001$).

Conclusions A *NOS3* polymorphism predicts endothelial response to folate in children with diabetes or obesity, with implications for vascular risk and folate intervention studies. *(J Pediatr 2015;166:319-25)*.

Endothelial dysfunction, a fundamental event in the development of atherosclerosis,[1] occurs early in both type 1 diabetes and obesity, before clinically detectable atherosclerosis[2] and is critical to the pathogenesis of micro- and macrovascular complications.[3] Endothelial dysfunction of the coronary arteries predicts atherosclerosis progression and cardiovascular events.[4] Endothelial function of the brachial artery can be measured noninvasively using flow-mediated dilatation (FMD).[5] Brachial artery FMD correlates well with coronary endothelial function[6] and intima-media thickness.[7] Endothelial function as measured by FMD is impaired in children with both type 1 diabetes[8] or obesity.[9]

Endothelial nitric oxide synthase (eNOS, E.C. 1.14.13.39), coded by the *NOS3* gene, is a key endothelial enzyme in maintaining vascular tone. A common polymorphismin intron 4 of endothelial nitric oxide synthase (eNOS4), influences nitric oxide (NO) production[10] and may predispose to diabetic nephropathy and retinopathy.[11] Another common polymorphism, Glu298Asp, also alters endothelial function in young healthy men.[12]

Endothelial function and eNOS itself are both directly affected by folate supplementation. Endothelial function improves within hours of oral folate[13] and within minutes of intravenous 5-methyl-tetrahydrofolate, its active form.[14] Folate affects eNOS function, both directly and by enhancing availability of its cofactor, tetra-hydro biopterin (BH_4).[15] In combination these data suggests endothelial response to folate may be affected by *NOS3* polymorphisms.

Two polymorphisms in the enzyme methylene tetrahydrofolate reductase (MTHFR, EC 1.5.1.20), 677C→T and 1298A→C, and a further polymorphism in methionine synthase reductase (MTRR, EC 2.1.1.135), 66 A→G,

BH_4	Tetra-hydro biopterin
eNOS	Endothelial nitric oxide synthase (enzyme)
eNOS4	Insertion/deletion polymorphism in intron 4 of endothelial nitric oxide synthase
FMD	Flow-mediated dilatation
GTN	Glyceryl trinitrate
MTHFR	Methylene tetrahydrofolate reductase
MTRR	Methionine synthase reductase
NO	Nitric oxide
RCF	Red cell folate
RFLP	Restriction fragment length polymorphism
tHcy	Total plasma homocyst(e)ine

From the [1]Department of Pediatrics and Child Health, University of Otago Wellington, Wellington, New Zealand; [2]Discipline of Pediatrics and Robinson Institute, University of Adelaide, Adelaide, Australia; [3]Diabetes and Endocrinology Department, [4]Genetics and Molecular Pathology Department, and [5]Medical Imaging, Women's and Children's Hospital, North Adelaide, South Australia, Australia

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are associated with altered folate and homocysteine metabolism and have been implicated as risk factors for vascular disease.[16,17] Limited evidence links these polymorphisms with increased risk of complications in type 1 and 2 diabetes.[18-20] We have shown an association between polymorphisms in MTHFR and MTRR and onset of elevated albumin excretion rate in children with type 1 diabetes.[21]

We have previously shown that endothelial dysfunction in children with type 1 diabetes relates to red cell folate (RCF),[8] and in intervention studies folate improves[22] or normalizes[23] endothelial function in children with type 1 diabetes, but not with obesity.[24] However, there are no studies assessing the interaction between endothelial response to folate and *NOS3* polymorphisms, which may explain individual variations in response.

We hypothesized that polymorphisms in *NOS3* would influence the endothelial response to folate and polymorphisms in folate pathway enzymes, or *NOS3* may influence vascular function in children with diabetes or obesity. We therefore, sought to determine in children with diabetes and obesity the interaction between polymorphisms in *NOS3*, vascular function, and endothelial responsiveness to folate and the influence of polymorphisms in MTHFR and MTRR on vascular function, to further investigate at a clinical level the interaction between folate, eNOS, folate gene polymorphisms, and vascular function.

Methods

A total of 244 subjects (age 13.8 ± 2.8 years, 125 males) were studied: 139 with type 1 diabetes (136 Caucasian, 1 African, 1 Latino, 1 Asian), 58 with obesity (55 Caucasian, 2 Australian Aboriginal, 1 Asian), and 47 controls (45 Caucasian, 2 Asian). Subjects had all participated in our previous studies of endothelial function in children with diabetes or obesity, conducted prior to mandatory folate fortification in Australia, including a cross-sectional study with age/sex-matched controls[9] and 3 separate randomized double-blind placebo-controlled intervention trials assessing the effect of folate on endothelial function.[22-24] All studies had the same senior investigators, the same dose of folate (5 mg/d), the same outcome measures measured at baseline and 8 weeks, the same inclusion (other than diabetes[22,23] or obesity[24]) and exclusion criteria, the same vascular function protocols, and the same ultrasound equipment and sonographer expertise. Exclusion criteria in all 4 studies were history of smoking, lipid lowering treatment, vitamin B12 deficiency, current or recent use of folate supplements, and subjects with syndromal or endocrinological causes of obesity. DNA was collected prospectively in all studies, to allow for pooling of polymorphism data. The Human Research Ethics Committee of the Women's and Children's Hospital, Adelaide, Australia approved the studies. Written informed consent was obtained from the parents/guardians and the patient if he/she was more than 16 years old.

All subjects were included in the baseline analysis. To assess endothelial response to folate, results from subjects who were randomized to receive active therapy in the intervention trials were analyzed. Subjects receiving placebo in the intervention trials could not be included in this analysis, as they did not have an endothelial response to folate. This analysis, therefore, included 24 subjects from the cross-over study of folate supplementation in type 1 diabetes,[22] 61 subjects who received folate in the parallel-arm study in type 1 diabetes[23] [31 received folate and placebo, 30 received folate and vitamin B6 (100 mg/d), subjects in these groups were combined as there was no difference in endothelial function at 8 weeks between them], and 28 subjects who received folate in the parallel arm study in obesity.[24] Thirty-one subjects who received vitamin B6 alone (and demonstrated an endothelial response) in the parallel arm study[23] acted as a treatment control group.

During all studies, subjects were instructed to avoid other vitamin preparations. Compliance, assessed by pill counting, was higher than 80%. At each assessment, vascular function [FMD and glyceryl trinitrate (GTN)-mediated dilatation], serum folate, RCF, total plasma homocyst(e)ine (tHcy), vitamin B12, fasting lipids, height, weight, and blood pressure were measured as previously described.[8,9,22-24]

Ultrasound Assessment of Vascular Function

In all studies, FMD and GTN-mediated dilatation were assessed by the same experienced sonographers using identical equipment after overnight fasting in a quiet and stable temperature environment, as previously described.[8,9,22-24] In brief, brachial artery diameter was measured in longitudinal section with a 10 MHz linear array transducer using HDI 3000 ultrasound system, with a simultaneous electrocardiogram. Each study included 4 scans: (1) resting scan; (2) reactive hyperemia scan (FMD) recorded between 45 and 75 seconds after deflation of a sphygmomanometer cuff which occluded arterial blood flow for 4 minutes; (3) re-control scan 10-15 minutes later; and (4) post-GTN scan, 4 minutes after the sublingual administration of 400 μg GTN. For each scan, average measurements were made by blinded observers over 4 consecutive cardiac cycles, incident with the electrocardiogram R wave. Our inter-assay coefficient of variation between 20 subjects studied twice was 3.9% for FMD and 4.0% for GTN-mediated dilatation[8] and interobserver coefficient of variation in 20 subjects was 3.85% for FMD and 4.2% for GTN-mediated dilatation.

Laboratory Tests

Triglycerides, total, high density lipoprotein, and low density lipoprotein cholesterol, hemoglobin A1c, glucose, and tHcy were measured as previously described.[8,9,22-24] Vitamin B12, serum folate, and RCF were measured using an Ion Capture reaction (AxSYM Folate System Abbott Laboratories, Abbott Park, Illinois) in the cross-sectional[9] and first 2 intervention studies[22,23] and by chemiluminescent microparticle binding protein assay (Architect