Endothelial Dysfunction Relates to Folate Status in Children and Adolescents With Type 1 Diabetes

Esko J. Wiltshire,[1] Roger Gent,[2] Craig Hirte,[3] Alexia Pena,[4] David W. Thomas,[5,6] and Jennifer J. Couper[4,6]

Endothelial dysfunction occurs early in the development of vascular disease in diabetes. Total plasma homocyst(e)ine (tHcy) is associated with endothelial dysfunction. We therefore aimed to assess endothelial function in children with type 1 diabetes in relation to tHcy and its determinants. Endothelial function was assessed in 36 children with type 1 diabetes aged 13.7 ± 2.2 years and 20 age- and sex-matched control subjects using ultrasound assessment of flow-mediated dilatation (FMD) and glyceryl trinitrate (GTN)-dependent brachial artery responses. von Willebrand factor (vWF) and thrombomodulin, markers of endothelial activation, were measured in 64 children with type 1 diabetes and 52 control subjects. Fasting glucose, tHcy, serum and red cell folate, vitamin B12, HbA_{1c}, creatinine, and lipids were also measured. FMD (5.2 ± 4.7 vs. 9.1 ± 4.0%, $P = 0.002$) and the ratio of FMD:GTN-induced dilatation (0.22 ± 0.39 vs. 0.41 ± 0.29%, $P = 0.008$) were significantly lower in diabetic subjects, indicating endothelial dysfunction. In diabetic subjects, red cell folate correlated independently with FMD ($\beta = 0.42$, $P = 0.028$) and the ratio of FMD:GTN-induced dilatation ($\beta = 0.59$, $P < 0.001$). Resting vessel diameter correlated independently with tHcy ($\beta = -0.51$, $P < 0.001$) and height ($\beta = 0.65$, $P < 0.001$). vWF correlated independently with HbA_{1c} ($\beta = 0.38$, $P = 0.003$), and thrombomodulin correlated independently with red cell folate ($\beta = -0.38$, $P = 0.005$), tHcy ($\beta = -0.37$, $P = 0.004$), diastolic blood pressure ($\beta = -0.28$, $P = 0.025$), and creatinine clearance ($\beta = 0.26$, $P = 0.033$). Children with type 1 diabetes have early endothelial dysfunction. Better folate status is associated with better endothelial function, as measured by higher FMD, higher FMD: GTN ratio, and lower thrombomodulin. Folate may therefore protect against endothelial dysfunction in children with diabetes. *Diabetes* 51:2282–2286, 2002

From the [1]Department of Paediatrics, Wellington School of Medicine and Health Sciences, Wellington, New Zealand; the [2]Department of Ultrasonography, Women's and Children's Hospital, Adelaide, Australia; the [3]Public Health Research Unit, Women's and Children's Hospital, Adelaide, Australia; the [4]Department of Diabetes and Endocrinology, Women's and Children's Hospital, Adelaide, Australia; the [5]Department of Clinical Pathology, Women's and Children's Hospital, Adelaide, Australia; and the [6]Department of Paediatrics, University of Adelaide, Adelaide, Australia.

Address correspondence and reprint requests to Esko J. Wiltshire, Department of Paediatrics, Wellington School of Medicine and Health Sciences, P.O. Box 7343, Wellington South, New Zealand. E-mail: esko@wnmeds.ac.nz.

Received for publication 3 January 2002 and accepted in revised form 12 April 2002.

CV, coefficient of variation; FMD, flow-mediated dilatation; GFR, glomerular filtration rate; GTN, glyceryl trinitrate; Lp(a), lipoprotein (a); tHcy, total plasma homocyst(e)ine; vWF, von Willebrand factor.

The endothelium is a key regulator of vascular function (1). Endothelial dysfunction is fundamental to the development of any vascular process and occurs early in the development of atherosclerosis (2).

Considerable laboratory and clinical evidence indicates that endothelial dysfunction is a critical part of the pathogenesis of microvascular and macrovascular complications in type 1 diabetes (3), and these complications develop early (4,5). Endothelial dysfunction precedes the development of microalbuminuria, the earliest sign of diabetic nephropathy (6). Therefore, understanding the determinants of endothelial dysfunction early in type 1 diabetes is critical to strategies aimed at preventing these complications.

Total plasma homocyst(e)ine (tHcy) is identified as an independent risk factor for atherosclerosis in epidemiological, clinical, and laboratory investigations (7,8) (tHcy refers to the combined plasma pool of homocysteine, homocystine, mixed disulfides involving homocysteine, and homocysteine thiolactone). Hyperhomocyst(e)inemia is associated with endothelial dysfunction and oxidative injury (8). Therefore, the potential exists for interactions between homocysteine and other factors implicated in the pathogenesis of vascular complications in patients with diabetes; such an interaction has been suggested in a clinical prospective study of mortality in type 2 diabetes (9).

Studies in adults with either type 1 or type 2 diabetes have shown higher tHcy in patients with microvascular (10) and macrovascular (11) complications. Reduced tHcy concentrations have been found in some populations of adults with type 1 diabetes (12,13). Glomerular hyperfiltration has been proposed as the mechanism (14). Despite these reduced tHcy values, the gradation of mortality risk associated with tHcy is preserved in diabetes (15).

We recently reported that children and adolescents with type 1 diabetes have lower tHcy values (16), in part because of better folate and vitamin B12 status and glomerular hyperfiltration. To assess the biological consequences of these lower tHcy values, we aimed to investigate endothelial function and its association with tHcy and its determinants in children and adolescents with type 1 diabetes.

RESEARCH DESIGN AND METHODS

Subjects. Children and adolescents with type 1 diabetes were recruited consecutively from the diabetes clinic at the Women's and Children's Hospital

TABLE 1
Results of ultrasound studies in the diabetic group and control subjects

	Diabetic group	Control subjects	P†
n	35	20	—
FMD (%)	5.2 ± 4.7	9.1 ± 4.0	0.002
GTN-induced dilatation (%)	19.5 ± 6.5	23.3 ± 7.4	0.07
FMD:GTN ratio‡	0.25 (−1.75 to −0.9)	0.41 (0.06 to 0.92)	0.008§
Resting vessel diameter (mm)	2.97 ± 0.42	2.94 ± 0.3	0.76
Reactive hyperemia (%)	418.6 ± 143.6	375.3 ± 128.2	0.27
Age (years)	13.7 ± 2.2	13.8 ± 2.5	0.87
Sex (M:F)	18:17	8:10	0.56‖
Height (cm)	157.4 ± 13.2	167.2 ± 13.4	0.025
Weight (kg)	54.2 ± 15.4	60.3 ± 11.7	0.18
Systolic blood pressure (mmHg)	118.3 ± 14.4	119.6 ± 13.9	0.78
Diastolic blood pressure (mmHg)	60.9 ± 6.4	61 ± 9.1	0.95
HbA_{1c} (%)	9.1 ± 0.9	—	—
Insulin dose (units/kg)	1.2 ± 0.35	—	—
Duration of diabetes (years)	5.7 ± 3.3	—	—

Data are means ± SD unless otherwise stated. †Student's t test unless otherwise indicated. ‡Median (range). §Mann-Whitney U test. ‖χ^2 test.

(Adelaide, Australia), which manages 90% of children with diabetes in South Australia. Subjects had had diabetes for at least 6 months and were well, without ketosis or hypoglycemia, and fasting at the time of sample collection. No subject had background retinopathy on direct fundoscopy through dilated pupils or microalbuminuria on measurement of overnight albumin excretion rate. In 64 subjects (of 110 children approached), markers of endothelial activation were assessed. Participants did not differ in any clinical characteristics from the whole clinic population of the same age. Of the subjects, 36 were studied with ultrasound assessment of endothelial function, of whom 20 were studied on two occasions to determine within-subject variation. Markers of endothelial activation were assessed in 52 healthy age- and sex-matched control subjects, recruited from two sources: friends of the participating patients (n = 37) and subjects attending the Women's and Children's Hospital for minor elective day surgery (n = 15). In the latter group, fasting blood was collected before induction of anesthesia. Ultrasound assessment of endothelial function was also performed in 20 healthy age- and sex-matched control subjects (all friends). Tanner stage was assessed by self-report using Tanner stage illustrations and confirmed by a pediatric endocrinologist during routine clinical follow-up in subjects with diabetes. Medication (including vitamin supplements) and smoking history were obtained from all subjects. Any subjects taking vitamin supplements were excluded. Baseline characteristics of the subjects are shown in Tables 1 and 2. The study was approved by the Human Research Ethics Committee, Women's and Children's Hospital. Written informed consent was obtained from parents/guardians of subjects <16 years of age and also from all subjects >12 years of age.

Ultrasound assessment of endothelial function. Flow-mediated dilatation (FMD) and glyceryl trinitrate (GTN)-induced dilatation were assessed as originally reported (17). Using this method, FMD and the ratio of FMD:GTN-induced dilatation provide the best measure of endothelial function (17), which is dependent on release of nitric oxide (18). The diameter of the brachial artery (2–15 cm above the elbow) was measured in a longitudinal section from two-dimensional ultrasound images, with a 10.0-MHz linear array transducer (Advanced Technology Laboratories [ATL], Bothel, WA), using an ATL HDI 3000 ultrasound system. All studies were performed by an experienced pediatric vascular ultrasonographer. The transmit (focus) zone was set to the depth of the vessel. Depth and gain settings were set to optimize images of the lumen/arterial wall interface. All images were obtained at the minimum field of viewing setting to maximize size of the vessel in the image. Machine operating parameters were not changed during any study. A suitable site for imaging the vessel was first selected, with reproducible ultrasonic markers, such as venous valves or vessel bifurcations, to ensure that measurement occurs at the same place for each scan. An electrocardiogram was recorded with the ultrasonic images. The first scan was taken at rest. Reactive hyperemia was induced by occluding arterial blood flow using a sphygmomanometer inflated to 250 mmHg for 4.5 min. Arterial flow velocity was measured by means of a pulsed Doppler signal at 60° to the vessel, during the resting scan, and for the first 15 s after deflation of the cuff. The second scan (reactive hyperemia) was taken for 30 s before and 90 s after cuff deflation. There was 15 min allowed for vessel recovery, and a resting (third, recontrol) scan was repeated. GTN spray (400 μg; Nitrolingual Spray, Rhone-Poulenc Rorer) was administered sublingually, and the last scan was recorded 4 min later.

All images were recorded onto high-quality VHS videotapes and analyzed subsequently. Vessel diameters were measured with ultrasonic calipers by observers blinded to the stage of experiment and subject (diabetes or control) and expressed as percentages of the first control (resting) scan: after reactive hyperemia and after GTN. For each scan, measurements were made over four cardiac cycles, and the measurements were averaged. All measurements were made incident with the electrocardiogram R wave (i.e., at end diastole).

TABLE 2
Results of endothelial activation in the diabetic group and control subjects

	Diabetic group	Control subjects	P†
n	64	52	—
vWF (%)‡	123 (57–477)	113 (54–294)	0.13§
Thrombomodulin (pg/l)‡	9.35 (0–1,127)	10.85 (0.1–950)	0.34§
Age (years)	13.6 ± 2.7	13.2 ± 2.4	0.45
Sex (M:F)	36:28	27:25	0.71‖
Height (cm)	156.8 ± 15	157.3 ± 13.4	0.84
Weight (kg)	54.7 ± 18.7	52.4 ± 16.1	0.5
Systolic blood pressure (mmHg)	123.1 ± 14.6	118.1 ± 16.9	0.14
Diastolic blood pressure (mmHg)	65.2 ± 8.4	64.7 ± 9.4	0.87
HbA_{1c} (%)	9.2 ± 1.7	—	—
Insulin dose (units/kg)	1.1 ± 0.4	—	—
Duration of diabetes (years)	5.4 ± 3.8	—	—

Data are means ± SD unless otherwise indicated. †Student's t test unless otherwise indicated. ‡Median (range). §Mann-Whitney U test. ‖χ^2 test.

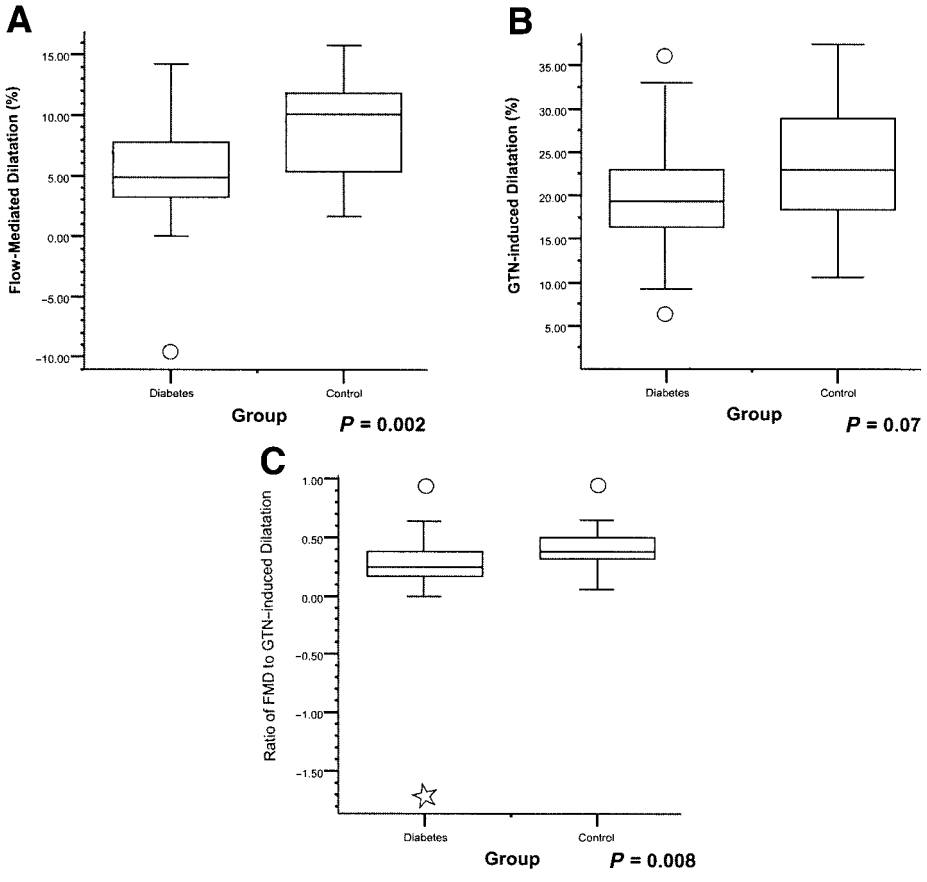

FIG. 1. ***A*: FMD in diabetes and control groups. *B*: GTN-induced dilatation in diabetes and control groups. *C*: Ratio of FMD:GTN-induced dilatation in diabetes and control groups. For each graph, the horizontal line is the median, the edges of the box represent the 25th and 75th centiles, the bars represent values within 1.5 × the interquartile range and outliers (○), and extreme values (☆) are shown.**

Reactive hyperemia was calculated as the flow in the first 15 s after cuff deflation divided by the flow during the resting scan. Scans in which the diameter of the artery differed by >3% between the first (resting) and third (recontrol) scans were excluded from the analysis.

Laboratory. Venous blood samples were collected after an overnight fast in all subjects and before insulin administration in the subjects with diabetes. Samples for von Willebrand factor (vWF) and thrombomodulin assay were collected in tubes containing 0.109 mol/l trisodium citrate and separated immediately, and the plasma was frozen at −70°C until sample assay, which was done in batches. Plasma vWF was measured using a commercially available immunoturbidometric assay (Diagnostica Stago, Asnieres, France). Intra-assay coefficient of variation (CV) is 1.9%, and interassay CV is 2.9%. Plasma thrombomodulin was measured using a commercially available sandwich enzyme-linked immunosorbent assay (Diagnostica Stago). tHcy, serum and red cell folate, and vitamin B12 were measured on the Abbott IMX analyzer, as previously reported (16). HbA_{1c} was measured using high-performance liquid chromatography. Creatinine was measured using an enzymatic method (19). Creatinine clearance was estimated using the formula glomerular filtration rate (GFR) (ml/min/1.73 m^2) = 38 × height (cm)/plasma creatinine (mmol/l) (20). Triglycerides, total cholesterol, and HDL cholesterol were measured using commercial enzyme-based assays on the Beckman Synchron CX5ce analyzer. LDL cholesterol was calculated using the Friedewald equation (21). Lipoprotein (a) [Lp(a)] was measured using end point nephelometry (Hyland laser nephelometry PDQ) and addition of specific monoclonal antibodies, as previously described (22).

Statistics. The data were analyzed using SPSS (version 10) software. Differences between groups were assessed using the Student's *t* test for normally distributed data and the Mann Whitney *U* test for data that were not normally distributed. Data were assessed as being normally distributed by plotting them against the normal distribution and using the Kolmogorov-Smirnov test. Correlations were determined using the Pearson's correlation coefficient for normally distributed data and the Spearman's rank correlation coefficient for nonnormally distributed data. Multiple linear regression analysis was undertaken to determine independent contributors to the dependent variables. Data that were not normally distributed were log transformed for regression analysis. β refers to the adjusted correlation coefficient in the regression analysis.

RESULTS

FMD. All scans were of sufficient technical quality to be analyzed. One FMD study in a subject with diabetes was excluded from the analysis because of a difference in vessel size between scan 1 (baseline) and scan 3 (recontrol) of >3%. CV in 20 subjects studied on two occasions was 3.9% for FMD and 4.0% for GTN-induced dilatation.

Subjects with diabetes had significantly lower FMD and FMD:GTN ratio–induced dilatation than control subjects. There was no difference in resting vessel diameter or reactive hyperemia between the two groups (Table 1 and Fig. 1).

Subjects with diabetes. Significant correlations on univariate analysis for FMD and FMD:GTN ratio in the diabetes group are shown in Table 3. Resting vessel diameter correlated with tHcy ($r = -0.42$, $P = 0.016$), Lp(a) ($r = 0.451$, $P = 0.004$), age ($r = 0.36$, $P = 0.03$), height ($r = 0.64$, $P < 0.001$), weight ($r = 0.56$, $P = 0.001$), and systolic blood pressure ($r = 0.38$, $P = 0.03$).

Multiple linear regression analysis was undertaken with the ultrasound measurements (FMD, FMD:GTN ratio, GTN dilatation, and baseline vessel diameter) in turn as the dependent variable. For FMD, independent variables were red cell folate, glucose, baseline vessel diameter, and serum folate. For the FMD:GTN ratio, independent variables were red cell folate and serum folate. For GTN-induced dilatation, independent variables were baseline vessel diameter, tHcy, glucose, and vitamin B12. For baseline vessel diameter, independent variables were

TABLE 3
Significant correlations in the diabetic group on univariate analysis

	FMD (n = 35)		FMD:GTN ratio (n = 35)		vWF (n = 64)		Thrombomoulin (n = 64)	
	r	P	r	P	r	P	r	P
Red cell folate	0.33	0.065	0.5	0.004	−0.1	0.41	−0.18	0.16
Serum folate	0.02	0.92	−0.01	0.96	−0.26	0.04	−0.02	0.89
tHcy	0.1	0.57	−0.11	0.52	−0.08	0.55	−0.24	0.06
HbA_{1c}	0.06	0.75	0.07	0.71	0.3	0.017	0.03	0.84
Glucose	0.33	0.08	0.25	0.2	—	—	—	—
Triglycerides	0.24	0.19	0.44	0.01	0.1	0.45	−0.02	0.87
HDL	−0.06	0.73	−0.14	0.45	0.22	0.08	0.25	0.051
Lp(a)	0.08	0.69	0.16	0.39	−0.24	0.06	−0.16	0.22
Creatinine clearance	—	—	—	—	−0.06	0.68	0.31	0.018

tHcy, height, age, weight, systolic blood pressure, and ln[Lp(a)]. Independent associations remained between *1*) FMD and red cell folate ($\beta = 0.42, P = 0.028$), *2*) FMD:GTN ratio and red cell folate ($\beta = 0.59$, $P < 0.001$), *3*) GTN-induced dilatation and vitamin B12 ($\beta = 0.45$, $P = 0.02$), and *4*) baseline vessel diameter and height ($\beta = 0.65$, $P < 0.001$) and tHcy ($\beta = -0.51$, $P < 0.001$).

Markers of endothelial activation

Subjects with diabetes. Correlations on univariate analysis for vWF and thrombomodulin in the diabetes group are shown in Table 3. Multiple regression analysis, with vWF as the dependent variable and HbA_{1c}, serum folate, HDL cholesterol, and logLp(a) as independent variables, indicated that vWF was independently associated with HbA_{1c} ($\beta = 0.38$, $P = 0.003$). With thrombomodulin as the dependent variable and red cell folate, tHcy, calculated creatinine clearance, creatinine, HDL cholesterol, serum folate, and diastolic blood pressure as the independent variables, thrombomodulin was independently associated with red cell folate ($\beta = -0.38$, $P = 0.005$), tHcy ($\beta = -0.37$, $P = 0.004$), diastolic blood pressure ($\beta = -0.28$, $P = 0.025$), and creatinine clearance ($\beta = 0.26$, $P = 0.033$).

DISCUSSION

We have confirmed that endothelial dysfunction is common in children and adolescents with type 1 diabetes of short duration. In addition, we have determined for the first time that folate status is an important determinant of endothelial function in these children, both when measured by FMD and markers of endothelial activation (vWF and thrombomodulin).

Impaired FMD has been reported in young adults with type 1 diabetes (23) and in a small number of adolescents (24). We have extended knowledge of factors that contribute to endothelial dysfunction in children with type 1 diabetes. In the diabetes group, we found an independent association between FMD and red cell folate. Red cell folate is likely to reflect endothelial cell folate status. The ratio of FMD:GTN-induced dilatation (the best measure of endothelial function per se) was also independently associated with red cell folate, again suggesting that folate status is an important factor protecting against endothelial dysfunction.

Of interest was the finding that resting vessel diameter correlated negatively with tHcy. This result is the reverse of that expected from the increase in both vessel diameter and tHcy with age. Regression analysis suggested that height and tHcy were independent predictors of resting vessel diameter. These results would be consistent with tHcy having an effect on vessel size by inducing vasoconstriction. The smaller vessel would then show greater FMD or GTN-induced dilatation. Therefore, tHcy may have induced vasoconstriction, which partially masks associated abnormal FMD.

Unlike Clarkson et al. (23), we did not find significant associations between any of the ultrasound measurements and either duration of diabetes or LDL cholesterol. We examined subjects with shorter duration of diabetes, and these associations may only become apparent with longer duration. It may also be because other variables (notably red cell folate and tHcy, as discussed above) are more important, particularly during the early stages of blood vessel dysfunction in diabetes.

We also examined endothelial function by assessing soluble markers of endothelial activation. There are a large number of such potential markers. We examined vWF because it rises before the development of microalbuminuria (6) and thrombomodulin because it is associated with tHcy in adults with diabetes (10). Neither vWF nor thrombomodulin were significantly different in diabetic and control subjects. However, vWF correlated strongly and independently with HbA_{1c}, suggesting poor metabolic control leads to endothelial activation, an observation previously made in adults (25). vWF also correlated negatively with serum folate, and although this relationship was not significant in the multiple regression analysis, serum folate may have a protective effect on the endothelium.

Thrombomodulin also had a negative independent association with red cell folate—further evidence of a protective effect of folate on endothelial function. The negative independent correlation between thrombomodulin and tHcy is the reverse of the relationship obtained in adults (10), in which higher tHcy is associated with higher thrombomodulin. These authors suggest that tHcy leads directly to endothelial dysfunction in diabetes, with elevated thrombomodulin as a consequence. Because the study of Hofmann et al. (10) included a significant number of subjects with nephropathy, it is difficult to determine whether their observed association between tHcy and thrombomodulin is primary or secondary. It may be that, early in diabetes, hyperfiltration is associated with elevated thrombomodulin, which remains elevated as renal disease progresses. Hyperfiltration is associated with

lower tHcy (14,16), whereas elevated tHcy occurs with progressive renal impairment. Together, these results and those of Hofmann et al. (10) would be consistent with this explanation: the differences in tHcy reflect different stages during the progression of nephropathy, whereas elevated thrombomodulin occurs at the early stage of hyperfiltration and then remains elevated.

Recently, Fiorina et al. (26) reported that patients with type 1 diabetes and nephropathy had near-normal endothelial function (assessed using FMD) after kidney-pancreas transplantation, whereas patients having kidney-alone transplantation had significantly impaired endothelial function. vWF was also lower in the kidney-pancreas transplantation group. In both groups, FMD correlated negatively with tHcy, but the association with folate was not reported.

We have shown that children with type 1 diabetes have early endothelial dysfunction, which relates to folate status when using biological and biochemical measures. Despite lower mean tHcy values in children with type 1 diabetes than in control subjects (16), there appear to be significant biological effects at these levels. An intervention trial to assess whether folate improves endothelial function in children and adolescents with type 1 diabetes is therefore justified.

ACKNOWLEDGMENTS

We thank Dr. J. Penfold, Dr. D. Corlis, and Dr. D.T. Thomas for referring patients and Dr. V. Williams for vWF and thrombomodulin assays.

E.J.W. was supported by a fellowship from Juvenile Diabetes Foundation Australia and the Royal Australasian College of Physicians. This work was supported by research grants from Channel 7, Australia, and an Aza Research Grant.

REFERENCES

1. Furchgott RF, Zawadzki JV: The obligatory role of the endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 288:373–376, 1980
2. Ross R: The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 362:801–809, 1993
3. Cohen RA: Dysfunction of the vascular endothelium in diabetes mellitus. *Circulation* 87:67–76, 1993
4. Rudberg S, Osterby R, Dahlquist G, Nyberg G, Persson B: Predictors of renal morphological changes in the early stage of microalbuminuria in adolescents with IDDM. *Diabetes Care* 20:265–271, 1996
5. Berg UB, Torbjörnsdotter TB, Jaremko G, Thalme B: Kidney morphological changes in relation to long-term renal function and metabolic control in adolescents with IDDM. *Diabetologia* 41:1047–1056, 1998
6. Stehouwer CDA, Fischer A, van Kuijk AWR, Polak BCP, Donker AJM: Endothelial dysfunction precedes development of microalbuminuria in IDDM. *Diabetes* 44:561–564, 1995
7. Boushey CJ, Beresford SAA, Omenn GS, Motulsky AG: A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. *JAMA* 274:1049–1057, 1995
8. Welch GN, Loscalzo J: Homocysteine and atherothrombosis. *N Engl J Med* 338:1042–1050, 1998
9. Hoogeveen EK, Kostense PJ, Jakobs C, Dekker JM, Nijpels G, Heine RJ, Bouter LM, Stehouwer CDA: Hyperhomocysteinemia increases risk of death, especially in type 2 diabetes: 5 year follow-up of the Hoorn study. *Circulation* 101:1506–1511, 2000
10. Hofmann MA, Kohl B, Zumbach MS, Borcea V, Bierhaus A, Henkels M, Amiral J, Schmidt AM, Fiehn W, Ziegler R, Wahl P, Nawroth PP: Hyperhomocyst(e)inemia and endothelial dysfunction in IDDM. *Diabetes Care* 21:841–848, 1998
11. Okada E, Oida K, Tada H, Asazuma K, Eguchi K, Tohda G, Kosaka S, Takahashi S, Miyamori I: Hyperhomocysteinemia is a risk factor for coronary arteriosclerosis in Japanese patients with type 2 diabetes. *Diabetes Care* 22:484–490, 1999
12. Robillon JF Canivet B, Candito M, Sadoul JL, Jullien D, Morand P, Chambon P, Freychet P: Type 1 diabetes mellitus and homocyst(e)ine. *Diabete Metab* 20:494–496, 1994
13. Cronin CC, McPartlin JM, Barry D, Ferriss JB, Scott JM, Weir DG: Plasma homocysteine concentrations in patients with type 1 diabetes. *Diabetes Care* 21:1843–1847, 1998
14. Wollensen F, Brattström L, Refsum H, Ueland PM, Berglund L, Berne C: Plasma total homocysteine and cysteine in relation to glomerular filtration rate in diabetes mellitus. *Kidney Int* 55:1028–1035, 1999
15. Kark JD, Selhub J, Bostom A, Adler B, Rosenberg IH: Plasma homocysteine and all-cause mortality in diabetes. *Lancet* 353:1936–1937, 1999
16. Wiltshire EJ, Thomas DW, Baghurst P, Couper JJ: Reduced total plasma homocyst(e)ine in children and adolescents with type 1 diabetes. *J Pediatr* 138:888–893, 2001
17. Celermajer DS, Sorensen KE, Gooch VM, Spiegelhalter DJ, Miller OI, Sullivan ID, Lloyd JK, Deanfield JE: Non invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. *Lancet* 340:1111–1115, 1992
18. Joannides R, Haefeli WE, Linder L, Richard V, Bakkali EH, Thuillez C, Lüscher TF: Nitric oxide is responsible for flow-dependent dilatation of human peripheral conduit arteries in vivo. *Circulation* 91:1314–1319, 1995
19. Tanganelli E, Prencipe L, Bassi D, Cambiaghi S, Murador E: Enzymic assay of creatinine in serum and urine with creatinine iminohydrolase and glutamate dehydrogenase. *Clin Chem* 28:1461–1464, 1982
20. Counahan R, Chantler C, Ghazali S, Kirkwood B, Rose F, Barratt TM: Estimation of glomerular filtration from plasma creatinine concentration in children. *Arch Dis Child* 51:875–878, 1976
21. Tietz NW: *Fundamentals of Clinical Chemistry.* 4th ed. Sydney, Australia, W.B. Saunders, 1996
22. Couper JJ, Bates DJ, Cocciolone R, Magarey AM, Boulton TJC, Penfold JL, Ryall RG: Association of lipoprotein (a) and puberty in IDDM. *Diabetes Care* 16:869–873, 1993
23. Clarkson P, Celermajer DS, Donald AE, Sampson M, Sorensen KE, Adams M, Yue DK, Betteridge J, Deanfield JE: Impaired vascular reactivity in insulin-dependent diabetes mellitus is related to disease duration and low density lipoprotein cholesterol levels. *J Am Coll Cardiol* 28:573–579, 1996
24. Donaghue KC, Robinson J, McCredie R, Fung A, Silink M, Celermajer DS: Macroangiopathy: does it play a role in young people? *Horm Res* 50:38–40, 1998
25. Stehouwer CDA, Stroes ESG, Hackeng WHL, Mulder PGH, den Ottolander GJH: von Willebrand factor and development of diabetic nephropathy in insulin-dependent diabetes mellitus. *Diabetes* 40:971–976, 1991
26. Fiorina P, La Rocca E, Venturini M, Minicucci F, Fermo I, Paroni R, D'Angelo A, Sblendido M, Di Carlo V, Cristallo M, Del Maschio A, Pozza G, Secchi A: Effects of kidney-pancreas transplantation on atherosclerotic risk factors and endothelial function in patients with uremia and type 1 diabetes. *Diabetes* 50:496–501, 2001