

## Brief Genetics Report

# Level of an Advanced Glycated End Product Is Genetically Determined

## A Study of Normal Twins

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Reducing sugars react with amino groups in proteins, lipids, and nucleic acids to produce advanced glycation end products (AGEs), including N<sup>ε</sup>-carboxymethyl lysine (CML), which have been implicated in oxidative stress and vascular damage. The aim of this study was to determine whether genetic factors influence serum CML levels in normal subjects. We performed a classical twin study of CML in healthy nondiabetic female twins, 39 monozygotic and 45 dizygotic pairs, aged 21–74 years. Serum CML levels were estimated by enzyme-linked immunosorbent assay. Twin correlations (*r*) for serum CML levels were higher in monozygotic (*r* = 0.71) compared with dizygotic (*r* = 0.50) twin pairs, suggesting a substantial genetic effect and confirmed by quantitative genetic model fitting. Additive genetic effects (heritability) explained 74% (95% CI 58–84) of population variance in CML. Heritability (%) of fasting glucose (51%) and HbA<sub>1c</sub> (62%) could not explain CML heritability, which was not associated with them. CML levels are, therefore, predominantly genetically determined and independent of genes influencing fasting glucose or HbA<sub>1c</sub>. Thus familial, largely genetic factors influence AGE implicating these glycoxidation products in the genetic contribution to macro- and microvascular disease. *Diabetes* 52:2441–2444, 2003

**T**he formation of advanced glycation end products (AGEs) by glycation and oxidation alters the functional property of matrix proteins and mediates sustained cellular changes by binding to AGE ligands (1). AGE formation has been implicated in widespread pathology, including the macro- and microvascular complications of diabetes (1–4). Factors determin-

ing AGEs in normal physiology are unclear. Tissue levels of AGE increase with age (5). The formation of AGEs is predominantly endogenous, but these products can also be derived from exogenous sources such as food and tobacco smoke (6,7). The nonenzymatic glycation of food leads to browning through the formation of AGE, which is known as the Maillard reaction (6). Heat-treated food contains substantial AGEs, which can promote inflammatory responses (6–8). Since the predominant source of AGEs is endogenous, AGE levels may be genetically determined.

The heterogeneity of AGEs implies that many products could be measured to estimate AGE formation. Of them, pentosidine and N<sup>ε</sup>-carboxymethyl lysine (CML) are the best characterized, and CML can serve as a biomarker of oxidative stress resulting from sugar and lipid oxidation (8–10). An assay for CML detects levels in normal and diabetic sera, and increased levels have been associated with diabetes and renal dysfunction (11). Since AGEs, including CML, are potentially important metabolic products, we aimed to determine whether population variation in CML levels could be due to genetic factors. We therefore studied a cohort of healthy nondiabetic female monozygotic and dizygotic twins to determine the impact of genetic and environmental factors on serum CML levels.

Table 1 shows the characteristics of the healthy female twin pairs. Creatinine levels were in the normal range for all twins, and the estimated creatinine clearance did not differ between monozygotic and dizygotic pairs. Mean CML values were similar in monozygotic and dizygotic twins, as were all other characteristics. CML was not significantly correlated with age (*r* = 0.07, NS), fasting glucose (*r* = 0.13, NS), or HbA<sub>1c</sub> levels (*r* = 0.05, NS). Twin correlations for CML levels were highly significant (*P* < 0.001 for both monozygotic and dizygotic pairs) and higher in monozygotic (*r* = 0.71) compared with dizygotic (*r* = 0.50) twin pairs, suggesting a substantial genetic effect (Fig. 1). This genetic effect was confirmed by univariate genetic model fitting.

Table 2 shows model fitting and standardized parameters for CML. Shared environment could be dropped from the full ACE model without deterioration in fit (ACE vs. AE:  $\Delta\chi^2 = 1.558 - 0.673 = 0.885$ , *P* > 0.05). Additive genetic influence (A) could not be dropped from the model

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AGE, advanced glycation end product; CML, N<sup>ε</sup>-carboxymethyl lysine; RAGE, receptor for AGE.

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TABLE 1  
Characteristics of healthy female twin pairs

	Monozygotic	Dizygotic
Pairs ( <i>n</i> )	39	45
Age (years)	53.3 ± 12.2	49.6 ± 15.5
Height (m)	1.61 ± 0.05	1.61 ± 0.06
Weight (kg)	66.3 ± 13.8	67.3 ± 12.1
BMI (kg/m <sup>2</sup> )	25.6 ± 4.8	25.8 ± 4.2
Current smoker (%)	22.0	20.0
Creatinine clearance (ml/min) <sup>†</sup>	88.4 ± 24.8	96.8 ± 28.4
Glucose (mmol/l)*	4.6 ± 0.5	4.5 ± 0.4
HbA <sub>1c</sub> (%)	5.7 ± 0.4	5.7 ± 0.4
CML (ng/ml)	781.5 ± 121.9	801.5 ± 131.9

Data are means ± SD, unless otherwise stated. \*Only for fasting twins ( $n_{MZ} = 35$  and  $n_{DZ} = 39$ ); <sup>†</sup>calculated with the Cockcroft-Gault formula: clearance =  $\{[(140 - \text{age}) \times \text{weight}/72]/\text{plasma creatinine}\} \times 0.85$ , available pairs  $n_{MZ} = 30$  and  $n_{DZ} = 43$ . DZ, dizygotic; MZ, monozygotic.

because the fit deteriorated (ACE vs. CE:  $\Delta\chi^2 = 4.958 - 0.673 = 4.285$ ,  $P = 0.04$ ) (Table 2). Thus, the AE model shows the best fit, confirmed by the lowest Akaike's Information Criterion (AIC). In this best-fitting model, additive genetic effects (heritability) explained 74% (95% CI 58–84) of the population variance in CML levels. The remainder was due to unique environment (26% [95% CI 16–42]). Shared environment and dominant genetic factors did not contribute significantly and were excluded from the model.

We previously showed (12) in these twins that genetic factors influenced fasting glucose levels (heritability 51%) and HbA<sub>1c</sub> (heritability 62%). However, bivariate modeling was not attempted as CML was not associated with either fasting glucose or HbA<sub>1c</sub>. Hence, CML heritability could not be explained by genes in common with those determining fasting glucose or HbA<sub>1c</sub>. That is, the genetic (and environmental) factors contributing to CML levels must be distinct from those contributing to fasting glucose and HbA<sub>1c</sub> levels.

This twin study established that a genetic effect accounts for 74% of normal population variance in CML levels; the remainder is due to unique environment (26%). Although genetic factors have a substantial influence on fasting glucose (51%) and HbA<sub>1c</sub> (62%), the heritability of CML could not be explained by genes in common with either. Neither age nor smoking was relevant to CML levels in these individuals, although both have been implicated in producing AGEs. While the study was performed in female twins, there is no evidence that CML levels are influenced by sex, so we have no reason to believe results in male twins would differ (13).

Several features implicate AGE in disease. Histological studies (3,4,14,15) have shown AGEs in a wide range of disease tissues. Moreover, CML modifications of proteins can engage receptors of signal transduction for AGE (RAGE), thereby activating key cell signaling pathways linked to accelerated vascular and inflammatory complications (6,9). CML serum levels are also increased in both

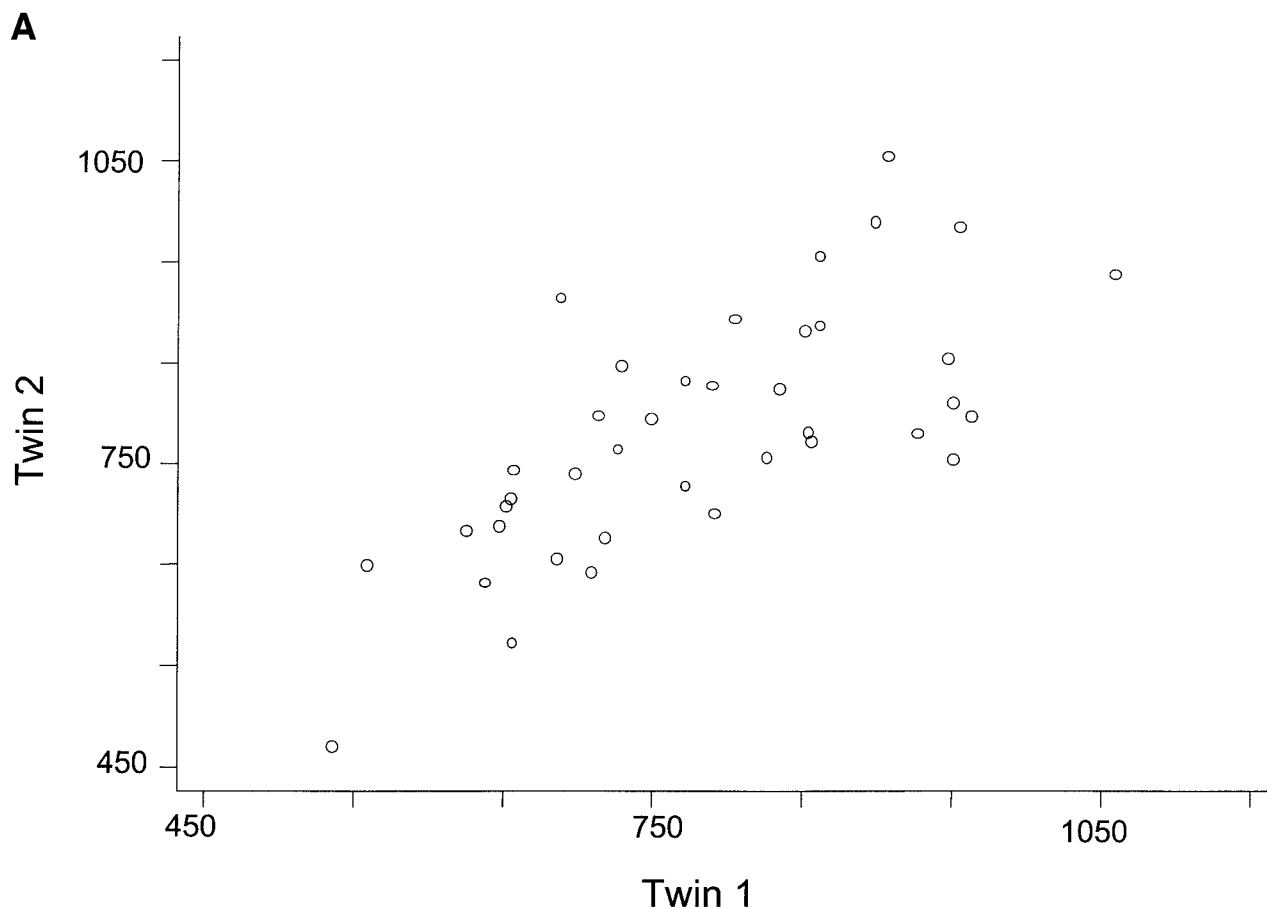


FIG. 1. Scatterplots of serum CML levels (ng/ml) for monozygotic pairs ( $r = 0.71$ ) (A) and dizygotic pairs ( $r = 0.50$ ) (B).

type 1 and type 2 diabetes as well as in diabetic nephropathy but did not correlate with parameters of blood glucose control (11,13,16). Finally, animal studies (17) limiting AGE formation can limit, or even reverse, the disease process.

Twin and family studies (18,19) have shown the importance of genetic effects in predisposing to vascular disease. Potential genes include determinants of cholesterol, blood pressure, and tissue oxidation. Our present study suggests that genes influencing levels of protein and lipid glycation and oxidation could also be important in healthy individuals and, by extrapolation, might also be relevant in disease (12). Whereas protein and lipid glycation and oxidation are nonenzymatically determined, it is possible that genetic factors influence events upstream or downstream of glycooxidation. For example, RAGE expression has been associated with induction of cellular oxidant stress and a diabetic microvascular complication; of four putative RAGE polymorphisms, a minor allele in the promoter region of one of the genes conferred a weak protective effect against developing diabetic nephropathy (20). However, there is no evidence that RAGE polymorphisms influence circulating CML levels. Since much of the normal variation in CML levels is inherited, it is possible that genetic factors determining differential levels could have a major impact on processes leading to pathology, including vascular disease and diabetes complications.

## RESEARCH DESIGN AND METHODS

**Subjects.** We studied 89 healthy, female, nondiabetic monozygotic and dizygotic twin pairs from the St. Thomas' U.K. Adult Twin Registry. Females were selected to avoid a sex effect. Twins from the registry are unselected volunteers ascertained from the general population through national media campaigns in the U.K. (12). Twin pairs selected for this study satisfied the following criteria: 1) European origin, 2) female, 3) no family or personal history of diabetes, 4) both twins of each pair available for study, 5) similar age range in monozygotic and dizygotic twin pairs, and 6) exclusion of diabetes at sampling by random whole blood glucose  $<10.0$  mmol/l or fasting blood glucose  $<6.1$  mmol/l.

The current twin sample is the same as described previously (12), apart from five pairs who were excluded; three because only one twin of each pair had CML levels measured and two additional pairs because one twin of each pair had extremely high CML values ( $>3$  SD above twin group mean). Thus a total of 84 pairs (39 monozygotic and 45 dizygotic; age range 21–74 years) were available for analysis. Subjects gave informed consent, and the St. Thomas' Hospital and St. Bartholomew's Hospital ethics committees approved the study.

**Biochemical analyses and confirmation of zygosity.** Zygosity was determined by standardized questionnaire and confirmed by DNA fingerprinting. Glucose was measured in whole blood on an Ektachem machine (Johnson & Johnson). High-performance liquid chromatography (BioRex 70; Biorad) determined HbA<sub>1c</sub>, with between-batch coefficient of variation  $<2.5\%$ . Plasma creatinine was measured using an enzymatic method (creatinine iminohydro-lase) on the Vitros 950 analyser.

**CML assay.** AGE-CML was determined with a novel competition-based enzyme-linked immunosorbent assay using CML-specific monoclonal antibody (mouse monoclonal 4G9; Alteon, New York, NY) (11). The assay is CML specific without cross-reaction with underivatized lysine, glycine, alanine, or  $\beta$ -alanine. The assay was calibrated with 6-(*N*-carboxymethylamino) caproate, which refers to the epitope recognized by mouse monoclonal antibody 4G9 (11). Results show CML levels expressed as the number of single CML

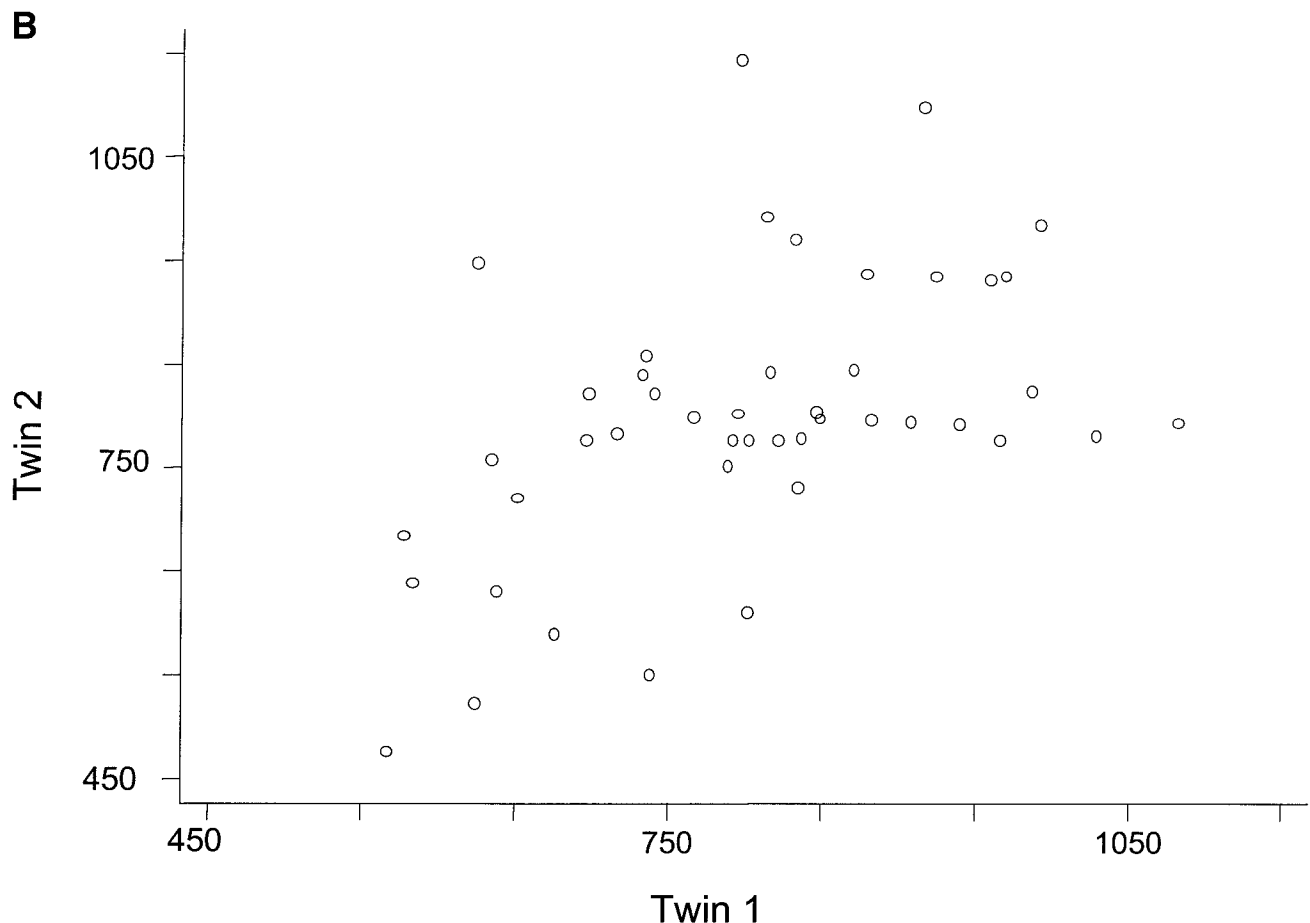


FIG. 1. Continued.

TABLE 2  
Model fitting results and standardized parameter estimates (95% CI) for CML

Model	$h^2$	$d^2/c^2$	$e^2$	$\chi^2$	df	P	AIC
ACE	0.50 (0.03–0.83)	0.23 (0.00–0.60)	0.27 (0.17–0.46)	0.673	3	0.879	–5.327
ADE	0.74 (0.04–0.84)	0.00 (0.00–0.70)	0.26 (0.16–0.42)	1.558	3	0.669	–4.442
AE	0.74 (0.58–0.84)		0.26 (0.16–0.42)	1.558	4	0.816	–6.442
CE		0.59 (0.43–0.72)	0.41 (0.28–0.57)	4.958	4	0.292	–3.042
E			1.00 (1.00–1.00)	38.67	5	0	28.666

AE is the best-fitting model. AIC, Akaike's Information Criterion;  $c^2$ , shared environmental variance component;  $d^2$ , dominance genetic variance component; df, degrees of freedom;  $e^2$ , unique environmental variance component;  $h^2$ , heritability.

epitopes in nanograms per milliliter of serum. Assay sensitivity was 5 ng CML/ml with intra- and interassay precision <4 and <5%, respectively.

**Analytical approach.** The aims of our analyses were to estimate the relative influence of genetic and environmental factors on CML levels and, in case of a significant association with fasting glucose and HbA<sub>1c</sub> levels, to estimate the extent to which genetic factors caused an association.

**Quantitative genetic model fitting.** We compared covariances (or correlations) in monozygotic and dizygotic twin pairs and quantified sources of individual differences by separation of observed phenotypic variance into additive (A) or dominant (D) genetic components and shared (C) or unique (E) environmental components (21). The latter also contains measurement error. Dividing each of these components by the total variance yields the different standardized components of variance, for example, heritability ( $h^2$ ).

Models were fitted to monozygotic and dizygotic variance/covariance matrices by the method of maximum likelihood. The significance of components A, C, and D was assessed by testing deterioration in model fit after each component was dropped from the full model (ACE or ADE), leading to the most parsimonious model wherein the pattern of variance/covariance is explained by as few parameters as possible. Standard hierarchic  $\chi^2$  tests were used to select the best-fitting model in combination with Akaike's Information Criterion ( $AIC = \chi^2 - 2 \text{ df}$ ) (21). The model with the lowest AIC reflects the best balance of goodness-of-fit and parsimony. Data handling and preliminary analyses were done with STATA. Quantitative genetic modeling was carried out using Mx software (22).

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## REFERENCES

- Brownlee M: Biochemistry and molecular cell biology of diabetic complications. *Nature* 414:813–820, 2001
- Singh R, Barden A, Mori T, Beilin L: Advanced glycation end-products: a review. *Diabetologia* 44:129–146, 2002
- Vitek MP, Bhattacharya K, Glendening JM, Stopa E, Vlassara H, Bucala R, Manogue K, Cerami A: Advanced glycation end products contribute to amyloidosis in Alzheimer disease. *Proc Natl Acad Sci U S A* 91:4766–4770, 1994
- Nakamura Y, Horii Y, Nishino T, Shiiki H, Sakaguchi Y, Kagoshima T, Dohi K, Makita Z, Vlassara H, Bucala R: Immunohistochemical localization of advanced glycosylation end products (AGEs) in coronary atheroma and cardiac tissue in diabetes. *Am J Pathol* 143:1649–1656, 1993
- Much G, Thome J, Foley P, Schinzel R, Riederer P: AGEs in aging and Alzheimers disease. *Brain Res Rev* 23:134–143, 1997
- Vlassara H, Cai W, Crandall J, Goldberg T, Oberstein R, Dardaine V, Pepp

- Rayfield EJ: Inflammatory mediators are induced by dietary glycotoxins, a major risk factor for diabetic angiopathy. *Proc Natl Acad Sci U S A* 99: 15596–15601, 2002
- Cerami C, Founds H, Nicholl I, Mitsuhashi T, Giordano D, Vanpatten S, Lee A, Al-Abed Y, Vlassara H, Bucala R, Cerami A: Tobacco smoke is a source of toxic reactive glycation end products. *Proc Natl Acad Sci U S A* 94:13915–13920, 1997
- Ikeda K, Higashi O, Sano H, Jinnouchi Y, Yoshida M, Araki T, Ueda S, Horiuchi S: N<sup>ε</sup>-[carboxy-methyl]lysine protein adduct is a major immunological epitope in proteins modified with advanced glycation end products of the Maillard reaction. *Biochemistry* 35:8075–8083, 1996
- Kislinger T, Fu C, Huber B, Ou W, Taguchi A, Du Yan S, Hofmann M, Yan SF, Pischetsrieder M, Stern D, Schmidt AM: N(epsilon)-(carboxymethyl)lysine adducts of proteins are ligands for receptor for advanced glycation end products that activate cell signalling pathways and modulate gene expression. *J Biol Chem* 274:31740–31749, 1999
- Reddy S, Bichler J, Wells-Knecht KJ, Thorpe SR, Baynes JW: N<sup>ε</sup>-[carboxy-methyl]lysine is a dominant advanced glycation end product (AGE) in tissue proteins. *Biochemistry* 34:10872–10878, 1995
- Wagner Z, Wittmann I, Mazak I, Schinzel R, Heidland A, Kientsch-Engel R, Nagy J: N(epsilon)-(carboxymethyl)lysine levels in patients with type 2 diabetes: role of renal function. *Am J Kidney Dis* 38:785–791, 2001
- Snieder H, Sawtell P, Ross L, Walker J, Spector TD, Leslie RDG: HbA<sub>1c</sub> levels are largely genetically determined even in type 1 diabetes: evidence from healthy and diabetic twins. *Diabetes* 50:2858–2863, 2001
- Berg TJ, Dahl-Jørgensen K, Clausen JT, Bangstad H-J, Torjesen PA, Hanssen KF: The advance glycation end product N<sup>ε</sup>-(carboxymethyl)lysine is increased in serum from children and adolescents with type 1 diabetes. *Diabetes Care* 21:1997–2002, 1998
- Horei K, Miyata T, Maeda K, Miyata S, Sugiyama S, Sakai H, van Ypersele de Strihou C, Monnier VM, Witztum JL, Kurokawa K: Immunohistochemical co-localization products in diabetic renal glomerular lesion: implication for glycooxydative stress in the pathogenesis of diabetic nephropathy. *J Clin Invest* 100:2995–3004, 1997
- Schleicher ED, Wagner E, Nerlich AG: Increased accumulation of the glycooxidation product N(epsilon)-(carboxymethyl)lysine in human tissues in diabetes and aging. *J Clin Invest* 99:457–468, 1997
- Kilhovd BK, Berg TJ, Birkeland KI, Thorsby P, Hanssen KF: Serum levels of advanced glycation end products are increased in patients with type 2 diabetes and coronary heart disease. *Diabetes Care* 22:1543–1548, 1999
- Park I, Raman KG, Lee KJ, Lu Y, Ferran LJ Jr, Chow WS, Stern D, Schmidt AM: Suppression of accelerated diabetic atherosclerosis by the soluble receptor for advanced glycation end products. *Nat Med* 4:1025–1031, 1998
- The Diabetes Control and Complications Trial Research Group: Clustering of long-term complications in families with diabetes in the Diabetes Control and Complications Trial. *Diabetes* 46:1829–1839, 1997
- Wagenknecht LE, Bowden DW, Carr JJ, Langedef CD, Freeman BI, Rich SS: Familial aggregation of coronary artery calcium in families with type 2 diabetes. *Diabetes* 50:861–866, 2001
- Poirier O, Nicaud V, Vionnet N, Raoux S, Tarnow L, Vlassara H, Parving HH, Cambien F: Polymorphism screening of four genes encoding advanced glycation end-product putative receptors: association study with nephropathy in type 1 diabetic patients. *Diabetes* 50:1214–1218, 2001
- Neale MC, Cardon LR: *Methodology for Genetic Studies in Twins and Families*. Dordrecht, the Netherlands, Kluwer Academic, 1992
- Neale MC, Boker SM, Xie G, Maes HH: *Mx: Statistical Modeling*. Richmond, VA, Virginia Commonwealth University, 1999