The *UCP1* −3826A/G Polymorphism Is Associated with Diabetic Retinopathy and Increased *UCP1* and *MnSOD2* Gene Expression in Human Retina

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Purpose. Uncoupling protein 1 (UCP1) reduces mitochondrial production of reactive oxygen species (ROS). ROS overproduction is related to diabetic retinopathy (DR), a chronic complication of diabetes mellitus (DM). Therefore, deleterious polymorphisms in the *UCP1* gene are candidate risk factors for DR. We investigated the relationships between the *UCP1* −3826A/G polymorphism and risk of DR and *UCP1* gene expression in human retina. Considering that superoxide dismutase-2 (MnSOD2) enzyme is the first line of defense against oxidative stress in mitochondria, we also analyzed *MnSOD2* gene expression in retinal samples according to different *UCP1* −3826A/G genotypes.

Methods. In a case-control study, frequencies of −3826A/G polymorphisms were analyzed in 257 type 1 DM patients (154 cases with DR and 103 controls without DR). In a cross-sectional study comprising cadaveric cornea donors, *UCP1* and *MnSOD2* gene expressions were evaluated in 107 retinal samples differentiated according to different −3826A/G genotypes.

Results. In the type 1 DM group, multivariate analysis confirmed that the G/G genotype was an independent risk factor for DR (OR = 3.503; $P = 0.043$). In cornea donors, G allele carriers had higher *UCP1* cDNA and protein concentrations than A/A carriers ($P = 0.034$ and $P = 0.039$, respectively). Interestingly, G allele carriers exhibited increased *MnSOD2* expression ($P = 0.001$).

Conclusions. This study suggests that the −3826A/G polymorphism is associated with DR in type 1 DM patients. This is the first report demonstrating *UCP1* gene expression in human retinas and indicates that the −3826A/G polymorphism influences its expression. In addition, the −3826G allele was associated with increased *MnSOD2* expression; thus, suggesting that this allele could be a marker of oxidative stress. (*Invest Ophthalmol Vis Sci.* 2012;53:7449–7457) DOI:10.1167/iovs.12-10660

Diabetic retinopathy (DR) is a common sight-threatening microvascular complication affecting patients with diabetes mellitus (DM) and is a major cause of new cases of blindness in adults.[1] Although the risk of developing DR increases with poor glycemic control, arterial hypertension, and long-term DM, its occurrence is also influenced by genetic factors.[2] Several studies have shown that the overproduction of reactive oxygen species (ROS) is a causal link between hyperglycemia and other important abnormalities involved in the development of DR.[3–5]

Uncoupling protein type 1 (UCP1) is a member of the family of anion-carrier proteins located in the inner mitochondrial membrane.[6] It uncouples substrate oxidation from ATP synthesis, dissipating the membrane potential energy and, consequently, reducing ATP production by the mitochondrial respiratory chain.[7,8] This uncoupling action means that UCP1 plays an important role in regulation of energy expenditure, in cold-induced and diet-induced thermogenesis, and in reducing mitochondrial ROS production.[9,10]

Mitochondria are the main source of superoxide production, which makes them the target of direct ROS attack.[11,12] There is a positive correlation between inner mitochondrial membrane potential and ROS production. At high membrane potentials, even a small increase in membrane potential stimulates significant hydrogen peroxide (H2O2) production. Therefore, it has been suggested that uncoupling of the mitochondrial proton gradient by UCP1 has a natural antioxidant effect.[13] Manganese superoxide dismutase (MnSOD) catalyzes the breakdown of superoxide into H2O2 scavenging superoxide and is considered as the first line of defense against oxidative stress in view of its location in the mitochondria.[4]

Until a few years ago, it was believed that UCP1 was expressed exclusively in brown adipose tissue, but it has recently been reported that *UCP1* expression has also been detected in other tissues and organs, such as white adipose tissue, pancreatic islets, skeletal muscle, rat and mouse thymus, and bovine retina.[14–19] Interestingly, Cui et al.[19] reported that endothelial cells from bovine retina incubated with high glucose levels increased *UCP1* expression, which protected them from glucotoxicity-induced ROS damage, suggesting that UCP1 plays a protective role in the pathogenesis of DR. On this

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basis, deleterious polymorphisms in the *UCP1* gene can be considered as candidate risk factors for DR.

The −3826A/G polymorphism is located in the promoter region of the *UCP1* gene and appears to be associated with type 2 DM, obesity, and other obesity-related parameters[20–24]; however, only two studies have investigated the association between this polymorphism and DR, and their results are inconclusive.[25,26] Therefore, in this study, we investigated whether the *UCP1* −3826A/G polymorphism was associated with DR in type 1 DM patients, and whether it had an effect on *UCP1* gene expression in human retina isolated from cadaveric cornea donors. We also investigated *MnSOD2* gene expression in retinal samples and analyzed the results with reference to different −3826A/G genotypes.

Subjects and Methods

Type 1 DM Patients, Nondiabetic Controls, and Phenotype Measurements

This was a case-control study designed to investigate whether the *UCP1* −3826A/G polymorphism is associated with DR. The sample comprised 257 unrelated type 1 DM patients from the outpatient clinic at the Hospital de Clínicas de Porto Alegre (Rio Grande do Sul, Brazil). Patients were considered to have type 1 DM if they had been diagnosed with hyperglycemia before the age of 40 years and required insulin for glycemic control within 1 year of diagnosis and treatment could not be interrupted thereafter.[27] Patients with DR were defined as cases (n = 154; 72 with light or moderate nonproliferative DR [NPDR] and 82 with severe NPDR or proliferative DR [PDR]) and patients free from any degree of DR and with confirmed type 1 DM for at least 10 years were defined as controls (n = 103).

All patients were of European ancestry (mostly of Portuguese, Spanish, Italian, or German descent) and self-defined as white. A standard questionnaire was used to collect information on age, age at DM diagnosis, and drug treatment and all patients underwent physical examination and laboratory evaluation. They were weighed unshod, wearing light outdoor clothes and their height was measured. Body mass index (BMI) was calculated as weight (kg)/height squared (meters). Blood pressure (BP) was measured twice using a mercury sphygmomanometer (Korotkoff phases I and V) with the subject seated and a 5-minute rest between measurements. The means of both measurements were used to calculate systolic and diastolic BP. Hypertension was defined as BP levels of 140/90 mm Hg or higher, or if the patient was taking antihypertensive drugs.

An experienced ophthalmologist assessed all patients for DR using fundoscopy through dilated pupils. DR was classified as "DR absent" (no fundus abnormalities), "nonproliferative DR" (microaneurysms, hemorrhage, and hard exudates), or "proliferative DR" (newly formed blood vessels and/or growth of fibrous tissue into the vitreous cavity).[28] DR classification was based on the most severe degree of retinopathy in the worst-affected eye.[29] For this study a single ophthalmologist, blind to patients' clinical data, classified all subjects. Diabetic nephropathy (DN) was diagnosed on the basis of urinary albumin excretion (UAE) in at least two of three consecutive 24-hour timed or random spot sterile urine samples over a 6-month period. Patients were classified as having normoalbuminuria (UAE $<$ 20 μg/min or $<$17 mg/L), microalbuminuria (UAE 20–200 μg/min or 17–174 mg/L), or macroalbuminuria (UAE $>$ 200 μg/min or $>$174 mg/L).

A serum sample was taken after 12 hours of fasting for laboratory analyses. Creatinine levels were determined using the Jaffe reaction; glycated hemoglobin (GHb) was quantified using an ion-exchange HPLC procedure (Merck-Hitachi L-9100 GhB Analyzer; Merck, Darmstadt, Germany; reference range: 4.7%–6.0%), total plasma cholesterol and triglycerides were assayed using enzymatic methods, and albuminuria was determined by immunoturbidimetry (Sera-Pak immuno microalbuminuria; Bayer, Tarrytown, NY; mean intra- and interassay coefficients of variance of 4.5% and 7.6%, respectively).

Finally, we genotyped 429 healthy blood donors of European ancestry who did not have DM or a family history of the disease (mean age = 44.0 ± 7.8 years; males = 55.0%) to estimate the allele frequencies of *UCP1* −3826A/G polymorphism in the general population. The research protocol was approved by the hospital's ethics commission and all subjects gave informed consent in writing.

Cornea Donor Samples and Phenotype Measurements

To investigate *UCP1* and *MnSOD2* gene expression in the presence of different −3826A/G genotypes, 332 eyes were obtained from 166 cadaveric cornea donors identified through the Central de Transplantes do Rio Grande do Sul (a Brazilian organization that regulates organ donations in Rio Grande do Sul, RS, Brazil). The eyes had been harvested at two hospitals in Porto Alegre, RS: the Hospital de Clínicas de Porto Alegre and the Hospital Santa Casa de Misericórdia. A standardized form was used to collect information from medical records about age, sex, presence of arterial hypertension and DM, smoking habits, occurrence of other diseases, and cause of death.

After enucleation and separation of corneas for donation, left-eye retinas were visually separated from the remaining intraocular structures, snap-frozen in liquid nitrogen, and stored at −80°C until analysis. Right-eye retinas were formalin-fixed for preparation of paraffin-embedded sections at a later date. Blood samples were also taken from each subject for DNA extraction and genotyping of the −3826A/G polymorphism. Following genotyping, subjects were divided into groups according to different genotypes of the polymorphism under investigation.

The protocol for this study was also approved by the Hospital Ethics Commissions and relatives of all donors gave their informed consent in writing authorizing the use of the retinas.

Genotyping

DNA was extracted from peripheral blood leukocytes using a standardized salting-out procedure. The *UCP1* −3826A/G polymorphism (rs1800592) was determined by digesting PCR products with the enzyme *BclI* (Invitrogen Life Technologies, Carlsbad, CA), as previously described,[30] and using the primers depicted in Table 1. Digestion fragments were resolved on 2% agarose gels containing GelRed Nucleic Acid Gel Stain (Biotium, Inc., Hayward, CA) and visualized under ultraviolet light. The genotyping success rate was better than 95%, with a calculated error rate based on PCR duplicates of less than 1%.

RNA Isolation

Retinal tissues (250 mg) were homogenized in phenol-guanidine isothiocyanate (Invitrogen Life Technologies). RNA was extracted with chloroform and precipitated with isopropanol by centrifugation (12,000*g*) at 4°C. The RNA pellet was washed twice with 75% ethanol and resuspended in 10 to 50 μL of water treated with diethylpyrocarbonate. The concentration and quality of total RNA samples were assessed using a NANODROP 2000 spectrophotometer (Thermo Scientific Inc., Newark, DE). Only RNA samples with adequate purity ratios (A260/A280 = 1.9–2.1) were used for subsequent analyses.[31] In addition, RNA integrity and purity were also checked on agarose gel containing GelRed Nucleic Acid Gel Stain (Biotium, Inc.). The mean RNA concentration (± SD) isolated was 14.9 ± 8.6 mg/250 mg retina.

Quantification of *UCP1* and *MnSOD2* Gene Expressions by Real-Time PCR

Real-time RT-PCR was performed in two separate reactions: first, RNA was reverse transcribed into cDNA, then cDNA was amplified by

Table 1. Primer Sequences Used to Genotype *UCP1* Gene Polymorphisms and to Analyze Gene Expression

	Sequences
–3826A/G polymorphism	F 5′ – CTTGGGTAGTGACAAAGTAT – 3′ R 5′ – CCAAAGGGTCAGATTTCTAC – 3′
UCP1 gene*	F 5′ – GCCATCTCCACGGAATCAAA – 3′ R 5′ – CCTTTCCAAAGACCCGTCAAG – 3′
MnSOD2 gene*	F 5′ – AAATTGCTGCTTGTCCAAATCAG – 3′ R 5′ – ATCAATCCCCAGCAGTGGAAT – 3′
β-actin gene*	F 5′ – GCGCGGCTACAGCTTCA – 3′ R 5′ – CTTAATGTCACGCACGATTTCC – 3′

F, forward primer; R, reverse primer.

* Primers were designed using published human gene sequences and Primer Express 3.0 software (Life Technologies - Applied Biosystems) and projected to target two consecutive exons, so as to prevent the amplification of any contaminating genomic DNA.

quantitative real-time PCR (RT-qPCR). Reverse transcription of 1 μg of RNA into cDNA was carried out using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies), following the manufacturer's protocol for the oligo $(dT)_{12-18}$ method.

RT-qPCR experiments were performed in a 7500 Fast Real-Time PCR System Thermal Cycler with 7500 FAST System Sequence Detection 1.4 Software (Life Technolgies - Applied Biosystems, Foster City, CA). Experiments were performed by real-time monitoring of the increase in fluorescence of SYBER Green dye.[32] Primers for *UCP1*, *MnSOD2,* and *β-actin* genes were designed using Primer Express 3.0 Software (Life Technologies - Applied Biosystems) and are depicted in Table 1. PCR reactions were performed using 10 μL of 2× Fast SYBER Green Master Mix (Life Technologies - Applied Biosystems); 1 μL (1 ng/μL) of forward and reverse primers for *UCP1*, *MnSOD2,* or *β-actin;* and 1 μL of cDNA template (0.25 μg/μL), in a total volume of 20 μL. Each sample was assayed in triplicate and a negative control was included in each experiment. The thermocycling conditions for these genes were as follows: an initial cycle of 95°C for 20 seconds, followed by 50 cycles of 95°C for 3 seconds and 60°C for 30 seconds. RT-qPCR specificity was determined using melting curve analyses and all primers generated amplicons that produced a single sharp peak during the analyses.

Quantification of *UCP1* and *MnSOD2* cDNA were performed by relative quantification using the comparative $\Delta\Delta C_q$ method[31,33] and expressed relative to the reference gene (*β-actin*). Validation assays were done by amplification of the target (*MnSOD2* or *UCP1*) and reference (*β-actin*) genes, separately, using serial dilutions of a cDNA sample. As a requirement of this method, both target and reference genes exhibited equal amplification efficiencies (E = 95% to 105%) in all experiments. The $\Delta\Delta C_q$ method calculates changes in gene expression as relative fold difference (n-fold change) between an experimental and an external calibrator sample.[31,33]

UCP1 cDNA expression was evaluated in retina samples from 74 cornea donors who had been genotyped for the –3826A/G polymorphism (34 A/A, 26 A/G, and 13 G/G). *MnSOD2* cDNA expression was evaluated in 94 retina samples that had been genotyped for the –3826A/G polymorphism (46 A/A, 37 A/G, and 11 G/G).

Immunohistochemistry for UCP1 Protein in Human Retina

UCP1 protein distributions and intensities were determined by immunohistochemistry in formalin-fixed, paraffin-embedded retina sections (Fig. 3A). An anti-UCP1 rabbit polyclonal antibody (Abcam, Cambridge, MA) was used to detect UCP1 protein expression in human retina tissue, with brown adipose tissue as the positive control. Immunohistochemical analyses were performed on 4-μm retina sections as described previously.[34] The routine immunohistochemical technique comprised deparaffinization and rehydration, antigenic recovery, inactivation of endogenous peroxidase, and blocking of nonspecific reactions. Slides were incubated with primary antibody and then incubated with a biotinylated secondary antibody, streptavidin horseradish peroxidase conjugate (LSAB; Dako Cytomation, Inc., Carpinteria, CA), and diaminobenzidine tetrahydrochloride (Kit DAB; Dako Cytomation, Inc.). Quantification of the UCP1 protein was performed by digital image analysis using Image Pro Plus software, version 4.5 (Media Cybernetics, Bethesda, MD). Images were visualized through a Zeiss microscope (model AXIOSKOP-40; Carl Zeiss, Oberkochen, Germany) and captured using the Cool Snap-Pro CS (Media Cybernetics) camera. Two independent researchers analyzed the intensity of brownish-colored immunostaining in pixels in 10 fields from each slide, achieving a Pearson's correlation between their results of $r^2 = 0.89$ ($P = 0.004$). We used the mean number of pixels (in logarithmic scale) identified by both researchers to quantify UCP1 in each sample. UCP1 protein expression was evaluated in retina samples from 46 cornea donors genotyped for the –3826A/G polymorphism (16 A/A, 15 A/G, and 15 G/G).

Statistical Analyses

Allele frequencies were determined by gene counting, and departures from the Hardy-Weinberg equilibrium (HWE) were verified using the χ^2 test. Allele and genotype frequencies were compared between groups using the χ^2 test. Clinical and laboratory characteristics, cDNA abundance, and UCP1 protein expression were compared between groups using the unpaired Student's *t*-test, one-way ANOVA, or the χ^2 test, as appropriate. Variables with normal distribution are presented as mean ± SD or percentage. Variables with skewed distribution were log-transformed before analyses and are presented as median (minimum – maximum values) or median ±2 SE.

The magnitudes of associations between *UCP1* –3826A/G genotypes and DR were estimated using odds ratios (OR) with 95% confidence intervals (CIs). Multivariate logistic regression analysis was performed to assess the independent association between the *UCP1* –3826A/G polymorphism and DR (dependent variable) and to control for possible confounding factors whenever a statistically significant association was detected by the univariate analyses. DM duration was not included as an independent variable in this analysis because the control group (without DR) was selected on the basis of this characteristic.

Pearson's correlation test was used to assess correlations between different quantitative variables. Multiple linear regression analyses was performed with *UCP1* or *MnSOD2* gene expression (logarithmic) as dependent variables and age, sex, DM diagnosis, smoking habits, and presence of *UCP1* –3826G allele as independent variables. Results for which *P* was less than 0.05 were considered statistically significant. These statistical analyses were performed using SPSS 18.0 (SPSS Inc., Chicago, IL).

Table 2. Clinical and Laboratory Characteristics of Type 1 DM Patients, Broken Down by Presence of DR (Cases) or Absence of DR (Controls)

	Cases	Controls	P*
n	153	104	—
Sex, % males	42.3	51.0	0.203
Duration of diabetes, y	20.3 ± 7.1	18.1 ± 8.5	0.036
Age, y	39.2 ± 12.1	33.32 ± 13.8	<0.001
BMI, kg/m^2	23.6 ± 4.9	22.9 ± 5.1	0.362
HDL cholesterol, mM	1.5 ± 0.5	1.6 ± 0.5	0.339
Cholesterol, mM	5.00 ± 1.44	4.58 ± 1.04	0.015
Triglycerides, mM	1.0 (0.4–6.3)	0.7 (0.3–7.4)	0.006
Creatinine, μM	88.4 (44.2–132)	83.9 (35.4–132)	<0.001
GHb, %	8.92 ± 2.07	9.13 ± 7.09	0.749
Systolic BP, mm Hg	129.6 ± 21.6	116.9 ± 17.8	<0.001
Diastolic BP, mm Hg	81.0 ± 13.2	75.3 ± 10.7	<0.001
DN, %	78	22	<0.001

Data are expressed as mean ± SD, median (minimum–maximum values), or %. The control group comprises patients free from any degree of DR and with DM for more than 10 years. The case group comprises patients with DR. HDL, high-density lipoprotein; *n,* sample size.

* *P* values were computed using a χ^2 test or Student's *t*-test as appropriate.

Results

Study of the Association between the *UCP1* −3826A/G Polymorphism and Diabetic Retinopathy

Type 1 DM patients with DR differed significantly from control patients for DM duration, age, serum total cholesterol, triglycerides, creatinine, systolic and diastolic BP levels, and occurrence of DN (Table 2).

Neither genotype nor allele frequencies of the *UCP1* −3826A/G polymorphism differed statistically between nondiabetic subjects and patients with type 1 DM (G/G frequency: 12.1% vs. 11.2%, $P = 0.808$; G allele frequency: 33% vs. 31%, $P = 0.548$; respectively) and all genotypes were in agreement with those predicted by the HWE in all groups ($P > 0.05$). Interestingly, type 1 DM patients with DR had a higher frequency of the −3826G allele than type 1 DM patients without DR ($P = 0.029$, Table 3). Likewise, the percentage of type 1 DM patients that had the G allele in homozygosis was significantly higher among cases than among controls ($P = 0.009$), suggesting a recessive model of inheritance. Moreover, in the multivariate analysis, the −3826G/G genotype remained significantly associated with DR after adjustment for age, presence of arterial hypertension, and serum creatinine (Table 3). It is worth mentioning that frequency of the G/G genotype was similar between patients with light or moderate NPDR and patients with severe NPDR or PDR (7.0% G/G in patients without DR, 20.8% G/G in patients with light or moderate NPDR, and 18.3% in patients with severe NPDR or PDR; $P = 0.07$).

UCP1 and *MnSOD2* Gene Expressions in Human Retina from Cornea Donors, Analyzed according to Different *UCP1* −3826A/G Genotypes

One hundred and sixty-six cornea donors were genotyped for the *UCP1* −3826A/G polymorphism. The main clinical characteristics of these subjects were as follows: mean age was 56.5 ± 15.3 years, males comprised 58.4% ($n = 97$) of the sample, 39.7% ($n = 66$) of all patients had arterial hypertension, and 54.8% ($n = 91$) were smokers. DM was diagnosed in 7.9% ($n = 15$) of all patients. Diabetic patients differed from nondiabetic subjects in terms of age (66.6 ± 6.8 vs. 55.5 ± 14.4 years; $P = 0.005$) and presence of arterial hypertension (71.4% vs. 24.2%; $P = 0.015$). Diabetic and nondiabetic subjects were similar in terms of sex (47.8% vs. 62.5% males; $P = 0.390$) and proportion of smokers (56.2% vs. 57.1%; $P = 0.999$). Genotype frequencies of the *UCP1* −3826A/G polymorphism in cornea donor samples were 48.2% A/A, 42.2% A/G, and 9.6% G/G, and all frequencies were in agreement with those predicted by the HWE ($P = 0.772$).

Mean ± SD *UCP1* cDNA concentration for the whole retina tissue group was 0.93 ± 1.35 n-fold. No significant difference was observed when *UCP1* gene expression was broken down by sex (men: 1.07 ± 1.41 versus women: 0.84 ± 1.29 n-fold; $P = 0.381$) or hypertension status (normotensives: 1.0 ± 1.23 versus hypertensives: 0.74 ± 1.24 n-fold; $P = 0.423$). However, *UCP1* cDNA concentrations were higher in smokers than nonsmokers (1.26 ± 1.43 vs. 0.44 ± 1.0 n-fold; $P = 0.042$). *UCP1* gene expression did not correlate with age ($r = 0.067$, $P = 0.462$) and did not differ statistically between cornea donors with DM and donors without DM (1.02 ± 1.42 vs. 0.96 ± 1.15 n-fold; $P = 0.740$).

UCP1 gene expression did not differ statistically between different genotypes of this polymorphism ($P = 0.068$; Fig. 1A). Notwithstanding, we did observe that G allele carriers (A/G + G/G) exhibited greater *UCP1* gene expression than A/A

Table 3. Genotype and Allele Distributions of the *UCP1* −3826A/G Polymorphism in a Sample of DM Patients, Broken Down by Presence/Absence of DR

	Cases ($n = 154$)	Controls ($n = 103$)	P*	Unadjusted OR (95% CI); P†	Adjusted OR (95% CI); P‡
Genotypes					
A/A	57 (37.0)	45 (43.7)	0.018	1	1
A/G	67 (43.5)	51 (49.5)		1.037 (0.608–1.770); 0.894	1.186 (0.628–2.241); 0.599
G/G	30 (19.5)	7 (6.8)		3.383 (1.361–8.412); 0.009	3.503 (1.040–11.80); 0.043
Alleles					
A	0.590	0.690	0.029	—	—
G	0.410	0.310		—	—

Data are presented as absolute total (%) or proportion. The control group comprises patients free from any degree of DR and with DM for more than 10 years. The case group comprises patients with DR.

* *P* values were computed using χ^2 tests to compare case and control groups.

† Unadjusted OR (95% CI) and *P* values for the comparison between case and control groups.

‡ Adjusted OR (95% CI) and *P* values for the comparison between case and control groups, controlling for age, presence of arterial hypertension, and serum creatinine.

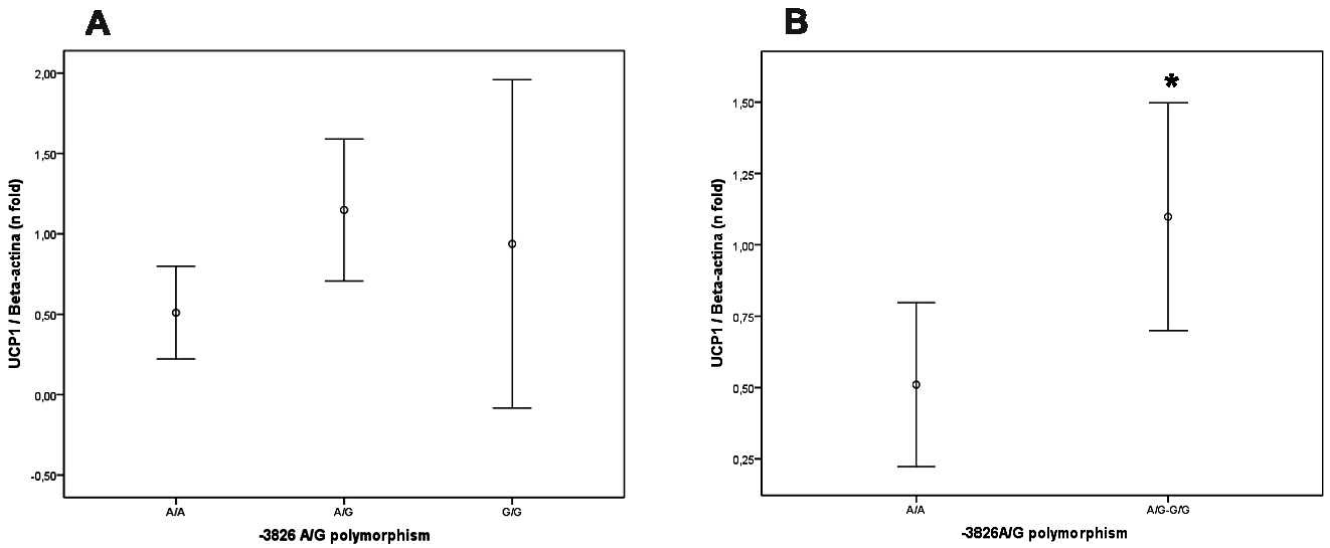

Figure 1. *UCP1* cDNA expression in human retina samples. (**A**) *UCP1* gene expression in samples stratified according to different –3826A/G genotypes. $P = 0.068$ (one-way ANOVA). $P = 0.057$ in relation to the A/A genotype group (Tukey's post hoc test). (**B**) *UCP1* gene expression in samples stratified according to presence/absence of the G allele (AA versus A/G–G/G). $^*P = 0.018$ (Student's *t*-test). Results are expressed as n-fold differences from the calibrator sample ($\Delta\Delta C_q$ method) and are presented as mean ±2 SE.

genotype carriers (1.10 ± 1.5 vs. 0.51 ± 0.99 n-fold; $P = 0.018$; Fig. 1B). After controlling for age, sex, DM diagnosis, and smoking habits in the linear regression analysis, presence of the G allele remained significantly associated with *UCP1* gene expression ($P = 0.034$).

The mean ± SD *MnSOD2* cDNA concentration for the entire retina tissue group was –0.82 ± 1.04 n-fold. No significant difference was observed when *MnSOD2* gene expression was analyzed broken down by sex (men: –1.0 ± 1.2 versus women: –0.8 ± 1.2 n-fold; $P = 0.393$), hypertension status (normotensives: –0.7 ± 1.4 versus hypertensives: –1.0 ± 1.1 n-fold; $P = 0.652$), smoking habits (nonsmokers: –0.77 ± 0.97 versus smokers: –0.66 ± 1.29; $P = 0.758$), or DM diagnosis (nondiabetic: –0.6 ± 0.9 versus DM subjects: –0.9 ± 1.0 n-fold; $P = 0.594$). *MnSOD2* gene expression did not correlate with age ($r = –0.152$, $P = 0.142$), but it did correlate weakly with *UCP1* cDNA concentrations ($r = 0.29$, $P = 0.015$).

MnSOD2 gene expression appeared to be greater in both A/G and G/G genotype carriers, when compared with A/A genotype subjects ($P = 0.004$; Fig. 2A); however, after application of Tukey's post hoc test, only the A/G genotype group retained conventional statistical significance ($P = 0.006$). Furthermore, G allele carriers (A/G + G/G) exhibited greater *MnSOD2* gene expression than A/A genotype carriers ($P = 0.001$; Fig. 2B). After controlling for age, sex, and DM diagnosis in the linear regression analysis, the G allele remained independently associated with increased *MnSOD2* gene expression ($P = 0.031$).

UCP1 Protein Immunohistochemistry in Human Retina from Cornea Donors, Analyzed according to Different *UCP1* –3826A/G Genotypes

The mean ± SD UCP1 protein concentration for the entire retina tissue group was 1.14 ± 0.3 pixels (logarithmic scale). No significant difference was observed when UCP1 protein immunoreactivity was analyzed by sex (men: 1.85 ± 0.30 versus women: 1.85 ± 0.31 pixels; $P = 0.988$), hypertension status (normotensives: 1.87 ± 0.28 versus hypertensives: 1.89 ± 0.42 pixels; $P = 0.652$), smoking habits (nonsmokers: 1.93 ± 0.29 versus smokers: 1.79 ± 0.37 pixels; $P = 0.418$), or DM diagnosis (nondiabetic: 1.84 ± 0.29 versus DM subjects: 1.93 ± 0.39 pixels; $P = 0.499$). UCP1 protein expression did not correlate with age ($r^2 = 0.087$; $P = 0.542$) or *MnSOD2* cDNA concentrations ($r^2 = 0.032$; $P = 0.872$), but it did exhibit a positive correlation with *UCP1* cDNA concentrations ($r^2 = 0.401$, $P = 0.038$).

In agreement with the *UCP1* gene expression analyses, immunohistochemical analyses showed that G allele carriers also had increased UCP1 protein expression compared with subjects with the A/A genotype, after adjustment for age, DM diagnosis, and smoking habits (1.98 ± 0.31 vs. 1.70 ± 0.33 pixels; $P = 0.039$; Fig. 3D). Moreover, UCP1 protein immunoreactivity was not exclusive to a specific retina cell layer (Figs. 3B, 3C).

Discussion

In this study, we investigated the frequencies of the –3826A/G polymorphism of the *UCP1* gene in a sample of type 1 DM patients subdivided according to presence/absence of DR. The G/G genotype was significantly associated with increased risk of DR. Although UCP1 plays a recognized role in protection against oxidative stress[9] and although oxidative stress is one of the major contributors to accelerated loss of retinal capillary cells in DM,[35] only two other studies have analyzed the association between the –3826A/G polymorphism and DR.[25,26] Rudofsky et al.[26] studied the association between different polymorphisms of the *UCP* genes and diabetic complications in 227 type 1 DM patients from Germany. They did not detect any association between the *UCP1* –3826A/G polymorphism and DR, DN, or diabetic neuropathy. Furthermore, Rudofsky et al.[25] did not observe any association between the *UCP1* –3826A/G polymorphism and DR in a sample of type 2 DM patients from Germany. It is worth noting that a recent study published by our group indicated that presence of the –866A/55Val/Ins haplotype of the *UCP2* gene was associated with an increased risk for proliferative DR in both type 2 and type 1 DM patients,[36] further indicating that UCPs might play an important role in the pathogenesis of DR.

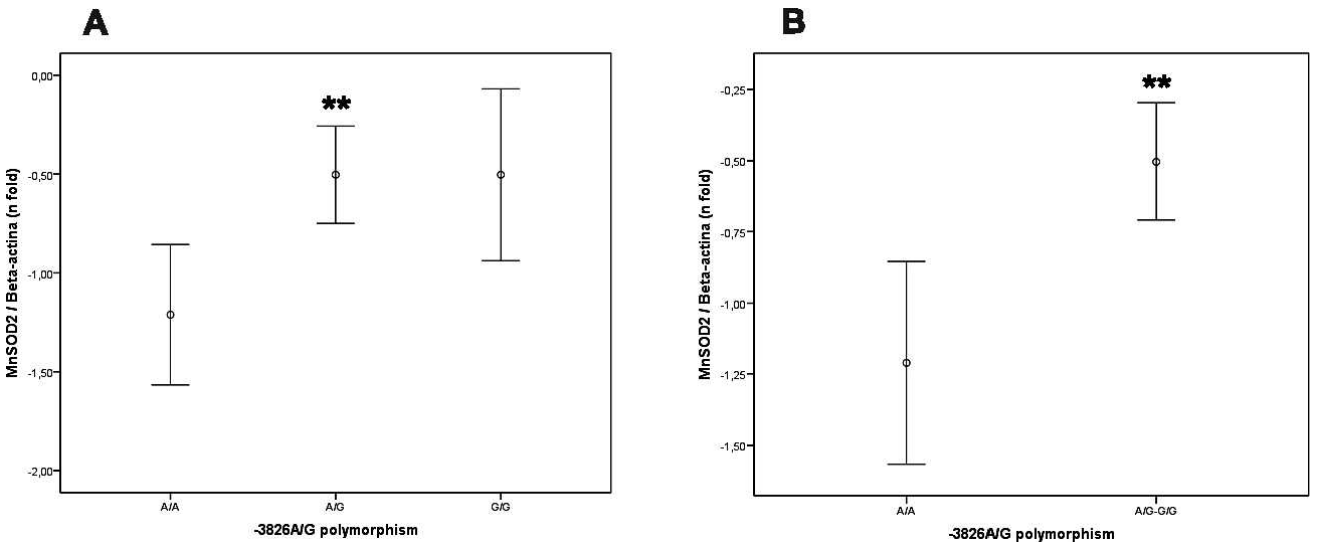

FIGURE 2. *MnSOD2* cDNA expression in human retina samples. (**A**) *MnSOD2* gene expression in samples stratified according to different −3826A/G genotypes. $P = 0.004$ (one-way ANOVA). $^{**}P = 0.006$ in relation to the A/A genotype group (Tukey's post-hoc test). (**B**) *MnSOD2* gene expression in samples stratified according to presence/absence of the G allele (AA versus A/G–G/G). $^{**}P = 0.001$ (Student's *t*-test). Results are expressed as n-fold differences from the calibrator sample ($\Delta\Delta C_q$ method) and are presented as mean ±2 SE.

Functional polymorphisms can influence gene expression and regulate the final quantity of protein in a given tissue. Accordingly, in this study, we found that retina samples from cornea donors carrying the −3826G allele exhibited a 2-fold increase in the *UCP1* gene expression compared with samples from A/A genotype carriers. Immunohistochemical analyses confirmed that the G allele was associated with increased UCP1 protein reactivity in retina samples. On the other hand, Esterbauer et al.[37] measured *UCP1* cDNA expression in intraperitoneal adipose tissue from obese individuals and found that it was lower in those with the −3826G allele than in A/A genotype carriers. Moreover, Rose et al.[38] explored the functional relevance of three different haplotypes constituted by the −3826A/G and −3737C/A polymorphisms of the *UCP1* gene, which are in strong linkage disequilibrium. Their transfection experiments in MCF-7 and T47D cell lines showed that the luciferase activity of the plasmidial construct containing the −3826A/−3737C haplotype was significantly higher than the activity of that containing the −3826G/−3737A haplotype. No activity was observed for the −3826G/−3737C haplotype in basal conditions.[38]

A possible explanation for the contrasting results mentioned above is that the −3826A/G polymorphism could be involved in putative binding sites for specific transcription factors and so preferential binding of some transcription factors to the A or G allele in the *UCP1* promoter sequence could confer tissue-specific advantages to either allele, as has already been demonstrated for the −866G/A polymorphism in the *UCP2* promoter sequence.[39] This hypothesis is reinforced by the knowledge that the −3826A/G polymorphism is located in proximity to a complex enhancer region (from positions −3820 to −3470, upstream of the *UCP1* transcription start site), which contains multiple and distinct cis-acting elements that appear to mediate strong drug-dependent transcriptional activation of the *UCP1* gene.[40] Indeed, using the MatInspector Online Software (Genomatix, Bayerstr, Munich), Rose et al.[38] found a putative retinoic acid response element in the *UCP1* promoter region (from positions −3842 to −3826) that included the −3826A/G polymorphism. Furthermore, an ATF/CREB binding element (from −3738 to −3733), a progesterone responsive element (PRE)-like sequence (from −3817 to −3804), and an estrogen responsive element (ERE)-like sequence (from −3713 to −3701) were also predicted in the vicinity of the −3826A/G and −3737C/A variants. Interestingly, different *UCP1* haplotypes constituted by the −3826A/G and −3737C/A polymorphisms responded differently after stimuli with progesterone or estradiol.[38] The authors concluded that it is likely that the real in vivo effect of the −3826A/G polymorphism is mediated by the intricacy of different cellular and physiological stimuli and is influenced by interactions with other *UCP1* polymorphisms or with other genes.[38]

Echtay et al.[41] have proposed a simple feedback cycle in which mitochondrial ROS overproduction acutely and chronically increases proton conductance through effects on UCP1-3, which results in decreased superoxide production by the mitochondrial respiratory chain. Furthermore, retinal mitochondria become dysfunctional in DM and production of superoxide radicals increases.[42,43] In this context, inhibition of oxidative stress through overexpression of the *MnSOD2* gene also prevents retinal cells from undergoing the accelerated apoptosis that precedes the onset of DR in diabetic mice.[43] Thus, one plausible explanation for the mechanism by which MnSOD2 ameliorates the development of DR is by protecting mitochondrial DNA from glucose-induced oxidative damage.[43] In the present study, *MnSOD2* cDNA concentrations were positively correlated with *UCP1* cDNA concentrations in human retina from cornea donors, and subjects carrying the *UCP1* −3826G allele exhibited greater *MnSOD2* gene expression than A/A genotype carriers. Our findings therefore suggest that *UCP1* −3826A/G genotypes may influence *MnSOD2* gene expression, possibly because MnSOD2 is the main scavenger of mitochondrial superoxide and, as mentioned earlier, this free radical directly activates *UCP1* gene expression.[41,44]

Interestingly, Cui et al.[19] have shown that increased mitochondrial ROS production could be induced by high glucose concentrations. At high glucose concentrations, endothelial cells from bovine retina increased *UCP1*, *UCP2,* and *MnSOD2* expression to compensate for the increased ROS production. However, this compensatory mechanism disappeared when glucose concentrations were too high (30 mM), suggesting that UCPs and MnSOD may exert a compensatory influence on oxidative stress only up to a certain point.[19] Thus, on the basis of our present results taken in conjunction with the findings of Cui et al.[19] in bovine retina, we hypothesize that

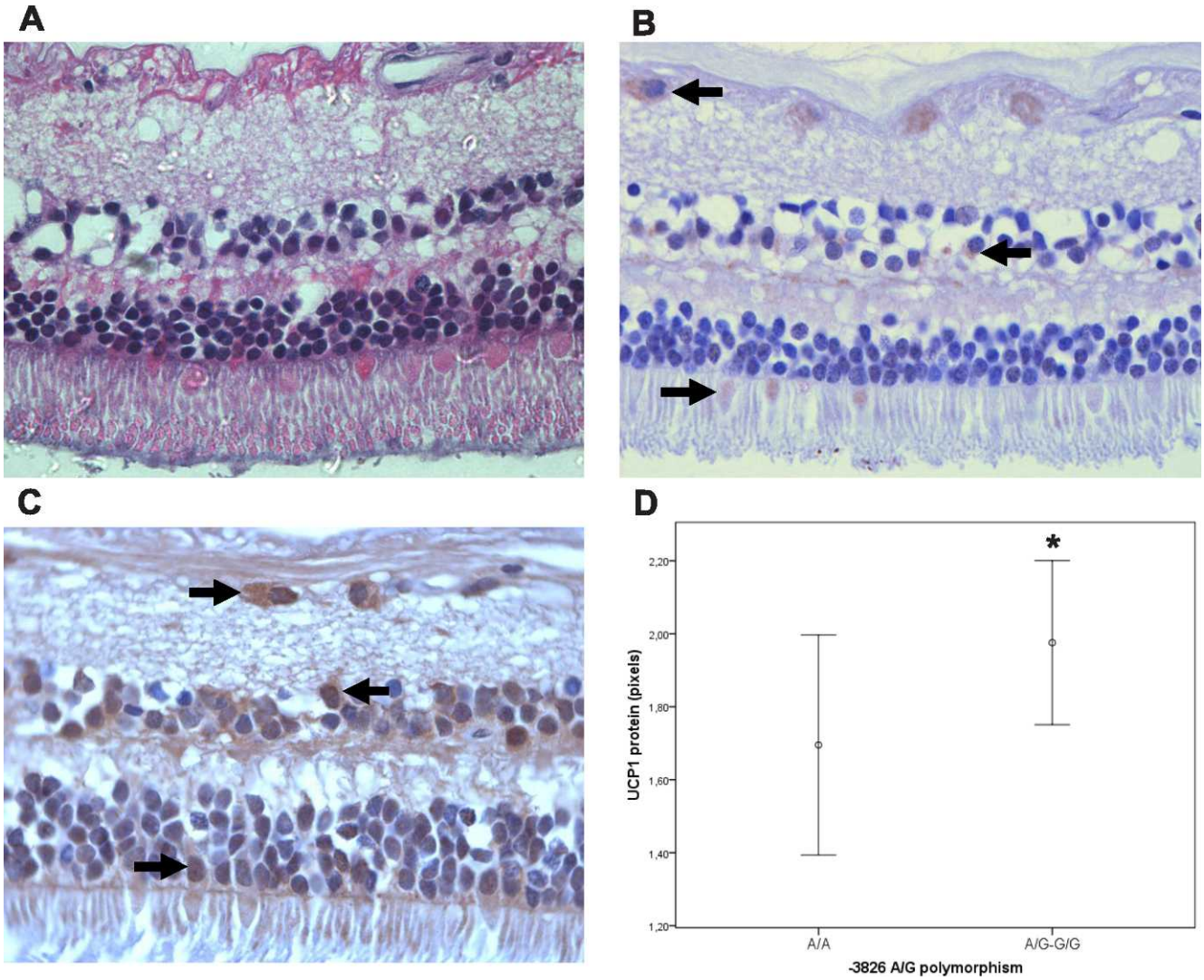

Figure 3. Representative photomicrographs of human retina. (**A**) Human retina stained with hematoxylin-eosin. (**B**, **C**) UCP1 immunostaining in human retina cell layers. *Arrows* indicate different areas of UCP1 immunostaining. Original magnification of all images = ×1000. (**D**) UCP1 protein expression in samples stratified according to presence/absence of the –3826G allele (A/A versus A/G–G/G). $^{*}P = 0.039$ (Student's *t*-test). Data are represented as mean number of pixels (logarithmic scale) of two counts conducted by two independent researchers and are shown as mean ±2 SE.

the increased *UCP1* and *MnSOD2* gene expression observed in G allele carriers could be a compensatory mechanism responding to possible elevated ROS production in the retina in response to increased glucose concentrations, as occurring in a diabetes milieu. Up to certain glucose levels, the increased *UCP1* and *MnSOD2* expression in G allele carriers would protect against the effects of elevated ROS and, consequently, DR. However, at higher glucose levels, increased UCP1 and MnSOD2 would no longer be able to compensate for increased ROS overproduction. In this glucotoxicity environment, the G allele would be a marker of excessive ROS production, which is the actual risk factor to DR.

The present results should be interpreted with caution, as the real effect of the –3826A/G polymorphism in human retina will be dependent on several physiological stimuli as well as interactions with other polymorphisms. Moreover, it may be argued that Bonferroni correction should be applied to the *P* values obtained for the comparisons of genotype frequencies of the –3826A/G polymorphism between diabetic patients with or without DR. If Bonferroni corrections were strictly used, some of our *P* values could not retain the statistical significance. Nevertheless, although there is a chance of type I error due to multiple comparisons, the association of the G/G genotype with DR might be worth noting, because it is biologically plausible, and because we showed that this polymorphism is also associated with changes in *UCP1* and *MnSOD2* gene expression.

In conclusion, the data presented here indicate that the *UCP1* –3826 G/G genotype is associated with an increased risk of DR in type 1 DM patients. Furthermore, the mutated G allele was associated with increased *MnSOD2* cDNA, *UCP1* cDNA, and UCP1 protein concentrations in human retina samples from cornea donors. To our knowledge, these are the first data demonstrating *UCP1* expression in human retinas. It is likely that interactions between UCP1 and MnSOD2 proteins in retinal cells as well as different hormonal stimuli of tissue-specific transcription factors may further influence the effect of *UCP1* in protection against ROS and, in turn, its impact on the risk of developing DR. Additional functional studies will be needed to confirm the association between the –3826A/G

polymorphism and DR and also to elucidate the mechanisms through which these interactions occur.

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