Universidade Federal do Rio Grande do Sul
Programa de Pós-Graduação em Ciências Médicas: Endocrinologia

POLIMORFISMO -3826A/G NO GENE *UCP1*: INVESTIGAÇÃO DE SUA POSSÍVEL ASSOCIAÇÃO COM RETINOPATIA DIABÉTICA EM PACIENTES COM DIABETES MELLITUS TIPO 1 E DE SEU EFEITO NA EXPRESSÃO DA UCP1 NA RETINA

Dissertação de Mestrado

Letícia de Almeida Brondani

Porto Alegre, Setembro de 2011

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Letícia de Almeida Brondani

Orientadora: Prof[a]. Dr[a]. Daisy Crispim Moreira

Dissertação de mestrado apresentada ao Programa de Pós-Graduação em Ciências Médicas: Endocrinologia, da Universidade Federal do Rio Grande do Sul (UFRGS) como requisito parcial para obtenção do título de Mestre em Endocrinologia.

Porto Alegre, setembro de 2011.

Dedico essa dissertação aos meus pais, meus exemplos de vida.

"Chegou a tua vez, oh! Natureza!
Eu desafio agora essa grandeza,
Perante a qual meus olhos se extasiam...
Eu desafio, desta cova escura,
No histerismo danado da tortura
Todos os monstros que os teus peitos criam!"

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- Artigo de revisão: "The role of the uncoupling protein 1 (UCP1) on the development of obesity and type 2 diabetes mellitus" (submetido a **Diabetology & Metabolic Syndrome**).

- Artigo original: "Association between the -3826A/G polymorphism in the uncoupling protein 1 (*UCP1*) gene and diabetic retinopathy and *UCP1* gene expression in human retina" (a ser submetido à revista **Diabetes**).

SUMÁRIO

LISTA DE ABREVIATURA

3'UTR	3' untranslated region
ADP	Adenine diphosphatase
ANT	Adenine nucleotide translocase
ATF	Activating transcription factors
ATP	Adenine triphosphatase
BAT	Brown adipose tissue
β3-AR	β3-adrenergic receptor
BMI	Body mass index
CoQ	Coenzyme Q
CREB	cAMP-response element binding
DM	Diabetes mellitus
DM1	Type 1 diabetes mellitus
DM2	Type 2 diabetes mellitus
DR	Diabetic retinopathy
FADH2	Flavin adenine dinucleotide reduced
FFA	Free fatty acid
GDP	Guanosine diphosphate
GSH	Glutathione
H_2O_2	Hydrogen peroxide
HNE	4-hydroxy-2-nonenal
HWE	Hardy-Weinberg equilibrium
IR	Insulin resistance
MAPK	Mitogen-activated protein kinase

MnSOD2	Manganese superoxide dismutase 2
MRC	Mitochondrial respiratory chain
mRNA	Messenger RNA
NADH	Nicotinamide adenine dinucleotide reduced
O_2^-	Superoxide anion
OR	Odds ratio
$^{\bullet}OH$	Hydroxyl radical
OXPHOS	Oxidative phosphorylation
PDR	Proliferative diabetic retinopathy
PGC-1α	PPAR-gama coactivator-1α
Pi	Inorganic phosphate
PKA	Protein kinase A
PPAR	Peroxisome proliferator-activated receptor
RMR	Resting metabolic rate
ROS	Reactive oxygen species
RT-PCR	Reverse transcription-polymerase chain reaction
SD	Standard deviation
SE	Standard error
UCP1	Uncoupling protein 1
UCPs	Uncoupling proteins
WAT	White adipose tissue

RESUMO

Está bem definido que fatores genéticos têm um papel importante no desenvolvimento do diabetes mellitus (DM) e de suas complicações crônicas. Sendo assim, grandes esforços têm sido feitos para se identificar os genes associados com estas doenças. A proteína desacopladora 1 (UCP1), principalmente expressa no tecido adiposo marrom, desacopla a oxidação dos substratos da síntese de ATP pela ATP-sintase, dessa forma, dissipando o potencial de membrana e, consequentemente, diminuindo a produção de ATP pela cadeia respiratória mitocondrial. Esse desacoplamento então leva à regulação do gasto energético, à termogênese e à proteção contra o estresse oxidativo. Um dos principais mecanismos pelo qual a hiperglicemia leva ao aparecimento das complicações crônicas do DM, como a retinopatia diabética (RD), é através do aumento da produção de espécies reativas de oxigênio pela mitocôndria. Portanto, é biologicamente plausível que o polimorfismo -3826A/G localizado na região promotora do gene *UCP1* possa estar associado com a RD em pacientes com DM.

No presente estudo, através de um delineamento de caso-controle, investigamos se o polimorfismo -3826A/G no gene *UCP1* está associado à RD em pacientes com diabetes mellitus tipo 1 (DM1). Além disso, em um estudo transversal realizado em doadores de córnea, avaliamos se a *UCP1* está expressa na retina humana e se o polimorfismo -3826A/G modifica a sua expressão nesse tecido. Também avaliamos a expressão do gene *MnSOD2*, o qual codifica uma importante enzima antioxidante, de acordo com os diferentes genótipos do polimorfismo -3826A/G.

No estudo de caso-controle, as frequências do polimorfismo -3826A/G foram avaliadas em 257 pacientes com DM1 diferenciados de acordo com a presença de RD

(154 casos com RD e 103 controles sem RD). O estudo transversal incluiu 166 doadores cadavéricos de córneas. Em um subgrupo de 107 amostras de retina diferenciadas de acordo com a presença do alelo de risco do polimorfismo estudado, as concentrações dos mRNAs de *UCP1* e *MnSOD2* foram avaliadas pela técnica de PCR em tempo real.

O alelo G do polimorfismo -3826A/G foi mais frequente em pacientes com RD do que em pacientes sem esta complicação (41,0% *vs*. 31,0%; P = 0,029). O genótipo G/G foi associado a um risco aumentado para RD, após o ajuste para idade, presença de hipertensão arterial e níveis de creatinina sérica (Razão de Chances = 3,503, IC 95% 1,04 – 11,80; P = 0,043). Nossos dados mostram pela primeira vez a expressão do mRNA de *UCP1* na retina humana (0,93 ± 1,35 *n fold*). Além disso, os portadores do alelo G apresentaram uma maior expressão gênica de *UCP1* do que os portadores do genótipo A/A (1,10 ± 1,50 *vs*. 0,51 ± 0,99 *n fold*; P = 0,034). Interessantemente, os portadores do alelo G também apresentaram uma expressão gênica aumentada de *MnSOD2* (P = 0,031). Adicionalmente, as concentrações de *UCP1* e de *MnSOD2* na retina se correlacionaram positivamente (r = 0,29, P = 0,015).

Em conclusão, nossos resultados mostram uma associação independente do polimorfismo -3826A/G com a RD em pacientes com DM1. Esse estudo é o primeiro a relatar que a UCP1 está expressa na retina humana e que o polimorfismo -3826A/G influencia a sua expressão nesse tecido. Possivelmente, a expressão de *MnSOD2* influencia o efeito da UCP1 na proteção contra o estresse oxidativo. Estudos funcionais adicionais serão necessários para avaliar qual o efeito da UCP1 na retina humana e confirmar se mudanças na expressão do gene *UCP1* também ocasionam mudanças nos níveis de proteína.

ABSTRACT

It is well established that genetic factors play an important role in the development of diabetes mellitus (DM) and its chronic complications. Therefore, great efforts have been made to identify genes associated with these diseases. The uncoupling protein 1 (UCP1), mainly expressed in brown adipose tissue, acts uncoupling the oxidation of substrates from ATP synthesis by ATP-synthase, thereby dissipating the membrane potential and, consequently, decreasing the ATP production by the mitochondrial respiratory chain. This uncoupling then leads to the regulation of energy expenditure, thermogenesis, and protection against oxidative stress. One of the main mechanisms by which hyperglycemia leads to the development of chronic diabetic complications, such as diabetic retinopathy (DR), is through the increased production of reactive oxygen species by mitochondria. Thus, it is biological plausible that the -3826A/G polymorphism located at the promoter region of the *UCP1* gene might be associated with DR in patients with DM.

In this study, through a case-control design, we investigated whether the -3826A/G polymorphism in the *UCP1* gene is associated with DR in patients with type 1 diabetes mellitus (DM1). In addition, in a cross-sectional study performed in cornea donors, we evaluated whether the *UCP1* gene is expressed in human retina, and whether the -3826A/G polymorphism modifies its expression in this tissue. We also evaluated the *MnSOD2* gene expression (which is a gene that codifies an important antioxidant enzyme) according to the different genotypes of the -3826A/G polymorphism.

In the case-control study, the frequencies of the -3826A/G polymorphism were evaluated in 257 patients with DM1 differentiated according to the presence of DR (154 cases with DR and 103 controls without DR). The cross-sectional study included 166

cadaveric donor corneas. In a subgroup of 107 retinal samples differentiated according to the presence of the risk allele of the analyzed polymorphism, *UCP1* and *MnSOD2* mRNA concentrations were evaluated by real time-PCR technique.

The G allele of the -3826A/G polymorphism was more frequent in patients with DR compared to patients without this complication (41.0% *vs*. 31.0%; P = 0.029). The G/G genotype was associated with an increased risk to DR, after adjusting for age, arterial hypertension and serum creatinine levels (Odds Ratio = 3.503; 95% 1.04 - 11.80; P = 0.043). Our data show for the first time the *UCP1* mRNA expression in human retina (1.35 ± 0.93 *n fold*). Moreover, G allele carriers had a higher *UCP1* mRNA expression than A/A genotype carriers (1.10 ± 1.50 *vs*. 0.51 ± 0.99 *n fold*, P = 0.034). Interestingly, G allele carriers also showed an increased *MnSOD2* gene expression (P = 0.031). Additionally, *UCP1* and *MnSOD2* concentrations in retina were positively correlated (r = 0.29, P = 0.015).

In conclusion, our results show an independent association between the -3826 A/G polymorphism and DR in DM1 patients. This is the first report showing that *UCP1* is expressed in human retina, and that the -3826A/G polymorphism influences its expression in this tissue. Possibly, *MnSOD2* expression might influence the UCP1 effect in the protection against oxidative stress. Further functional studies will be needed to evaluate the effect of UCP1 in human retina, and to confirm that changes in the *UCP1* gene expression also cause changes in protein levels.

PARTE I

Artigo de revisão

THE ROLE OF THE UNCOUPLING PROTEIN 1 (UCP1) ON THE DEVELOPMENT OF OBESITY AND TYPE 2 DIABETES MELLITUS

O PAPEL DA PROTEÍNA DESACOPLADORA 1 (UCP1) NO DESENVOLVIMENTO DA OBESIDADE E DIABETES MELLITUS TIPO 2

The role of the uncoupling protein 1 (UCP1) on the development of obesity and type 2 diabetes mellitus

Letícia de Almeida Brondani, Taís Silveira Assmann, Guilherme Coutinho Kullmann Duarte, Luís Henrique Canani, Daisy Crispim*

Endocrinology Division, Hospital de Clínicas de Porto Alegre, Universidade Federal do Rio Grande do Sul. Porto Alegre, RS, Brazil. Emails: lestrega@hotmail.com (LAB); taisassmann@hotmail.com (TSA); gui_couto@hotmail.com (GCKD); luishenriquecanani@gmail.com (LHC); daisy_crispim@hotmail.com (DC).

*** Correspondence:** Dr. Daisy Crispim. Rua Ramiro Barcelos 2350; prédio 12; 4° andar. CEP 90035-003, Porto Alegre, RS, Brazil.

ABSTRACT

It is well established that genetic factors play an important role in the development of both type 2 diabetes mellitus (DM2) and obesity, and that genetically susceptible subjects can develop these metabolic diseases after being exposed to environmental risk factors. Therefore, great efforts have been made to identify genes associated with DM2 and/or obesity. Uncoupling protein 1 (UCP1) is mainly expressed in brown adipose tissue, and acts in thermogenesis, regulation of energy expenditure, and protection against oxidative stress. All these mechanisms are associated with the pathogenesis of DM2 and obesity. Hence, *UCP1* is a candidate gene for the development of these disorders. Indeed, several studies have reported that polymorphisms -3826A/G, -1766A/G and -112A/C in the promoter region, Ala64Thr in exon 2 and Met299Leu in exon 5 of *UCP1* gene are possibly associated with obesity and/or DM2. However, the results are still controversial in different populations. Thus, the aim of this study was to review the role of UCP1 in the development of these metabolic diseases.

Keywords: UCP1, obesity, type 2 diabetes mellitus, DNA polymorphisms, brown adipose tissue.

Introduction

Type 2 diabetes mellitus (DM2) and obesity are common, multifactorial conditions for which susceptibility is determined by the joint actions of genetic and environmental factors [1-3]. The prevalence of obesity and DM2 is increasing worldwide at an alarming rate, and both traits are associated with increased morbidity and mortality [4-6]. The dramatic increase in the prevalence of these disorders over the past two decades is mostly likely due to changes in diet and physical activity [7]. However, it is believed that these environmental changes would only lead to DM2 and/or obesity under a permissible genetic background [2]. Therefore, great efforts have been made to identify genes associated with these disorders, and a number of studies have been focused on the genes related to energy expenditure, such as those encoding adrenergic receptors and mitochondrial uncoupling proteins (UCPs) [8-10].

Uncoupling protein 1 (UCP1) plays important roles in metabolic and energy balance and regulation, cold- and diet-induced thermogenesis, and in decreasing reactive oxygen species (ROS) production by mitochondria, which are mechanisms associated with the pathogenesis of obesity and/or DM2 [8, 9]. Thus, the aim of the present study was to review the role of UCP1 in relation to the development of these conditions.

Mitochondrial Respiratory Chain

Mitochondria are organelles in all eukaryotic cells and are involved in many processes essential for cell survival and function, including energy production, redox control, calcium homeostasis, and certain metabolic and biosynthetic pathways. In addition, mitochondria are the main sources of ROS and often play a key role in physiological cell death mechanisms [11].

The main source of cell energy is the synthesis of ATP from ADP and inorganic phosphate (Pi) by oxidative phosphorylation (OXPHOS) carried out in the mitochondrial respiratory chain (MRC) [12]. MRC is located in the inner mitochondrial membrane, and is constituted by four multienzymatic complexes, an oligomeric protein complex (ATP-synthase), and two proteins responsible for electron transport, coenzyme Q (CoQ) and cytochrome *c* (**Figure 1**). OXPHOS involves the coupling of electron transport, through the complexes I-IV of the MRC, to the active pumping of protons across the inner mitochondrial membrane and ATP formation by ATP-synthase [11, 13].

Oxidation of reduced nutrient molecules through cellular metabolism yields electrons in the form of reduced hydrogen carriers (NADH and $FADH_2$), which donate electrons to the MRC. The movement of electrons through MRC is driven by a redox potential that is present across the chain. Complexes I, III and IV pump protons across the inner membrane as electrons pass down the MRC. This produces an electrochemical potential difference across the inner membrane, known as proton-motive force, consisting mostly of an electrochemical gradient (membrane potential) and a chemical gradient (pH difference). The energy that is conserved in the proton gradient across the inner membrane is used by ATP-synthase to synthesize ATP as protons are transported back from the intermembrane space into the mitochondrial matrix. The final destination for the electrons is the generation of molecular oxygen, which is reduced to water by complex IV, in the last step of the MRC. Therefore, the process of substrate oxidation and oxygen reduction is also called respiration [11, 13-15].

The coupling of respiration to ATP synthesis is not 100% efficient and some of the energy is dissipated as heat. Partial uncoupling of respiration from ATP synthesis, also known as proton-leak, can be mediated by UCPs and by other mitochondrial inner

membrane proteins as for example the adenine nucleotide translocase (ANT); thus, preventing the inhibition of MRC by exaggerated ATP levels [16-18].

Although OXPHOS constitutes a vital part of cellular metabolism, the MRC is probably the most important site of ROS production [19]. ROS correspond to a variety of molecules and free radicals (chemical species with one unpaired electron) derived from the metabolism of molecular oxygen. Superoxide anion ($O_2^{-\bullet}$) is the precursor of most ROS and a mediator in oxidative chain reactions [19, 20]. Dismutation of $O_2^{-\bullet}$ (either spontaneously or through a reaction catalyzed by superoxide dismutases) produces hydrogen peroxide (H_2O_2), which in turn may be fully reduced to water or, in the presence of ferrous or cuprous ions, may form the highly reactive hydroxyl radical ($^{\bullet}OH$) [11]. ROS normally exist in all aerobic cells in balance with biochemical antioxidants. Oxidative stress occurs when this critical balance is disrupted because of excess ROS, antioxidants depletion, or both. This stress causes damage to cellular macromolecules, such as nucleic acids, proteins, lipids and structural carbohydrates [21]. Moreover, oxidative stress can also lead to cell death by necrosis or apoptosis, mechanisms involved in the pathogenesis of ageing and some disorders such as DM2 and its chronic complications [22, 23].

Uncoupling of the Mitochondrial Respiratory Chain, Changes in Energy Expenditure, and Adaptive Thermogenesis

Total body energy expenditure represents the conversion of oxygen and food (or storable forms of energy) to carbon dioxide, water, heat and "work" on the environment [24]. Energy expenditure in humans can be subdivided into: 1) basal energy expenditure or resting metabolic rate (RMR), measured under resting conditions and required for

normal functioning of cells; 2) energy expenditure resulting from physical activity; and 3) energy expenditure attributed to adaptive thermogenesis (**Figure 2**) [24, 25].

Uncoupling (proton-leak) of MRC constitutes a considerable part of the RMR [25]. Approximately 20-50% of total energy expenditure is due to proton-leaks, with the skeletal muscle as the main contributor [26]. Variations in the RMR are due to several determinants, including body composition (fat *vs*. fat free mass), concentrations of steroid and thyroid hormones, genetic factors, and the activity of the sympathetic nervous system [25]. It is known that a low energy expenditure could predict future weight gain [27], and that only a slight imbalance between energy intake and energy expenditure is necessary for a weight gain if it persists over several years [28]. Thus, increasing the energy expenditure by increasing the proton-leak in mitochondria has been recognized as an effective way to achieve weight loss [25].

Brown adipose tissue (BAT) is found in newborns, rodents and hibernating mammals, and is the main site of adaptive thermogenesis, which is defined as non-shivering heat production in response to environmental temperature or diet [9, 17, 29]. As a result, thermogenesis in BAT has important roles in thermal and energetic balance and, when deficient, can lead to obesity [30]. BAT is a metabolically active tissue, which consists of adipocytes rich in mitochondria and numerous small lipid droplets, and is heavily innervated by sympathetic nerves [25]. This tissue differs from the white adipose tissue (WAT), which contains large lipid droplets and few mitochondria [17, 25].

In fetuses and newborn infants, BAT has traditionally been regarded as occurring in specific depots, such as axillary, interscapular, perirenal and periadrenal [31]. At birth, human newborns have considerable amount of BAT, corresponding to 1-5% of the total body weight. This amount is able to take care of the heat generation for

the body when the skeletal muscles are yet not able to make any controlled movements and thus, produce heat. Children have highly active functional BAT until 13-15 years of age; but, until a few years ago, it was thought that the quantity of BAT declines after puberty, being rare in adults [30]. Nevertheless, nowadays it is known that BAT can be found in adults in the presence of catecholamine-secreting tumors, such as pheochromocytomas and paragangliomas [32]. Besides, some recent studies have shown that BAT in adults is functionally and metabolically highly active, especially after chronic exposure to cold [33, 34].

In 1978, Himms-Hagen and Desautels [35] showed that BAT metabolism played a role in obesity development, and that obese mice had a defect in the mechanisms necessary for the activation of BAT thermogenesis. After this pioneering work, many studies have also shown that defective BAT thermogenesis is involved in the development of obesity in most rodent models, and activation of BAT thermogenesis reduces weight gain in these animals [36, 37]. Studies in humans show that although the amount of BAT is reported to be decreased in healthy adults, it is still responsible for 1-2% of the energy expenditure, preventing a weight gain of 1-2 kg per year [38-40]. Interestingly, healthy men that has BAT when exposed to cold (19°C) have a 30% increase in energy expenditure when compared to thermoneutrality (27°C), in contrast to those men with almost no BAT that did not show any increase in cold-induced energy expenditure [41].

It has been suggested that development of ectopic BAT within the WAT may play an important role in preventing obesity [16]. In agreement with this hypothesis, transgenic mice overexpressing UCP1 in their skeletal muscle or WAT develop a resistance to diet-induced obesity and DM2, and also have a marked stimulation of fatty acid oxidation in muscles [29, 42]. In addition, Tiraby *et al*. [43] reported that the

adenovirus-mediated expression of human PGC-1α (PPARγ-coactivator-1α) increased the expression of UCP1, respiratory chain proteins, and fatty acid oxidation enzymes in human subcutaneous white adipocytes. Changes in the expression of other genes were also consistent with brown adipocyte mRNA expression profile. The authors concluded that human white adipocytes can therefore acquire typical features of brown fat cells following proper stimulation [43]. These data indicate that a moderate induction of UCP1 in WAT may be used to increase metabolic energy expenditure in obese subjects. Thus, specific uncoupling of adipocyte mitochondria remains an attractive target for the development of anti-obesity drugs [16].

Mitochondrial Uncoupling Proteins (UCPs)

UCPs 1, 2, 3, 4 and 5 are members of an anion-carrier protein family and are located in the inner mitochondrial membrane [44]. These proteins have similarities in their structures, but different tissue expression in mammals. The original UCP, UCP1, is mainly expressed in BAT [45]. UCP2 is widely distributed, whereas UCP3 is mainly restricted to the skeletal muscle and UCP4 and 5 are mainly expressed in the brain [44, 45].

Over the last few years, several studies have shown that UCPs decrease metabolic efficiency by uncoupling substrate oxidation in mitochondria from ATP synthesis by MRC. This is thought to be accomplished by promoting net translocation of protons from the intermembrane space, across the inner mitochondrial membrane, to the mitochondrial matrix, thereby dissipating the potential energy available for ATP synthesis, and consequently, decreasing ATP production [44, 46]. This uncoupling effect then leads to homologue- and tissue-specific functions, such as thermogenesis and energy expenditure (UCP1), regulation of free-fatty acids (FFAs) metabolism

(UCP2 and UCP3), reduction in ROS formation (UCP1-3 and UCP5), and regulation of ATP-dependent processes (UCP2) [45, 47].

Uncoupling Protein 1 (UCP1)

Thermogenesis in BAT is due to UCP1, also called thermogenin or SLC25A7 [25, 48]. In 1985, Aquila *et al*. [49] cloned the *UCP1* coding DNA sequence, and also determined its amino acid sequence. *UCP1* gene covers a 9 kb region on chromosome 4 (region 4q28-q31) and contains 6 exons and 5 introns **(Figure 3)** [50]. UCP1 is a 33-kDa dimeric protein that dissipates the pH-gradient generated by OXPHOS **(Figure 1**), releasing chemical energy as heat [18, 25].

UCP1 gene expression is increased by cold, adrenergic stimulation, β3-agonists, retinoid and thyroid hormones and cAMP [25, 51, 52]. Its expression is activated by non-sterified fatty acids and inhibited by purine nucleotides (GDP, ATP and ADP) [11, 25, 44]. Many studies based on the use of drugs activating β3-adrenergic receptor (β3-AR) confirmed that the sympathetic nervous system was the main trigger of UCP1 activation and induction [53]. Moreover, uncoupling of MRC through UCP1 action is only observed when the cells are properly stimulated, for example, by norepinephrine [46]. Norepinephrine stimulation of β3-AR results in three concerted processes: 1) activation of p38 mitogen-activated protein kinase (MAPK) pathways that upregulate UCP1 synthesis; 2) activation of protein kinase A (PKA)-mediated pathways that initiate lipolysis and release of acute regulators of UCP1, such as FFAs; and 3) inhibition of lysosomal pathways that degrade UCP1 [17, 18]. In addition, it is well known that PGC-1α plays a pivotal role in the regulation of *UCP1* gene expression, following adrenergic stimulation, through a

MAPK pathway that is associated with the JNK-interacting protein (JIP) family of scaffold proteins [46].

The uncoupling activity of UCP1 is explained by its ability to transport protons across the inner mitochondrial membrane, in particular when FFAs bind to the protein. However, although CoQ has been described as a cofactor essential for its activity, the precise mechanism by which FFAs regulate transport through UCP1 is still a matter of intense debate [54, 55]. The main proposed mechanisms include the fatty acids protonophore (or *flip-flop*) model and the channel (or *proton buffering*) model. In the *flip-flop* model, UCP1 is a carrier of fatty acid anions, which are transported by this protein from the matrix side to the intermembrane space. In this model, each fatty acid anion combines with a proton, becomes electrically neutral and flips back through the membrane, releasing the proton in the matrix [56]. The channel model predicts a two-domain structure of UCP1 with a pore domain and a gating domain, which allows protons to pass through the UCP1. In this model, fatty acid carboxy groups are involved in the proton transport by providing H^+-buffering capacity [57]. The arguments for and against each model were reviewed by Brand *et al*. [26] in 1999.

Transgenic and knockout rodent models are common approaches for investigating the impact of overexpression or the absence of a particular gene on the mouse phenotype. The UCP1 knockout mice did not become obese, and merely showed an increased sensitivity to cold exposure [58]. On the other hand, transgenic mice with increased UCP1 expression in WAT were obesity-resistant after being fed with a diet rich in saturated fat [59]. UCP1 has also been ectopically expressed in skeletal muscle of mice, which showed an improved glucose tolerance when compared with wild-type mice, after a high-fat diet [60]. In humans, UCP1 expression in the intraperitoneal fat of obese subjects is 50% lower than in normal weight subjects, in spite of the amount of

BAT interspersed in WAT depots in adult individuals is relatively low (approximately 1 brown adipocyte / 200 white adipocytes) [38, 61].

Until a few years ago, it was believed that UCP1 was expressed exclusively in BAT; but it was recently reported that UCP1 mRNA expression and/or protein were also observed in the WAT of mouse and humans, in mammalian pancreatic islets, in human skeletal muscle, in bovine retina, in human longitudinal smooth muscle layers, and in rat and mouse thymocytes [62-66]. However, the physiological role of UCP1 in these tissues is still a matter of debate [25]. As already mentioned, uncoupling of MRC due to UCP1 activity allows a more rapid flux of electrons through the inner mitochondrial membrane, reducing membrane potential and, consequently, decreasing ROS production [44]. Therefore, the main role of UCP1 in these other tissues seems to be a protection against oxidative stress [67, 68]. Superoxide anions could activate UCPs through lipid peroxidation products, such as 4-hydroxy-2-nonenal (HNE), which is a marker of oxidative stress and a direct activator of UCP1 [68-70]. On the other hand, superoxide dismutase enzymes inhibit the UCP1 activity [68]. Interestingly, Cui *et al*. [65] reported that endothelial cells from bovine retina incubated with high glucose levels increased UCP1 and UCP2 expression, which protected them from ROS damage derived from glucotoxicity, suggesting a protective role of these UCPs in the pathogenesis of diabetic retinopathy, a chronic diabetic complication.

UCP1 Gene Polymorphisms Associated with Obesity and/or Type 2 Diabetes Mellitus

Because UCP1 has been found to decrease membrane potential and increase energy expenditure, *UCP1* gene is regarded as a candidate gene for obesity, DM2 or

related-traits [25]. For that reason, in a number of genetic studies, the relationship between *UCP1 locus* and susceptibility to these disorders has been investigated, with particular attention being paid to -3826A/G, -1766A/G and -112A/C polymorphisms in the promoter region, Ala64Thr polymorphism in exon 2, and Met299Leu polymorphism in exon 5 of the *UCP1* gene (**Figure 3**). Results of these studies have been variable (**Table 1**): while some studies showed an association of one or more of these polymorphisms with obesity, DM2, body fat accumulation, body mass index (BMI) or other characteristics of metabolic syndrome [40, 62, 71-91], other studies were unable to find any association between these polymorphisms and these characteristics [92-102].

Most association studies about the effects of *UCP1* gene polymorphisms were focused on the -3826A/G (rs1800592) polymorphism. The -3826G allele has been associated with reduced *UCP1* mRNA expression in intraperitonial adipose tissue of obese subjects, indicating that this polymorphism has functional importance [103]. Several independent studies support the association between the -3826G allele and obesity, BMI or other obesity-related parameters [75-77, 81-84, 87, 90]. Additionally, other studies indicate that the -3826G allele might be associated with reduced HDL-cholesterol levels [62, 74, 78, 85], increased triglycerides [78, 84] or LDL-cholesterol levels [80, 85], and increased systolic and/or diastolic blood pressure [76, 85].

Conversely, studies on the association of the effects of *UCP1* gene polymorphisms on DM2 have shown controversial results: a few studies reported an association between the -3826G allele and DM2, insulin resistance (IR) or increased insulin or glucose levels [78, 82]; whereas other studies indicated that this polymorphism is not associated with these characteristics [73, 77, 81, 82, 93, 95-97,

100, 101]. A number of studies analyzed the association between other *UCP1* gene polymorphisms and DM2. Mori *et al*. [79] reported that the C allele of -112A/C polymorphism and Leu allele of Met299Leu polymorphism were associated with susceptibility to DM2 in Japanese subjects. A recent study showed that the -3826A/-112A/Met229 *UCP1* haplotype was associated with increased risk for DM2 in Indian subjects [91]. In addition, the study of Fukuyama et al. [73] indicated that the -112A/C polymorphism was associated with both increased insulin resistance and hepatic lipid content in Japanese subjects with DM2.

Like UCP1, β3-AR is expressed in BAT and WAT and plays an important role in the induction of lipolysis and in the regulation of energy homeostasis [10]. In addition, it is the main adrenoreceptor that stimulates UCP1 expression [52]. The Trp64Arg polymorphism in the β*3-AR* gene has been associated with weight gain and other obesity-related indexes as well as with insulin resistance in different populations (reviewed in [10]). Interestingly, some studies have shown that a synergistic effect between the -3826A/G polymorphism (*UCP1* gene) and the Trp64Arg polymorphism (*β3-AR* gene) is associated with an increased tendency to weight gain [87], lower RMR [99], resistance to weight loss [98, 104] or subsequent weight-maintenance after a low-calorie diet [98]. In contrast, other studies did not found any influence of the interaction between these two polymorphisms on the resistance to a low-calorie diet [105], BMI and triglyceride levels [81, 84] or several metabolic parameters related to obesity and DM2 [101]. The ethnical difference, the age and environmental factors as well as synergetic effect with other genes might explain the controversial results among different investigations [10].

In brief, studies on these associations cited here indicated that the -3826A/G polymorphism contributes to the susceptibility of obesity. On the other hand, results

reported by other studies related to the effects of -3826A/G polymorphism and other *UCP1* gene polymorphisms on lipid profile, blood pressure or DM2 are still inconclusive.

Conclusion

Several studies have contributed to the understanding of the mechanisms underlying BAT function and UCP1 activity in this tissue. Interestingly, recent studies have shown that UCP1 can also be detected in pancreatic islets, WAT, skeletal muscle, longitudinal smooth muscle layers, retina and thymus. However, the physiological functions of UCP1 in these tissues are not established as well as in BAT, and future studies will determine the role of UCP1 in these tissues.

Obesity and DM2 are multifactorial diseases associated with both genetic and environmental factors. Knowledge on factors associated with these disorders will allow us to better understand them, and may provide us with more effective approaches to treatment and prevention. UCP1 plays important roles in thermogenesis, regulation of energy expenditure, and in decreasing oxidative stress, which are mechanisms associated with the pathogenesis of obesity and DM2. Thus, *UCP1* gene is an excellent candidate for these disorders. Indeed, numerous studies strongly suggest that the *UCP1* -3826A/G polymorphism is associated with obesity. Further studies are needed to investigate *UCP1* gene polymorphism in different populations in order to confirm the association between these polymorphisms and DM2, and also to elucidate the molecular mechanisms of association between *UCP1* polymorphisms and obesity, DM2, and related-phenotypes.

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Authors' Contributions

All authors have contributed equally to the concept of the review, the literature search, and the writing. All authors have read and approved the final manuscript.

Conflict of Interests

The authors declare that they have no competing interests.

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Table 1 Studies of the association between *UCP1* gene polymorphisms and obesity, type 2 diabetes mellitus or related-traits

Polymorphism	Population and Design	Characteristics	Results	Ref.
-3826A/G	Colombian (455 patients with DM2 and 449 non-diabetic controls).	DM2.	Association between the A allele and DM2 (OR = 0.78, P = 0.02).	[88]
-3826A/G	Korean (40 obese women). Randomized clinical trial of low-calorie meals (white *vs*. mixed rice), in a 6-week follow-up.	Weight, BMI, lipid profile, and blood glucose.	A/A genotype was associated with significant weight loss in the mixed rice group.	[89]
-3826A/G	Japanese (32 obese women). Low-calorie diet intervention in a 2-month follow-up.	Obesity- and lipid-related parameters.	The degree of reduction in the HDL levels was significantly smaller in G allele carriers than in A/A carriers.	[74]
-3826A/G	239 African-Americans and 583 Hispanics.	BMI, WHR, lipid profile, blood glucose, IR, and AIRg.	A/A genotype was associated with AIRg in African-Americans and HDL levels in Hispanics.	[62]
-3826A/G	Chinese (127 obese and 257 non-obese subjects).	Obesity.	No association.	[92]
-3826A/G	Swedish (292 obese and 481 non-obese women).	IR and obesity.	No association.	[93]
-3826A/G	Czech (295 DM2 patients, 113 offspring of DM2 patients, and 120 healthy adults).	Anthropometric parameters, lipid profile, and blood glucose.	No association with DM2. In the offspring of DM2 patients, the A/G genotype was associated with higher BMI and subcutaneous fat mass compared with A/A carriers.	[75]

-3826A/G	Spanish (159 obese and 154 non-obese subjects).	MetS related-traits.	Within the obese group, the G allele was associated with greater BMI, greater percentage of body fat and higher DBP and SBP values than A/A carriers.	[76]
-3826A/G	Spanish (160 men and 172 women with and without obesity).	BMI, WHR, insulin, blood glucose, leptin, and lipid profile.	The G allele was more frequent in obese than in non-obese women (0.31 *vs*. 0.17, P = 0.008).	[77]
-3826A/G	Polish (118 obese subjects).	Lipid profile, blood glucose, insulin, and leptin.	G/G genotype carriers had higher triglyceride levels and decreased HDL and insulin levels than A allele carriers.	[78]
-3826A/G	German (154 obese and 154 non-obese subjects).	Obesity.	No association.	[94]
-3826 A/G	379 Danish subjects.	Obesity, WHR, IR, blood glucose, and lipid profile.	No association.	[95]
-3826A/G	Finnish (70 DM2 patients and 123 non-diabetic subjects), in a 10-year follow-up.	BMI, blood glucose, insulin, and BP.	No association.	[96]
-3826A/G	Turkish (271 obese and non-obese subjects).	BMI, BP, blood glucose, and lipid profile.	G/G genotype carriers showed BMI-associated increases of cholesterol levels which were more marked than in A allele carriers.	[80]
-3826A/G	214 Japanese men.	BMI and IR.	BMI was higher in subjects with the G allele *vs*.	[81]

			those without it.	
-3826A/G	Indian (89 DM2 patients and 100 non-diabetic controls).	DM2.	No association.	[97]
-3826A/G	251 Japanese men.	BMI, blood glucose, and lipid profile.	Men with the A/G genotype had higher BMI than those with the A/A genotype.	[83]
-3826A/G	Japanese (182 postmenopausal and 99 premenopausal women), in a 4-year follow-up.	BMI and lipid profile.	In the premenopausal women, G allele carriers had higher BMI than A/A genotype carriers. In the postmenopausal women, the 4-year change in triglyceride levels was higher in G allele carriers than in non-carriers.	[84]
-3826A/G	Korean (190 obese subjects).	Lipid, blood glucose, and BP.	The G allele was associated with higher DBP and LDL levels and with lower HDL levels compared with A/A genotype carriers.	[85]
-3826A/G	Swedish (674 obese and 311 non-obese subjects).	Obesity.	No association.	[102]
-3826A/G	Finish (170 obese women). Treatment with low-calorie diet, in a 12-week follow-up.	Weight loss and RMR.	No association.	[98]
-3826A/G	Australian (526 obese or overweight women).	BMI, DM2, blood glucose, lipid profile, insulin.	The G allele was associated with higher BMI and glucose levels than A/A genotype carriers.	[82]

-3826A/G	French (238 morbidly obese and 91 non-obese subjects).	Obesity and weight gain.	The G allele was associated with high weight gain during adult life (OR = 1.4, P = 0.02).	[87]
-3826 A/G	Finish (170 obese subjects).	RMR.	No association.	[99]
-3826 A/G	German (236 morbidly obese and 198 non-obese subjects).	Obesity and DM2.	No association.	[100]
-3826 A/G	1020 German subjects.	BMI, DM2, blood glucose, and lipid profile.	No association.	[101]
-3826A/G -412A/C	367 Korean women.	Body fat distribution.	-3826G and -412C alleles were individually associated with larger areas of abdominal subcutaneous fat. The [GC] haplotype enhanced the significance of this association.	[90]
-3826A/G Ala64Thr	162 German subjects.	BMI and WHR.	The 64Thr allele was significantly associated with higher WHR.	[71]
-3826A/G -112A/C	Japanese (93 DM2 patients).	DM2-related clinical characteristics.	IR and hepatic lipid content were significantly greater in -112C allele carriers than in non-carriers.	[73]
Met229Leu -112A/C	Japanese (320 DM2 patients and 250 non-diabetic controls).	DM2.	Leu229 and -112C allele frequencies were higher in DM2 patients than in the control group.	[79]
-1176A/G	387 Korean women.	BMI, WHR, percentage of	WHR, body fat mass and percentage of body fat	[72]

		body fat.	were significantly higher in G allele carriers compared to A/A genotype carriers.	
Met229Leu Ala64Thr	German (293 obese and 134 non-obese children)	Obesity.	Thr/Thr genotype was associated with risk for obesity.	[40]
-3826A/G -1776A/G Ala64Thr	Korean (453 overweight women).	Body fat distribution.	The [GAA] haplotype was associated with decreased abdominal fat tissue area, body fat mass and WHR.	[86]
-3826A/G -112A/C Met229Leu	Indian (812 DM2 patients and 990 non-diabetic subjects).	DM2.	Association between the -3826A/-112A /Met229 haplotype and risk for DM2 (OR = 1.82, P = 0.009).	[91]

AIRg = acute insulin response to glucose; BMI = body mass index; DBP = diastolic blood pressure; SBP = systolic blood pressure; DM2 = type 2 diabetes mellitus; IR = insulin resistance; MetS = metabolic syndrome; WHR = waist-to-hip ratio.

Figure 1

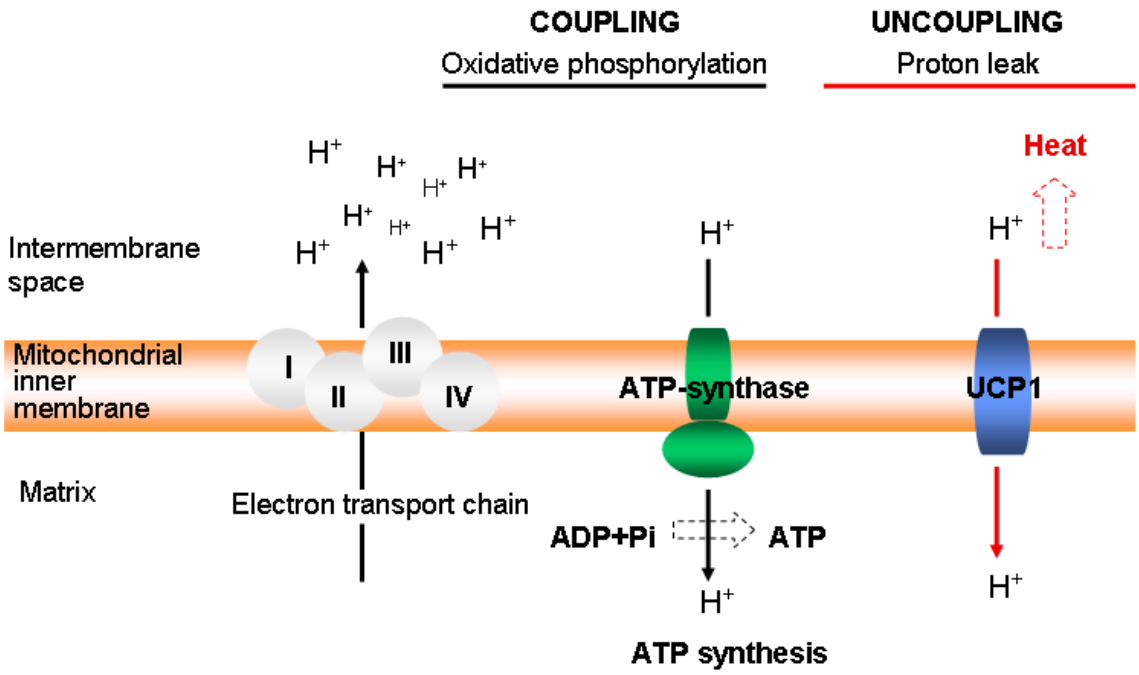

Figure 1 UCP1 location and function in the mitochondrial respiratory chain (MRC). Numbers I-IV corresponds to MRC complexes. ATP-synthase is the fifth complex of MRC. During respiration, protons are pumped by MRC complexes and a proton gradient is generated. The energy of the proton gradient drives the synthesis of ATP by the ATP-synthase complex. UCP1 catalyzes a regulated re-entry of protons into the matrix, uncoupling MRC and, consequently, reducing ATP synthesis and generating heat.

Figure 2

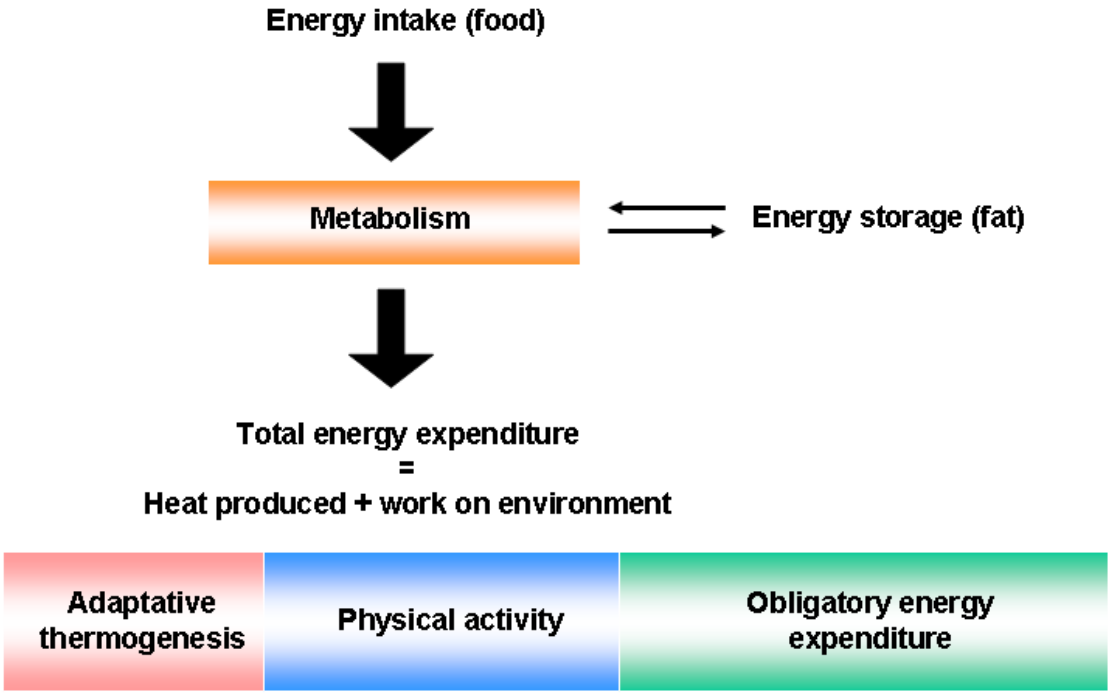

Figure 2 A thermodynamic perspective of energy expenditure. Energy enters into a body as food and leaves it as heat and "work". Energy can also be obtained from adipose stores. Total energy expenditure can be divided into: obligatory energy expenditure or resting metabolic rate, measured under resting conditions and required for functioning of cells; energy expenditure resulting from physical activity (variable); and energy expenditure attributed to adaptive thermogenesis (variable, regulated by the brain), which is defined as non-shivering heat production in response to environmental temperature or diet. Adaptive thermogenesis mainly occurs in brown adipocytes mitochondria and skeletal muscles. Adapted from Lowell and Spiegelman [24].

Figure 3

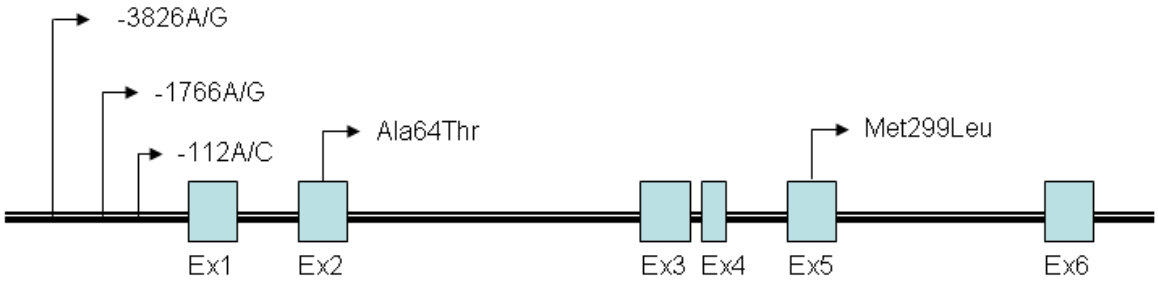

Figure 3 Map of *UCP1 locus* on chromosome 4 (region 4q28-q31). The six exons (boxes) are numbered from left to right according to the transcriptional region. The vertical arrows show the main common polymorphisms associated with obesity or type 2 diabetes mellitus. Adapted from Jia *et al*. [10].

PARTE II

Artigo Original

ASSOCIATION BETWEEN THE -3826A/G POLYMORPHISM IN THE UNCOUPLING PROTEIN 1 (*UCP1*) GENE ON DIABETIC RETINOPATHY AND *UCP1* GENE EXPRESSION IN HUMAN RETINA

ASSOCIAÇÃO ENTRE O POLIMORFISMO -3826A/G NO GENE DA PROTEÍNA DESACOPLADORA 1 (UCP1) E A RETINOPATIA DIABÉTICA E A EXPRESSÃO GÊNICA DA UCP1 NA RETINA HUMANA

Association between the -3826A/G polymorphism in the uncoupling protein 1 (*UCP1*) gene and diabetic retinopathy and *UCP1* gene expression in human retina

Short title: *UCP1* gene expression in human retina.

Letícia A. Brondani[1,3], Bianca M. de Souza[1,3], Guilherme C. K. Duarte[1], Lúcia M. Kliemann[2], Alexandre S. Marcon[4], Jorge L. Gross[1,3], Luís H. Canani[1,3], Daisy Crispim[1,3]

1 - Endocrinology Division, 2 - Pathology Service, Hospital de Clínicas de Porto Alegre; 3 – Post-Graduation Program in Medical Sciences: Endocrinology, Universidade Federal do Rio Grande do Sul; 4 - Cornea Bank, Hospital Santa Casa de Misericórdia de Porto Alegre. Porto Alegre, RS, Brazil.

Corresponding author:

Dr. Daisy Crispim. Rua Ramiro Barcelos 2350; prédio 12; 4° andar. CEP 90035-003 Porto Alegre, RS, Brazil. Phone number: +55 51 33598318. Fax number: +55 51 33598777. E-mail: daisy_crispim@hotmail.com

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Objective **-** Uncoupling protein 1 (UCP1) decreases the reactive oxygen species (ROS) formation by mitochondria. ROS overproduction is related to diabetic retinopathy (DR), a chronic complication of diabetes mellitus. Therefore, *UCP1* is a candidate gene for the development of DR. Here, we investigated the effects of the *UCP1* -3826A/G polymorphism on risk of DR and *UCP1* gene expression in human retina. In addition, *MnSOD2* gene expression was also investigated in retinal samples according to -3826A/G genotypes.

Research design and methods – Frequencies of the -3826A/G polymorphism were analyzed in 257 type 1 diabetes patients (154 with DR and 103 without DR), and in 166 cadaveric cornea donors. In a subset of 107 retinal samples differentiated according to -3826A/G genotypes, *UCP1* and *MnSOD2* gene expressions were measured by RT-qPCR.

Results – In the type 1 diabetes 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, -3826G allele carriers (A/G + G/G) had a higher *UCP1* gene expression than A/A carriers (1.1 ± 1.5 *vs*. 0.5 ± 0.9 n fold; P = 0.034). Also, G allele carriers showed increased *MnSOD2* expression (P = 0.031).

Conclusions – This study suggests that the -3826A/G polymorphism is associated with DR in type 1 diabetes patients. This is the first report that shows *UCP1* expression in human retina, and indicates that the -3826A/G polymorphism influences its expression. Possibly, *MnSOD2* expression might influence the UCP1 effect in the protection against oxidative stress.

Keywords: *UCP1* gene expression, -3826A/G polymorphism, human retina, diabetic retinopathy.

Diabetic retinopathy (DR) is a common sight-threatening microvascular complication affecting patients with diabetes mellitus and it represents a major cause of new cases of blindness in adults (1). Although the risk of developing this complication increases with poor glycemic control, arterial hypertension and long-term duration of diabetes, its occurrence is also influenced by genetic factors (2). Several studies show 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 (UCP) 1 is a member of an anion-carrier protein family and is located in the inner mitochondrial membrane (6). UCP1 uncouples substrate oxidation from ATP synthesis, thereby dissipating the membrane potential energy and consequently decreasing ATP production by mitochondrial respiratory chain (7, 8). This uncoupling then leads to important roles in energy expenditure regulation, cold- and diet-induced thermogenesis, and in decreasing ROS formation by mitochondria (9, 10).

Mitochondria are the main source of superoxide production and this makes them the target of direct attack of ROS (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 causes a large stimulation of hydrogen peroxide (H_2O_2) production. Therefore, uncoupling of the mitochondrial proton gradient by UCP1 has been suggested to have a natural antioxidant effect (13). Manganese superoxide dismutase (MnSOD) catalyzes the breakdown of superoxide into H_2O_2 scavenging superoxide, and, because of its mitochondrial localization, this enzyme is considered as the first line of defense against oxidative stress (4).

Until a few years ago, it was believed that UCP1 was expressed exclusively in brown adipose tissue; but it was recently reported that UCP1 expression was also

observed in other tissues and organs, such as white adipose tissue, pancreatic islets, skeletal muscle, 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 ROS damage derived from glucotoxicity, suggesting a protective role of UCP1 in the pathogenesis of DR. Therefore, *UCP1* is a candidate gene for the development of DR.

The -3826A/G polymorphism is located in the promoter region of the *UCP1* gene, and it seems to be associated with type 2 diabetes, body mass index (BMI), obesity or other obesity-related parameters (20-25). However, only two studies evaluated the association between this polymorphism and DR, showing inconclusive results (26, 27). Therefore, in the present study we investigated whether the *UCP1* -3826A/G polymorphism is associated with DR in type 1 diabetes patients, and if this polymorphism has an effect in the *UCP1* gene expression in human retina isolated from cadaveric cornea donors. In addition, *MnSOD2* gene expression was also investigated in retinal samples according to different -3826A/G genotypes.

RESEARCH DESIGN AND METHODS

Type 1 diabetes samples and phenotype measurements

The case-control study designed to investigate whether the *UCP1* -3826A/G polymorphism is associated with DR was carried out in 257 unrelated type 1 diabetes patients from the out-patient clinic of Hospital de Clínicas de Porto Alegre (Rio Grande do Sul, Brazil). Patients were considered to have type 1 diabetes if hyperglycemia was diagnosed before the age of 40 years, its control required insulin treatment within 1 year of diagnosis, and this treatment

could not be interrupted thereafter (28). Cases were defined by the presence of DR (n = 154). Patients without any degree of DR and with known type 1 diabetes duration of at least 10 years were considered as controls (n = 103).

All patients were of European ancestry (mostly descendants of Portuguese, Spanish, Italians and Germans). The ethnic group was defined on the basis of self-classification and subjective classification (skin color, nose and lip shapes, hair texture, and family ancestry). A standard questionnaire was used to collect information about age, age at diabetes diagnosis and drug treatment, and all patients underwent physical and laboratory evaluations. They were weighed without shoes and in light outdoor clothes and had their height measured. BMI was calculated as weight (kg)/height^2 (meters). Blood pressure (BP) was measured twice after a 5-min rest in the sitting position using a mercury sphygmomanometer (Korotkoff phases I and V). The mean value of two measurements was used to calculate systolic and diastolic BP. Hypertension was defined as BP levels ≥ 140/90 mmHg, or if the patient was taking antihypertensive drugs.

Assessment of DR was performed in all patients by an experienced ophthalmologist using fundoscopy through dilated pupils. DR was classified as 'absent DR' (no fundus abnormalities), 'nonproliferative DR' (microaneurysms, haemorrhage, and hard exudates) or presence of 'proliferative DR' (newly formed blood vessels and/or growth of fibrous tissue into the vitreous cavity) (29). The DR classification was based on the most severe degree of retinopathy in the worst affected eye. We have previously described an excellent agreement of DR classification (95.3%) carried out by different trained ophthalmologists from our group (30). In this study, a single ophthalmologist, unaware of the patients' clinical data, classified all the subjects. The diagnosis of diabetic nephropathy (DN) was based on the albumin excretion rate (AER) in at least two out of three consecutive 24-h timed or random spot sterile

urine collections in a 6-month period. Patients were classified as having normoalbuminuria (AER < 20 μg/min or <17 mg/L), microalbuminuria (AER 20–200 μg/min or 17–174 mg/L) or macroalbuminuria (AER > 200 μg/min or >174 mg/L).

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

The protocol was approved by the Hospital ethical committees, and all patients gave their written informed consent.

Cornea donor's samples and phenotype measurements

For evaluating the *UCP1* and *MnSOD2* gene expression according to 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), and collected at two Porto Alegre (RS) hospitals, namely Hospital de Clínicas de Porto Alegre and Hospital Santa Casa de Misericórdia. A standard questionnaire was used to collect information from medical records about age, gender, presence of arterial hypertension and diabetes, occurrence of other diseases and cause of death.

After enucleation and separation of corneas for donation, retinas were visually separated from the remaining intraocular structures, snap-frozen in liquid nitrogen and stored

at -80°C until analysis. Blood samples were also collected 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 analyzed polymorphism.

The protocol of this study was also approved by the Hospital Ethics committees, and relatives of all donors gave their written informed consent authorizing the use of the retinas.

Genotyping

DNA was extracted from peripheral blood leukocytes using a standardized salting-out procedure. The -3826A/G polymorphism (rs1800592) in the promoter region of the *UCP1* gene was determined by digesting polymerase chain reaction (PCR) products with the enzyme *Bcl*I (Invitrogen Life Technologies, CA, USA), as previously described (31) and using primers depicted in **Table 1**. Digestion fragments were resolved on 2% agarose gels containing GelRed^{TM} Nucleic Acid Gel Stain (Biotium Inc., CA, USA) and visualized under ultraviolet illuminations.

RNA isolation

Retinal tissues (250 mg) were homogenized in phenol-guanidine isothiocyanate (Invitrogen Life Technologies, CA, USA). RNA was extracted with chloroform and precipitated with isopropanol by centrifugation (12,000 x g) at 4°C. RNA pellet was washed twice with 75% ethanol and ressuspended in 10-50 μL of diethylpyrocarbonate treated water. Concentrations of isolated RNAs were assessed using NANODROP 2000 spectrophotometer (Thermo Scientific Inc., DE, USA).

Quantification of *UCP1* and *MnSOD*2 gene expressions by real-time PCR

Reverse transcription real time-PCR was performed in two separate reactions: first, RNA was reverse transcribed into cDNA, then cDNA was amplified by real-time PCR. 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 oligo $(dT)_{12-18}$ method.

Real-time PCR experiments were done in a 7500 Fast Real-Time PCR System thermal cycler (Applied Biosystems) using SYBER® Green dye [32]. Primers and probes for *UCP1*, *MnSOD2* and *β-actin* were designed using Primer Express 3.0 Software (Applied Biosytems) and are depicted in **Table 1**. PCR reactions were performed using 10 µL of 2x Fast SYBER® Green Master Mix (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 20s, followed by 50 cycles of 95°C for 30s and 60°C for 30s. The specificity of the RT-PCR was determined using melting curve analyses.

Quantification of the *UCP1* and *MnSOD2* mRNA were performed by relative quantification using the comparative C_T method (33), and human *β-actin* as an endogenous control. Validation assays were done by amplification of the target (*MnSOD2* or *UCP1*) and control (*β-actin*) genes, separately, using serial dilutions of a cDNA sample. Both target and control genes presented equal efficiencies of amplification. The comparative C_T method calculates changes in gene expression as relative fold difference (n fold) between an experimental and an external calibrator sample (33).

Statistical analyses

Allele frequencies were determined by gene counting, and departures from the Hardy-Weinberg equilibrium (HWE) were verified using χ^2 tests. Comparisons of allelic and genotypic frequencies among groups of subjects were evaluated using χ^2 test. Clinical and laboratory characteristics and mRNA abundance were compared between groups by using unpaired Student's t-test, One-way ANOVA or χ^2, as appropriate. Variables with normal distribution are presented as mean ± 2 standard deviations (SD) or percentage. Variables with skewed distribution were logarithmically transformed before analyses and are presented as median (minimum – maximum values) or median ± standard error (SE).

The magnitude of association between *UCP1* -3826A/G genotypes and DR was estimated using odds ratio (OR) test with 95% CI. Multivariate logistic regression analysis was performed to assess the independent association between the *UCP1* -3826A/G polymorphism and DR (dependent variable) as well as to control for possible confounding factors whenever a statistically significant association was found in the univariate analyses. DM duration was not included as an independent variable in this analysis because the control group (without DR) was selected based on this characteristic.

Pearson's correlation tests were used to assess the correlation between *UCP1* and *MnSOD2* expression or between *UCP1* or *MnSOD2* expression and age. Multiple linear regression analysis was performed with *UCP1* or *MnSOD2* mRNAs (logarithmic) as dependent variables and age, gender, diabetes diagnosis and presence of *UCP1* -3826G allele as independent variables. $P < 0.05$ was considered statistically significant. These statistical analyses were done with SPSS 18.0 (Chicago, IL, USA).

RESULTS

Association study between the *UCP1* -3826A/G polymorphism and diabetic retinopathy

The main clinical features of type 1 diabetes patients stratified according to the presence or absence of DR are depicted in **Table 2**. Type 1 diabetes patients with DR differed from control patients for diabetes duration, age, serum total cholesterol, triglycerides, creatinine, systolic and diastolic BP levels and occurrence of DN.

Table 3 shows the distribution of the *UCP1* -3826A/G polymorphism in type 1 diabetes patients grouped according to the presence of DR. Genotypic frequencies of this polymorphism were in agreement with those predicted by the HWE in both case and control groups (P = 0.230 and P = 0.126, respectively). Interestingly, type 1 diabetes patients with DR presented a higher frequency of the -3826G allele than type 1 diabetes patients without DR (P = 0.029). Likewise, the percentage of type 1 diabetes patients that presented the G allele in homozygosis was significantly higher in the case group than in the control group (P = 0.009). Moreover, multivariate analysis, adjusting for age, presence of arterial hypertension and serum creatinine, confirmed that the -3826 G/G genotype is an independent risk factor for DR (**Table 3**). It is worth mentioning that the magnitude of this association was similar in patients with proliferative DR or nonproliferative DR (data not shown).

UCP1 and *MnSOD2* gene expression in human retina from cornea donors 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. The mean

age was 56.5 ± 15.3 years, males comprised 58.4% (n = 97) of the sample, and 39.8% (n = 66) of all patients had arterial hypertension. Diabetes was diagnosed in 9,0% (n = 15) of all patients. Diabetic patients differed from non-diabetic subjects regarding 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). Both diabetic patients and non-diabetic subjects were similar regarding gender (data not shown). The genotypic 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).

The mean ± SD *UCP1* mRNA concentration in the whole retinal tissue group was 0.926 ± 1.35 n fold. No significant difference was observed when *UCP1* gene expression was analyzed by gender (men: 1.07 ± 1.41 *vs*. women: 0.84 ± 1.29 n fold; P = 0.381) or hypertension status (normotensives: 1.0 ± 1.23 *vs*. hypertensives: 0.74 ± 1.24 n fold; P = 0.423). *UCP1* gene expression did not correlate with age (r = 0.067, P = 0.462), and it was not statistically different between cornea donors with or without diabetes (1.02 ± 1.42 *vs*. 0.96 ± 1.15 n fold; P = 0.740).

UCP1 gene expressions in retinal samples stratified according to different genotypes or alleles of the -3826A/G polymorphism are depicted in **Figure 1.** *UCP1* gene expression did not differ statistically among different genotypes of this polymorphism (P = 0.068; **Figure 1A**). Nevertheless, we observed that G allele carriers (A/G + G/G) presented a higher *UCP1* gene expression than A/A genotype carriers (1.10 ± 1.5 *vs*. 0.51 ± 0.99 n fold; P = 0.018). In the linear regression analysis, the presence of the G allele remained significantly associated with *UCP1* gene expression after controlling for age, gender and diabetes diagnosis (standardized coefficient β for the presence of the G allele = 0.22; P = 0.034).

The mean ± SD *MnSOD2* gene expression in the whole retinal tissue sample was -0.82 ± 1.04 n fold. No significant difference was observed when *MnSOD2* gene expression was analyzed by gender (men: -1.0 ± 1.2 *vs*. women: -0.8 ± 1.2 n fold; P = 0.393), hypertension status (normotensives: -0.7 ± 1.4 *vs*. hypertensives: -1.0 ± 1.1 n fold; P = 0.652) or diabetes diagnosis (non-diabetic: -0.6 ± 0.9 *vs*. diabetic 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 correlated weakly with *UCP1* mRNA concentrations (r = 0.29, P = 0.015).

MnSOD2 gene expression in retinal samples stratified according to different genotypes and alleles of the -3826A/G polymorphism are depicted in **Figure 2.** *MnSOD2* gene expression seemed to be higher in both A/G and G/G genotype carriers when compared with A/A genotype subjects (P = 0.004; **Figure 2A**); however, after Tukey's post-hoc test, only the A/G genotype group reached conventional statistical significance (P = 0.006). Furthermore, G allele carriers (A/G + G/G) showed higher *MnSOD2* gene expression than A/A genotype carriers (P = 0.001; **Figure 2B**). In the linear regression analysis, the G allele remained independently associated with increased *MnSOD2* gene expression after controlling for age, sex and diabetes diagnosis (standardized coefficient β for the presence of G allele = 0.215; P = 0.031).

DISCUSSION

In this study, we investigated the frequencies of the -3826A/G polymorphism in the promoter region of the *UCP1* gene in type 1 diabetes patients divided according to the presence of DR. The G/G genotype was significantly associated with an increased risk for DR after adjusting for age, presence of arterial hypertension and serum creatinine. Notwithstanding the

recognized role of UCP1 in the protection against oxidative stress (8), and that oxidative stress is one of the major contributors to the accelerated loss of retinal capillary cells in diabetes (34), only two other studies analyzed the association between the -3826A/G polymorphism and DR (26, 27). Rudofsky *et al.* (27) studied the association between different polymorphisms in the UCP genes and diabetic complications in 227 type 1 diabetes patients from Germany. No effect of the *UCP1* -3826A/G polymorphism on DR, DN or diabetic neuropathy was found. Nevertheless, the same study reported that patients carrying the *UCP2* -866G allele or the *UCP3* -55T allele had a reduced prevalence of diabetic neuropathy (*UCP2*: OR 0.44, P = 0.007; *UCP3*: OR 0.48, P = 0.031) compared with patients carrying the -866A or -55C alleles (27). In addition, in a sample of type 2 diabetes patients from Germany, Rudofsky *et al.* (26) did not observe any association between the *UCP1* -3826A/G polymorphism and DR, DN or diabetic neuropathy. It is worth noting that a recent study from our group indicated that the 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 diabetes patients (35), further indicating that UCPs might have an important role in the pathogenesis of DR.

It is well known that functional polymorphisms can influence gene expression and regulate the final quantity of protein in a given tissue. Accordingly, in the present study, we reported that retinal samples from cornea donors carrying the -3826G allele showed *UCP1* gene expression twice as higher as that presented by A/A genotype carriers. On the other hand, Esterbauer *et al.* (36) demonstrated that the -3826G allele was associated with a lower *UCP1* mRNA expression in intraperitoneal adipose tissue of obese individuals when compared with A/A genotype carriers. Moreover, Rose *et al*. (37) 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 that containing the -3826G/-3737A haplotype. No activity was observed for the -3826G/-3737C haplotype in basal conditions (37).

A possible explanation for the contrasting results mentioned above is that the -3826A/G polymorphism might be involved in putative binding sites for specific transcription factors, and that the 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 already demonstrated for the -866G/A polymorphism in the *UCP2* promoter sequence (38). This hypothesis is reinforced by the knowledge that the -3826A/G polymorphism is located in proximity of 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 seem to mediate strong drug-dependent transcriptional activation of the *UCP1* gene (39). Accordingly, Rose *et al*. (37), using the MatInspector Online Software, found a putative retinoic acid response element (from positions -3842 to -3826), which included the -3826A/G polymorphism, in the *UCP1* promoter region. 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 (37). Interestingly, different *UCP1* haplotypes constituted by the -3826A/G and -3737C/A polymorphisms responded differently after stimuli with progesterone or estradiol (37). 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 influenced by interactions with other *UCP1* polymorphisms or other genes (37).

Echtay *et al*. (40) have proposed a simple feedback cycle in which mitochondrial ROS overproduction acutely and chronically increases proton conductance through effects on UCP1-3, which consequently decrease superoxide production by the mitochondrial respiratory chain. Besides, it is well known that retinal mitochondria become dysfunctional in diabetes and the production of superoxide radicals is increased (41, 42). In this context, the inhibition of oxidative stress through overexpression of *MnSOD2* gene also prevents retinal cells from undergoing the accelerated apoptosis that precedes the onset of DR in diabetic mice (42). Thus, the protection of mitochondrial DNA from glucose-induced oxidative damage is one of the plausible mechanisms by which MnSOD2 ameliorates the development of DR (42). Cui *et al*. (19) showed that increased mitochondrial ROS production could be induced by high glucose concentrations. At high levels of glucose, endothelial cells from bovine retina increase UCP1, UCP2 and MnSOD2 expression to accommodate ROS production compensatively. Nevertheless, this compensative mechanism disappeared when glucose concentration was too high (30 mM), suggesting that UCPs and MnSOD could exert compensative actions against oxidative stress only to a certain extent (19). In the present study, *MnSOD2* mRNA concentrations positively correlated with *UCP1* mRNA concentrations in human retina from cornea donors, and those subjects carrying the *UCP1* -3826G allele showed higher *MnSOD2* gene expression compared to A/A genotype carriers. Thus, our findings suggest that MnSOD levels could influence *UCP1* gene expression, possibly because MnSOD is the major scavenger of mitochondrial superoxide and, as already mentioned, this free radical directly activates *UCP1* gene expression (40, 43).

In conclusion, the data presented here indicated that the *UCP1* -3826G/G genotype is associated with an increased risk to DR in type 1 diabetes patients. Furthermore, the mutated G allele was associated with increased *UCP1* and *MnSOD2* mRNA concentrations in human retina from cornea donors. To our knowledge, these are the first data showing *UCP1* gene expression in human retina. So, based on these results and in the study of Cui *et al*. (19) on bovine retina, we therefore hypothesized that the increased *UCP1* and *MnSOD2* gene expression observed in G allele carriers might be a compensatory mechanism in response to a possible elevated ROS production in retina. However, in a glucotoxicity context, such as occurring in some type 1 diabetes patients, the G/G genotype might no longer be able to compensating ROS overproduction and, consequently, could predispose their carriers to the development of DR. Likely, interactions between UCP1 and MnSOD2 proteins in retinal cells as well as different hormonal stimuli on tissue-specific transcription factors may influence the *UCP1* effect in the protection against ROS and the risk of DR development. However, further functional studies will be necessary to confirm the association between the -3826A/G polymorphism and DR, and also to elucidate how these interactions occur. In addition, therapeutic strategies to counteract ROS through reinforcing complementary action of UCPs and MnSOD2 should be explored.

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TABLE 1

Primer and probe sequences used for genotyping of *UCP1* gene polymorphisms or expression analyses

	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 and R = reverse primer. * Primers were designed using published human gene sequences and the Primer Express 3.0 Software (Applied Biosystems), and they were projected to target two consecutive exons of a gene in order to prevent the amplification of any contaminating genomic DNA.

TABLE 2

Clinical and laboratory characteristics of patients with type 1 diabetes according to the presence of diabetic retinopathy (cases) or absence of diabetic retinopathy (controls)

	Cases	Controls	P*
n	153	104	
Gender (% male)	42.3	51.0	0.203
Duration of diabetes (years)	20.3 ± 7.1	18.1 ± 8.5	0.036
Age (years)	33.8 ± 14.7	38.9 ± 12.4	0.025
BMI (kg/m^2)	23.6 ± 4.9	22.9 ± 5.1	0.362
HDL cholesterol (mmol/l)	1.49 ± 0.49	1.56 ± 0.46	0.339
Cholesterol (mmol/l)	5.0 ± 1.44	4.58 ± 1.04	0.015
Triglycerides (mmol/l)	1.0 (0.35 – 6.27)	0.71 (0.33 – 7.44)	0.006
Creatinine (µmol/l)	88.4 (44.2 – 981.2)	83.9 (35.4 – 707.2)	<0.001
GHb (%)	8.92 ± 2.07	9.13 ± 7.09	0.749
Systolic blood pressure (mmHg)	129.6 ± 21.6	116.9 ± 17.8	< 0.001
Diastolic blood pressure (mmHg)	81.0 ± 13.2	75.3 ± 10.7	< 0.001
Hypertension (%)	25	44.4	0,003
Diabetic nephropathy (%)	82.9	17.1	< 0.001

Data are presented as mean ± SD or %. The control group is constituted by patients without any degree of diabetic retinopathy and with at least 10 years of DM. The case group is composed of patients with DR. * P values were computed by χ^2 or Student´s t-test, as appropriate. *n* = sample number; BMI = body mass index. GHb = glycated haemoglobin.

TABLE 3

Genotypic and allelic distributions of the *UCP1* -3826A/G polymorphism in diabetic patients according to the presence of diabetic retinopathy

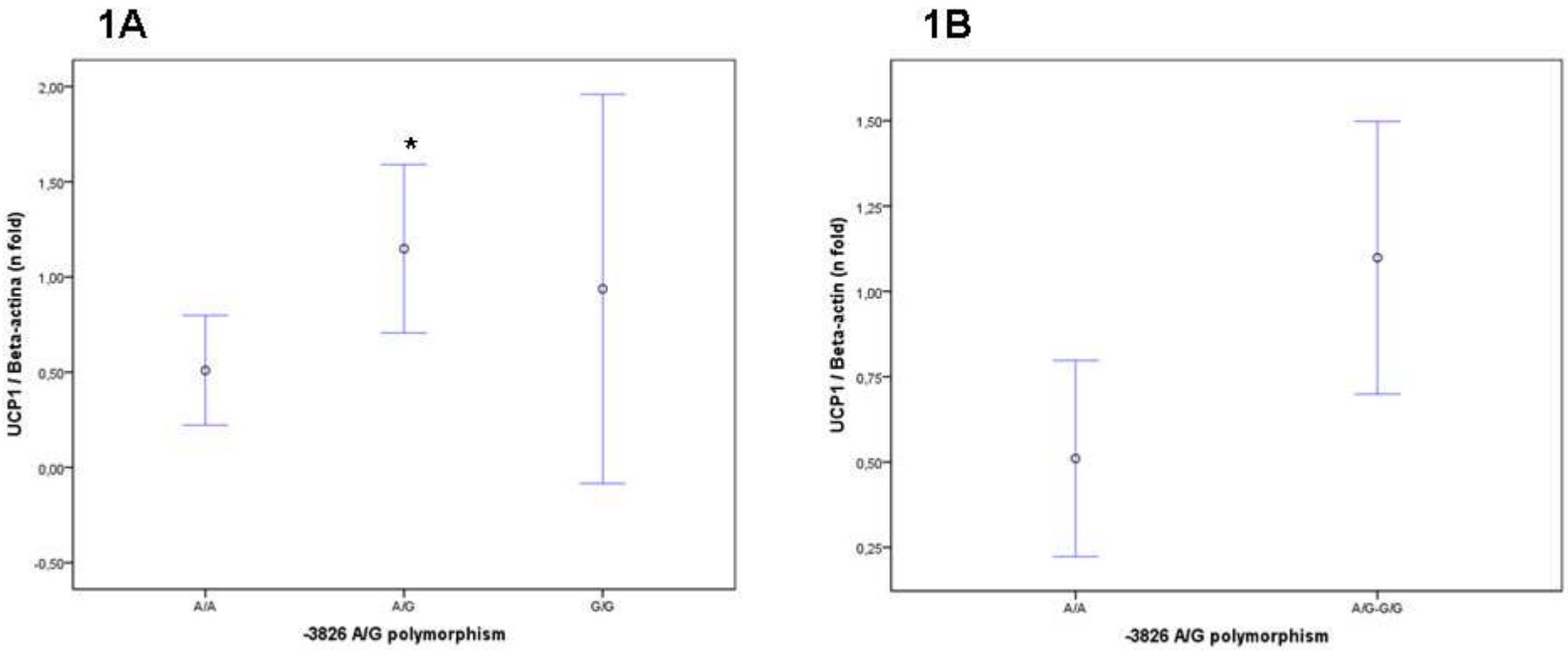
Genotypes	Cases (n = 154)	Controls (n = 103)	P*	Unadjusted OR (95% CI); P†	Adjusted OR (95% CI); P‡
A/A	57 (37.0)	45 (43.7)	0.012	1	1
A/G	67 (43.5)	51 (49.5)		1.037 (0.608 – 1.770); 0.894	1.186 (0.628 – 2.241); 0.599
G/G	30 (19.5)	7 (6.8)		3.383 (1.361 – 8.412); 0.009	3.503 (1.040 - 11.80); 0.043
Alleles					
A	0.590	0.690	0.029	-	-
G	0.410	0.310		-	-

Data are presented as number (%) or proportion. The control group is constituted by patients without any degree of diabetic retinopathy and with at least 10 years of DM. The case group is composed of patients with DR. * P values were computed by χ^2 tests comparing 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.

FIG. 1 *UCP1* mRNA expression in human retinal samples. **A)** *UCP1* gene expression in samples stratified according to different -3826A/G genotypes. P = 0.068 (One-way ANOVA test). * P = 0.057 in relation to the A/A genotype group (Tukey post-hoc test). **B)** *UCP1* gene expression in samples stratified according to the presence of the G allele (AA *vs*. A/G+G/G). P = 0.018 (Student's t-test). Data are represented as n fold differences to the calibrator sample ($\Delta\Delta C_T$ method), and are presented as mean ± 2 SE.

FIG. 2 *MnSOD2* mRNA expression in human retinal samples. **A)** *MnSOD2* gene expression in samples stratified according to different -3826A/G genotypes. P = 0.004 (One-way ANOVA test). * P = 0.006 in relation to the A/A genotype group (Tukey post-hoc test). **B)** *MnSOD2* gene expression in samples stratified according to the presence of the G allele (AA *vs*. A/G+G/G). P = 0.001 (Student's t-test). Values are represented as n fold differences to the calibrator sample ($\Delta\Delta C_T$ method), and are presented as mean ± 2 SE.

Figure 1


1A
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1,00
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-0,50
UCP1 / Beta-actina (n fold)
*
A/A
A/G
G/G
-3826 A/G polymorphism
1B
1,50
1,25
1,00
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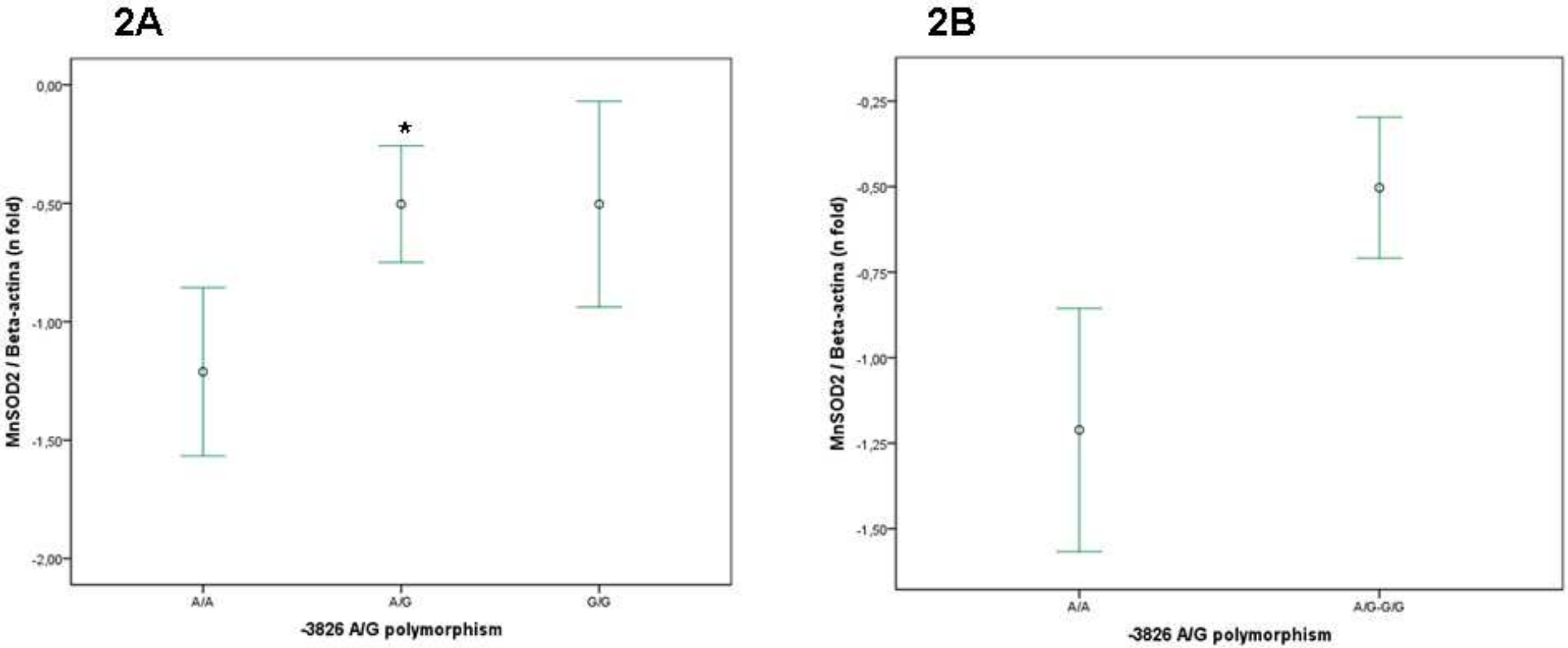
UCP1 / Beta-actin (n fold)
A/A
A/G-G/G
-3826 A/G polymorphism

Figure 2


2A
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MnSOD2 / Beta-actina (n fold)
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A/A
A/G
G/G
-3826 A/G polymorphism
2B
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-0,50
-0,75
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-1,25
-1,50
MnSOD2 / Beta-actina (n fold)
A/A
A/G-G/G
-3826 A/G polymorphism