UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS MÉDICAS:

ENDOCRINOLOGIA

EFEITO DO HAPLÓTIPO -866A / 55VAL / INS CONSTITUÍDO POR TRÊS POLIMORFISMOS NO GENE DA PROTEÍNA DESACOPLADORA 2 (*UCP2*) NA EXPRESSÃO DESTE GENE NA RETINA HUMANA

DISSERTAÇÃO DE MESTRADO

BIANCA MARMONTEL DE SOUZA

Porto Alegre, fevereiro de 2011

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS MÉDICAS: ENDOCRINOLOGIA

EFEITO DO HAPLÓTIPO -866A / 55VAL / INS CONSTITUÍDO POR TRÊS POLIMORFISMOS NO GENE DA PROTEÍNA DESACOPLADORA 2 (*UCP2*) NA EXPRESSÃO DESTE GENE NA RETINA HUMANA

BIANCA MARMONTEL DE SOUZA

Orientadora: Profa. Dra. Daisy Crispim Moreira

Co-orientador: Prof. Dr. Luís Henrique Canani

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, fevereiro de 2011

Dedico essa dissertação à minha avó

Maria Carmem Rosa de Souza, minha

Mestra de vida, minha saudade e meu

amor eternamente!

“Se as coisas são inatingíveis...

ora!

não é motivo para não querê-las...

que triste os caminhos,

se não fora

a presença distante das estrelas”

Mário Quintana

AGRADECIMENTOS

Ao meu pai Silvério, minha mãe Flávia, meu irmão, minha irmã e a todos da minha família, não menos importantes neste feito. Agradeço pelo carinho, amor, cuidado e dedicação pelo estímulo dado às minhas escolhas e por todos os ensinamentos de vida.

À Dra. Daisy Crispim, minha orientadora, pela oportunidade, confiança, respeito, carinho, preocupação, valorização e principalmente pela dedicada e competente orientação.

Ao meu co-orientador, Dr. Luís Henrique Canani pela oportunidade e orientação.

Ao Dr. Jorge Gross, Dr. Alexandre Marcon, Dra. Lúcia Kliemann, Dra. Rosana Nothen e Fernando Pagnussato pela fundamental colaboração com este trabalho.

À Taís Assmann, pela dedicação e contribuição dada a este trabalho.

À Denise Sortica, pelo apoio e amizade.

A todos os colegas e amigos do Serviço de Endocrinologia, pelo ótimo ambiente de trabalho, companheirismo e momentos de descontração.

As pessoas que fazem parte do meu lar recente, meu irmão Haroldo e minha amiga Carolina, pela paciência, companheirismo, amizade e ensinamentos de vida.

A todos meus amigos, que assim como eu, lutam cada um em sua área, por uma vida em um mundo melhor.

Finalmente, agradeço a todos, que direta ou indiretamente colaboraram na elaboração deste trabalho.

Esta dissertação de mestrado segue o formato proposto pelo Programa de Pós-Graduação em Ciências Médicas: Endocrinologia, Metabolismo e Nutrição, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, sendo apresentada na forma de um artigo de revisão e um artigo original sobre o tema da dissertação.

- Artigo de revisão: "The role of the uncoupling protein 2 (UCP2) on the development of type 2 diabetes mellitus and its chronic complications" (submetido aos Arquivos Brasileiros de Endocrinologia e Metabologia).

- Artigo original: "The presence of the -866A / 55Val / Ins haplotype in the *uncoupling protein 2* (*UCP2*) gene is associated with decreased *UCP2* expression in human retinal tissue" (a ser submetido à revista Clinical Endocrinology).

SUMÁRIO

LISTA DE ABREVIATURAS

3`UTR	3' untranslated region
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
AU	Arbitrary units
BMI	Body mass index
DM	Diabetes mellitus
DM2	Type 2 diabetes mellitus
DN	Diabetic nephropathy
DPN	Diabetic peripheral neuropathy
DR	Diabetic retinopathy (Retinopatia diabética)
FADH2	Flavin adenine dinucleotide reduced
FFA	Free fatty acid
FOXA1	Forkhead box A1
GDP	Guanosine diphosphate
GLUT4	Glucose transporter type 4
GSH	Glutathione
H_2O_2	Hydrogen peroxide
HIF-1α	Hypoxia-inducible factor-1α
HNE	4-hydroxy-2-nonenal
HWE	Hardy-Weinberg equilibrium
IR	Insulin resistance
IRS-1	Insulin receptor-1
JNK	Jun N-terminal kinase

LD	Linkage disequilibrium
MAPK	Mitogen-activated protein kinase
MnSOD2	Manganese superoxide dismutase 2
MRC	Mitochondrial respiratory chain
mRNA	Messenger RNA
mtDNA	Mitochondrial deoxyribonucleic acid
NADH	Nicotinamide adenine dinucleotide reduced
NRF-1	Nuclear respiratory factor-1
O_2^-	Superoxide anion
OD	Odds ratio
OH	Hydroxyl radical
PAX6	Paired box gene 6
PDR	Proliferative diabetic retinopathy
PGC-1α	PPAR-gama coactivator-1
Pi	Inorganic phosphate
PI3K	Phosphoinositide 3-kinase
PPAR	Peroxisome proliferator-activated receptor
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SD	Standard deviation
SE	Standard error
SIRT1	Sirtuin 1
SREBP-1c	Sterol regulatory element binding protein-1c
UCP2	Uncoupling protein 2 (Proteína desacopladora 2)
UCPs	Uncoupling proteins

RESUMO

Está bem definido que fatores genéticos têm um papel importante no desenvolvimento do diabetes mellitus (DM) tipo 2 e de suas complicações crônicas. Sendo assim, grandes esforços têm sido feitos para identificar os genes associados com estas doenças. A proteína desacopladora 2 (UCP2) é expressa em diversos tecidos e atua na proteção contra o estresse oxidativo e na regulação da secreção de insulina pelas células-beta pancreáticas e do metabolismo dos ácidos graxos, mecanismos associados com o DM tipo 2, bem como com suas complicações crônicas. Dessa forma, o gene *UCP2* é um gene candidato ao desenvolvimento destas doenças. Recentemente, nós relatamos que o haplótipo -866A / 55Val / Ins, constituído pelos polimorfismos -866G/A, Ala55Val e Ins/Del no gene *UCP2*, foi associado com risco aumentado para retinopatia diabética (RD) proliferativa, a forma mais severa de RD, em pacientes com DM tipo 1 e 2.

No presente estudo, nós avaliamos os efeitos deste haplótipo, bem como dos polimorfismos que o constituem, na expressão do gene *UCP2* na retina humana. Nós também avaliamos a expressão do gene *MnSOD2*, o qual codifica uma enzima antioxidante, de acordo com os diferentes haplótipos e polimorfismos estudados no gene *UCP2*.

Este estudo transversal incluiu 188 doadores cadavéricos de córneas, todos não-diabéticos. Em um subgrupo de 91 amostras de retina diferenciadas de acordo com a presença do haplótipo mutado (-866A / 55Val / Ins) e dos alelos de risco dos três polimorfismos estudados, as concentrações dos RNAm da UCP2 e MnSOD2 foram avaliadas pela técnica de PCR em tempo real.

Portadores do haplótipo mutado (homozigotos + heterozigotos) apresentaram uma menor expressão do RNAm da UCP2 do que portadores do haplótipo de referência (8,4 ± 7,6 *vs*. 18,8 ± 23,7 unidades arbitrárias; p = 0,046). De acordo com este resultado, os níveis do RNAm da UCP2 também foram menores em portadores dos alelos de risco -866A e 55Val quando comparados aos portadores dos outros genótipos destes polimorfismos (p = 0,010 e p = 0,003, respectivamente). A expressão do gene *UCP2* não diferiu significativamente entre portadores do alelo de risco Ins e portadores do genótipo Del/Del (p = 0,556). Indivíduos portadores do haplótipo heterozigoto (-866A 55Val Ins / -866G 55Ala Del), bem como heterozigotos para os três polimorfismos estudados, apresentaram uma expressão gênica aumentada de *MnSOD2* (p < 0,050).

Nossos resultados indicam que a presença do haplótipo mutado -866A / 55Val / Ins está associada com expressão gênica diminuída de UCP2 na retina humana. Possivelmente, os níveis de expressão gênica da MnSOD2 na retina podem influenciar o efeito da UCP2 na patogênese da RD proliferativa. Entretanto, estudos funcionais adicionais serão necessários para confirmar se mudanças na expressão do gene *UCP2* também ocasionam mudanças nos níveis de proteína.

ABSTRACT

It is well established that genetic factors play an important role in the development of type 2 diabetes mellitus (DM) and its chronic complications. Therefore, grate efforts have been made to identify genes associated with these diseases. Uncoupling protein 2 (UCP2) is expressed in several tissues, and acts in the protection against oxidative stress and in the regulation of both insulin secretion by pancreatic beta-cells and fatty acid metabolism, mechanisms associated with type 2 DM pathogenesis as wells as with its chronic complications. So, *UCP2* gene is a candidate gene to the development of these diseases. Recently, we reported that the -866A / 55Val / Ins haplotype (constituted by the -866G/A, Ala55Val and Ins/Del polymorphisms in the *UCP2* gene) was associated with increased risk for proliferative diabetic retinopathy (DR), which is the most severe form of DR, in both type 1 and type 2 DM patients.

In the present study, we evaluated the effects of this haplotype as well as the polymorphisms which constitute it on *UCP2* gene expression in human retina. We also evaluated *MnSOD2* gene expression, which codifies an antioxidant enzyme, accordingly to different haplotypes and studied polymorphisms in the *UCP2* gene.

This cross-sectional study included 188 cadaveric cornea donors, all nondiabetic. In a subset of 91 retinal samples differentiated according to the presence of the mutated haplotype (-866A / 55Val / Ins) and risk alleles of the three studied polymorphisms, *UCP2* and *MnSOD2* mRNA concentrations were measured by real-time PCR technique.

Mutated haplotype carriers (homozygous + heterozygous) presented a lower *UCP2* mRNA expression than reference haplotype carriers (8.4 ± 7.6 *vs*. 18.8 ± 23.7 arbitrary units; P = 0.046). Accordingly, *UCP2* mRNA levels were also lower in -866A and 55Val allele carriers when compared with carriers of other genotypes of these

polymorphisms (P = 0.010 and P = 0.003, respectively). *UCP2* gene expression did not differ between Ins allele carriers and Del/Del carries (P = 0.556). Subjects carrying the heterozygous haplotype (-866A 55Val Ins / -866G 55Ala Del) as well as heterozygous for the three studied polymorphisms showed increased *MnSOD2* gene expression (P < 0.050).

Our results indicate that the presence of the -866A / 55Val / Ins haplotype is associated with decreased *UCP2* mRNA expression in human retina. Possibly, *MnSOD2* expression levels in retina might influence the UCP2 effect on the pathogenesis of proliferative DR. However, further functional studies will be necessary to confirm if changes on *UCP2* gene expression also generate changes on protein levels.

Parte I

Artigo de revisão

THE ROLE OF THE UNCOUPLING PROTEIN 2 (UCP2) ON THE DEVELOPMENT OF TYPE 2 DIABETES MELLITUS AND ITS CHRONIC COMPLICATIONS

PAPEL DA PROTEÍNA DESACOPLADORA 2 (UCP2) NO DESENVOLVIMENTO DO DIABETES MELLITUS TIPO 2 E DE SUAS COMPLICAÇÕES CRÔNICAS

THE ROLE OF THE UNCOUPLING PROTEIN 2 (UCP2) ON THE DEVELOPMENT OF TYPE 2 DIABETES MELLITUS AND ITS CHRONIC COMPLICATIONS

PAPEL DA PROTEÍNA DESACOPLADORA 2 (UCP2) NO DESENVOLVIMENTO DO DIABETES MELLITUS TIPO 2 E DE SUAS COMPLICAÇÕES CRÔNICAS

Bianca Marmontel de Souza[1,3]

Taís Silveira Assmann[1]

Lúcia Kliemann[2]

Jorge Luiz Gross[1,3]

Luís Henrique 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. Porto Alegre, RS, Brazil.

Correspondence:

Dr. Daisy Crispim

Rua Ramiro Barcelos 2350; prédio 12; 4° andar. CEP 90035-003, Porto Alegre, RS, Brasil. E-mail: daisy_crispim@hotmail.com

Short title: Role of UCP2 in type 2 diabetes.

ABSTRACT

It is well established that genetic factors play an important role in the development of type 2 diabetes mellitus (DM2) and its chronic complications, and that genetically susceptible subjects can develop this disease after being exposed to environmental risk factors. Therefore, great efforts have been made to identify genes associated with DM2. Uncoupling protein 2 (UCP2) is expressed in several tissues, and acts in the protection against oxidative stress, in the negative regulation of insulin secretion by beta-cells, and in the fatty acid metabolism, mechanisms associated with DM2 pathogenesis as wells as with its chronic complications. So, *UCP2* gene is a candidate gene to the development of these diseases. Indeed, several studies have been reported that three common polymorphisms in the *UCP2* gene are associated with DM2 and/or obesity. Only a few studies investigated these polymorphisms in relation to diabetic chronic complications, obtaining inconclusive results.

Keywords: UCP2, type 2 diabetes mellitus, diabetic retinopathy, diabetic nephropathy, DNA polymorphisms, haplotype.

RESUMO

Está bem estabelecido que fatores genéticos têm um papel importante no desenvolvimento do diabetes mellitus tipo 2 (DM2), bem como de suas complicações crônicas, e que indivíduos geneticamente suscetíveis podem desenvolver esta doença após exposição a fatores de risco ambientais. Sendo assim, grandes esforços têm sido feitos para a identificação de genes associados ao DM2. A proteína desacopladora 2 (UCP2) é expressa em diversos tecidos e atua na proteção contra o estresse oxidativo, na regulação negativa da secreção de insulina pelas células-beta e no metabolismo dos ácidos graxos, mecanismos associados tanto à patogênese do DM2 como a suas complicações crônicas. Portanto, o gene *UCP2* é um gene candidato para o desenvolvimento dessas doenças. De fato, diversos estudos têm relatado que três polimorfismos comuns no gene *UCP2* estão associados ao DM2 e/ou obesidade. Apenas poucos estudos investigaram esses polimorfismos em relação às complicações crônicas do diabetes, obtendo resultados pouco conclusivos.

Descritores: UCP2, diabetes mellitus tipo 2, retinopatia diabética, nefropatia diabética, polimorfismos de DNA, haplótipo.

INTRODUCTION

It is estimated that 7.6% of the Brazilian population have diabetes mellitus (DM) (1). This disease constitutes a serious public health problem because of its high prevalence, increased morbidity and mortality rates, and the economic and social repercussions arising from the impact of its chronic complications, which compromise both quality of life and productivity of the affected subjects and have high costs for their treatment (2).

Type 2 DM (DM2) accounts for 90-95% of DM cases worldwide, usually occurs in obese subjects over 40 years of age, and is characterized by hyperglycemia due to a combination of insulin resistance (IR) and an inadequate compensatory insulin secretory response (2). The chronic hyperglycemia is associated with long-term structural damage, dysfunction, and failure of several organs and tissues, which consequently lead to the development of diabetic chronic complications (2). Among the diabetic chronic microvascular complications, diabetic retinopathy (DR) is the leading cause of blindness in adults (3), diabetic nephropathy (DN) is the most common cause of end-stage chronic renal disease and kidney transplants in many countries (4), and diabetic peripheral neuropathy (DPN) is responsible for 50-75% of non-traumatic lower limb amputations (5).

Uncoupling protein 2 (UCP2) plays important roles in decreasing reactive oxygen species (ROS) formation by mitochondria, regulation of insulin secretion by pancreatic beta-cells and regulation of free fatty acid (FFA) metabolism, which are mechanisms directly associated with the pathogenesis of DM or its chronic complications (6). Thus, the aim of this paper was to review the role of UCP2 in relation to the development of DM2 or its chronic microvascular complications (DR, DN and DPN).

THE MITOCHONDRIAL RESPIRATORY CHAIN (MRC)

Mitochondria are essential organelles in all eukaryotic cells and regulate a number of key processes vital for cell survival and function, including energy production, redox control, calcium homeostasis, and certain metabolic and biosynthetic pathways. In addition, mitochondria are the main source of ROS and often play an essential role in physiological cell death mechanisms (7).

The main source of cell energy is the synthesis of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and inorganic phosphate (Pi) by oxidative phosphorylation carried out in the mitochondrial respiratory chain (MRC) (8). The MRC is located in the mitochondrial inner membrane, and has five multienzymatic complexes and two proteins responsible for electron transport, cytochrome *c* and coenzyme Q (**Figure 1**). Oxidative phosphorylation involves the coupling of electron transport, through the MRC, to the active pumping of protons across the mitochondrial inner membrane and ATP formation (7).

The oxidation of reduced nutrient molecules, such as carbohydrates, lipids, and proteins, through cellular metabolism generates electrons in the form of reduced hydrogen carriers (NADH and FADH2), which are referred as reduced cofactors. These reduced cofactors donate electrons to the MRC. The movement of electrons through the components of the 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 chain complexes. This produces an electrochemical potential difference across the inner membrane, known as proton-motive force, Δp, consisting mostly of an electrochemical gradient (membrane potential) and a small chemical gradient (pH difference). The energy that is conserved in the proton gradient across the inner membrane is used by complex V (F_1F_0-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 formation 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 (7, 8).

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 ($O_2^{-\bullet}$), the product of an one-electron reduction of oxygen, is the precursor of most ROS and a mediator in oxidative chain reactions (7). 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, it may form the highly reactive hydroxyl radical ($^{\bullet}OH$) (7). 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, structural carbohydrates, and lipids. It is well established that there is a strong positive correlation between mitochondrial inner membrane potential and ROS production. At high membrane potentials, even a small increase in membrane potential gives rises to a large stimulation of H_2O_2 production. Therefore, "mild uncoupling", i.e., a small decrease in membrane potential, has been suggested to have a natural antioxidant effect (9).

MITOCHONDRIAL DYSFUNCTION AND TYPE 2 DIABETES MELLITUS

In the past decade, several clinical and experimental studies have strengthened the hypothesis that mitochondrial dysfunction, including the reduction in mitochondrial number and oxidative phosphorylation efficiency, is closely associated with DM2 (10).

Microarray analyzes performed in skeletal muscle biopsies of DM2 patients and nondiabetic subjects from different populations have shown that PGC-1α (PPAR-γ coactivator-1), the master control in the regulation of mitochondrial biogenesis, and NRF-1 (nuclear respiratory factor-1) as well as their downstream target genes in oxidative metabolism are decreased in DM2 patients (11, 12). These findings were confirmed by enzymatic studies that reported that DM2 patients, or their insulin-resistant offspring, show a decline in mitochondrial oxidative activity (13), in insulin-stimulated ATP synthesis and fatty acid oxidation (14, 15), and also in the number of mitochondria present in skeletal muscle as compared with age-matched insulin-sensitive subjects (13).

Mitochondrial dysfunction and increased ROS production are involved in reduced lipid oxidation and increased lipid content within muscle cells, exacerbating IR (16). The decrease in fatty acid oxidation produces increased intracellular levels of fatty acetyl-CoA and diacylglycerol. These molecules activate protein kinase C, p38/MAPK (mitogen-activated protein kinase) and JNK (jun N-terminal kinase), which, in turn, trigger a cascade of serines, leading to increased phosphorylation of serine residues of the insulin receptor-1 (IRS-1). An increase in serine phosphorylation blockades the tyrosine residue phosphorylation of the IRS-1, which inhibits its capacity to phosphorylate the downstream target phosphoinositide 3-kinase (PI3K). The inactivation of PI3K impairs the translocation of glucose transporter type 4 (GLUT4) to the plasma membrane, and, consequently, leads to a decrease in the glucose uptake of muscle cells upon insulin stimulation (16).

Furthermore, the decreased ATP synthesis resulted from mitochondrial dysfunction also plays an important role in decreasing insulin secretion in patients with

DM2, since it may impair the regulation of K^+ and Ca^+ channels present in the cell membrane, and thereby inhibits the exocytosis of insulin granules in beta-cells (10, 17).

UNCOUPLING PROTEINS (UCPs)

UCPs 1, 2, 3, 4 and 5 are members of an anion carrier protein family located in the mitochondrial inner membrane (6). These proteins have similarities in their structures, but a differential tissue expression in mammals. The original UCP, UCP1, is mainly expressed in brown adipose tissue, which is responsible for thermogenesis in newborns (18). It was recently shown that under certain physiological conditions such as hyperglycemia, UCP1 is also expressed in skeletal muscle, white adipose tissue, retinal cells and pancreatic beta-cells (19, 20). UCP2 is widely distributed, whereas UCP3 is mainly restricted to the skeletal muscle, and UCP4 and UCP5/BMCP1 are mainly expressed in the brain (6, 21).

Over the last few years, several studies have shown that UCPs decrease metabolic efficiency by dissociating substrate oxidation in the 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 conversion of ADP to ATP, and consequently decreasing ATP production (6, 18, 21). This uncoupling effect then leads to homologue- and tissue-specific functions such as thermogenesis (UCP1), regulation of FFAs metabolism and transport (UCP2 and UCP3), decreasing ROS formation (UCP1-3 and UCP5/BMCP1), and regulation of ATP-dependent processes (UCP2) (21, 22).

UNCOUPLING PROTEIN 2 (UCP2)

In 1997, Fleury *et al.* (23) cloned and sequenced a gene homologous to *UCP1* gene, later called *UCP2*. *UCP2* gene covers a 6.3 kb region on chromosome 11 (region 11q13), and has eight exons and seven introns (**Figure 2**). In humans, region 11q13 is linked to basal metabolic rate and body fat percentage (24). The transcriptional gene unit is constituted by two non-coding exons followed by six exons that encode the 308 amino acids of the protein (23). Human UCP2 share 57% amino acid-sequence identity with human UCP1, and it is 71% identical to human UCP3 (8). In addition, the amino acid sequence of human UCP2 is 95% identical to mouse UCP2 (23).

UCP2 is expressed in a wide range of tissues and cell types, including brown and white adipose tissues, skeletal muscle, heart, kidneys, liver, lungs, spleen, thymus, bone marrow, macrophages, brain, gastrointestinal tract, pancreatic islets and retinal cells (6, 8, 19, 21, 23, 25). Although *UCP2* is well expressed in many tissues at the mRNA level, it would seem that the UCP2 protein level is not simply proportional to the mRNA concentration. For example, whereas *UCP2* mRNA is found in heart, skeletal muscle, and brown adipose tissue, no UCP2 protein could be detected in these tissues (26). Thus, changes in the amount of *UCP2* mRNA do not always reflect the expression of the protein itself, which may be explained by a differential translational regulation of *UCP2* gene among tissues, providing a mechanism by which its expression can be rapidly and strongly induced under stress conditions (26). PPAR (peroxisome proliferator-activated receptor)-α, PPAR-γ, PGC-1α and SREBP-1c (sterol regulatory element binding protein-1c) transcription factors increase the *UCP2* gene expression, while SIRT1 (sirtuin 1) and FOXA1 (forkhead box A1) factors inhibit its expression (27). UCP2 is also translationally regulated by an inhibitory upstream open reading frame (ORF), which, when mutated, results in maximal *UCP2* mRNA translation (28).

Glutamine, an amino acid that has been implicated in insulin secretion pathway, overcomes ORF inhibition and increases UCP2 efficiency (28).

It is proposed that both UCP2 and UCP3 only mildly uncouple respiration, slightly dissipating the membrane potential energy, and thus slightly decreasing ATP production (8, 22) (**Figure 1)**. Nonetheless, unlike UCP1, UCP2 and UCP3 only uncouple the MRC after suitable induction by cold, ROS (particularly superoxide), ubiquinone, high levels of glucose and/or non-esterified fatty acids, high impact exercise, sepsis and hyperthyroidism. Their activities are inhibited by purine, such as ATP and guanosine diphosphate (GDP), and by interleukin-1β (29-31). As already mentioned, MRC uncoupling generated by UCP2 leads to protection from excessive ROS production, while it also seems to be associated with inhibition of insulin secretion by beta-cells and regulation of FFAs metabolism and transport (18, 22, 27, 32).

The "mild uncoupling" of MRC due to UCP2 activity allows a more rapid electron flux through the mitochondrial inner membrane, thus reducing membrane potential and thereby decreasing ROS production (6, 29). Since even a "mild uncoupling" has a large effect on reducing ROS production, the hypothesis that UCP2 protects against oxidative stress has strong support and is now generally accepted (6). Accordingly, UCP2 knockout mice have elevated ROS production in macrophages (33) and pancreatic beta-cells (34). Moreover, rat beta-cells clonal line (INS-1E) genetically modified to overexpress the *UCP2* gene presented an increased survival after treatment with the free radical H_2O_2 (35). Likewise, beta-cells exposed to oxidative stress attempt to overcome the toxicity caused by H_2O_2 through UCP2 mRNA induction (30). More recently, it was demonstrated that endothelial cells from bovine retinal cells incubated with high glucose levels increase the UCP2 expression, which protects them from ROS

damage derived from glucotoxicity, and suggests a protective role of this protein also in the pathogenesis of DR or other diabetic chronic complications (19).

An important coupling signal between glucose sensing and insulin secretion by pancreatic beta-cells is the rise in the ATP/ADP ratio. Increases in the ATP/ADP ratio close the ATP-sensitive K^+ channel in the mitochondrial inner membrane. This causes membrane depolarization, opening of voltage-gated calcium channels, and the influx of Ca^{2+} into the cytosol of the beta-cells, which subsequently triggers the exocytosis of granules containing insulin. UCP2, by virtue of its proton-leak activity, decreases the generation of ATP from glucose metabolism in beta-cells, which consequently impairs the glucose-stimulated insulin secretion (6, 7, 22, 24). Accordingly, several studies have been confirmed that UCP2 acts as a negative regulator of insulin secretion. For example, *UCP2* overexpression in rat islets totally suppresses the glucose-stimulated insulin secretion (36). Additionally, pancreatic islets from UCP2 knockout mice ($UCP2^{-/-}$) have increased insulin secretion in response to glucose and these mice have higher blood insulin and lower blood glucose than wildtype mice (32). Interestingly, double mutant $Lep^{ob/ob}$ / $UCP2^{-/-}$ mice (i.e., obese and with the *UCP2* gene deleted) have improved beta-cell function independent of obesity (32).

FFAs are endogenous physiological regulators associated with increased UCP2 and UCP3 expression in a tissue-specific manner (22, 31). In preadipocyte cell lines, unsaturated fatty acids markedly induce UCP2 mRNA expression (37). Other cell lines derived from heart, liver and pancreatic islets also respond to the addition of different FFAs to the culture medium with an increase in UCP2 mRNA levels (31). FFAs seem to regulate the UCP2 and UCP3 expression probably via PPAR-α, PPARγ and SREBP-1c transcription factors (31).

Studies in proteoliposomes, isolated mitochondria and intact cells suggest that both UCP2 and UCP3 have a role in transporting FFAs or fatty acid hydroperoxides (30, 38). These proteins seem to export fatty acid anions outside of the mitochondrial matrix when a large excess of fatty acids is inside mitochondria, protecting cells from the oxidative damage caused by excessive fatty acid peroxidation (lipotoxicity), mainly polyunsaturated fatty acids of the phospholipidic membrane. These fatty acids can bind to the superoxide produced by mitochondria, which converts them to 4-hydroxy-2-nonenal (HNE), an UCP2 activator. After activation by HNE, UCP2 is able of transporting protons across the mitochondrial inner membrane, thus increasing the uncoupling of the MRC, and consequently decreasing ROS formation (18, 29).

Beta-cell dysfunction is an event secondary to prolonged exposure of these cells to elevated glucose (glucotoxicity) and lipids (lipotoxicity) levels, conditions often associated with DM2. The exact mechanisms by which glucolipotoxicity triggers beta-cell dysfunction are not well known. However, evidences indicate that ROS production plays an important role in these mechanisms (6). As already mentioned, it is known that beta-cells exposed for a prolonged time to glucolipotoxicity increase UCP2 expression as a form of protection against damage caused by oxidative stress (27). Besides, it is clear that beta-cells display low expression and activity of many of the enzymes involved in antioxidant defense. Thus, an antioxidant effect of UCP2 is of special importance in this cell type (35). However, increased UCP2 production leads to a decreasing in insulin secretion, predisposing to DM2 (6, 27, 29, 38). Therefore, the *UCP2* gene is a candidate gene for the development of DM2, since altered expression of this gene can explain some key defects involved in this disease or its chronic complications.

Polymorphisms in the *uncoupling protein 2* gene associated with type 2 diabetes mellitus

In a number of genetic studies, the relationship between the *UCP2* locus and susceptibility for DM2 or obesity have been investigated, with specific attention being paid to the -866G/A (rs659366) polymorphism in the promoter region (39); the Ala55Val (C/T; rs660339) polymorphism in the exon 4 (40); and the Ins/Del polymorphism, which is an insertion/deletion of 45bp in the 3' UTR region (3' *untranslated region*) of exon 8 (41). The results of these studies have been variable (**Table 1**): while some studies showed an association of one or more of these polymorphisms with obesity, reduced levels of insulin secretion by beta-cells, IR and/or DM2 (39, 42-50), other studies were unable to find any association between these polymorphisms and these characteristics (40, 41, 51-53).

The A allele of the -866G/A polymorphism has been reported to increase the UCP2 transcriptional activity in transfected INS-1E cells derived from rat beta-cells (41). However, data in human tissues have been conflicting, reporting either increased (41, 54, 55) or decreased (49, 56) *UCP2* mRNA levels to be associated with the -866A allele. Computational analyzes demonstrated that this polymorphism is involved in putative binding sites for specific transcription factors, such as PAX6 (paired box gene 6) and HIF-1α (hypoxia-inducible factor-1α) (54). Esterbauer *et al*. (54) hypothesized that preferential binding of some transcription factors to the G or A allele in the promoter sequence may confer tissue-specific advantages to either allele.

Nondiabetic subjects carrying the -866A allele have been shown to have a decreased insulin secretion response to intravenous and oral glucose and, therefore, to have an increased risk of developing DM2 (43, 45, 46, 48). Similarly, DM2 patients carrying the A allele have been shown to have significantly lower insulin secretion

during an intravenous glucose tolerance test and to require insulin therapy more frequently and earlier after diagnosis of the disease than patients carrying the G/G genotype (39). In addition, some studies also suggest that A allele carriers also seem to have a higher waist-to-hip ratio, increased risk of metabolic syndrome, and higher levels of plasma markers of oxidative stress (55).

The Ins/Del polymorphism is located in the 3' UTR region of the gene, only 158pb from the transcriptional stop codon, and it also appears to be functional because mRNA transcribed from the sequence containing the insertion allele displayed a shorter half-life in a foetal myoblast cell line than mRNA transcribed from the sequence containing the deletion allele (57). In the other hand, the Ala55Val (C/T) polymorphism causes a conservative amino acid change (alanina / valina) at position 55 of exon 4, and until now, there has been no evidence that it generates a functional change in the protein. Thus, considering that the Ala55Val (C/T) polymorphism is tightly linked to the -866G/A polymorphism (|D'| = 0.991) and partially linked to the Ins/Del polymorphism (|D'| = 0.879), it is probable that this polymorphism is not a real disease-causing variant, but could be simply reflecting the -866G/A and Ins/Del polymorphism effects (58).

Contrasting to the -866G/A polymorphism, for which most studies suggest that it actually contributes to the biological variation of insulin secretion and to the susceptibility to DM2, association studies regarding the effects of Ala55Val and Ins/Del polymorphisms on DM2 or associated characteristics have obtained more controversial results. Subjects carrying the Val/Val genotype of Ala55Val polymorphism seem to have an increased risk of developing DM2 and obesity, and a higher body mass index (BMI) than subject with Ala/Val or Ala/Ala genotypes (42, 47, 55, 59). However, other studies indicated that this polymorphism is not individually associated with BMI,

obesity, metabolic syndrome, DM2, and insulin secretion (40, 49, 52, 53, 55). Likewise, some studies did not found any individual association between the Ins/Del polymorphism and obesity, DM2 and/or insulin secretion (49-51, 55), while others studies reported the association between the insertion allele and obesity (41, 55).

Haplotype combinations constituted by *UCP2* gene polymorphisms have also been found to be associated with BMI, obesity, insulin secretion and IR (49, 50, 53). Wang *et al*. (49) reported that the G Val Ins / A Val Del haplotype (-866G/A, Ala55Val, and Ins/Del polymorphisms) is associated with higher BMI (P = 0.028), IR (P = 0.031) and higher plasma insulin levels (P = 0.029) in northern European Caucasians with and without DM2. Ochoa *et al*. (50) investigated, in a group of Spanish obese and non-obese children, the association of -866G/A and Ins/Del polymorphisms in the *UCP2* gene and -55C/T (rs1800849) in *UCP3* gene with obesity and IR. Although they observed no association of individual polymorphisms with these characteristics, the authors observed that the -866G Del -55T / -866G Del -55T haplotype was associated with increased risk for obesity (OR = 1.9, 95% CI 1.4 - 2.6) and IR (OR = 9.5, 95% CI 2.3 - 39.9).

Association between *UCP2* gene polymorphisms and diabetic chronic complications

One of the main mechanisms linking hyperglycemia to diabetic microvascular complications is the mitochondrial overproduction of ROS (3, 4). However, despite the recognized role of UCP2 in the protection against oxidative stress, only a few studies evaluated the association between *UCP2* gene polymorphisms and the occurrence of diabetic chronic complications.

Rudofsky *et al*. (60) observed that type 1 DM patients carrying the -866A allele had a significantly reduced prevalence of DPN when compared with patients

homozygous for the -866G allele (OR = 0.44, 95% CI 0.2 - 0.8, P = 0.007). Interestingly, these authors also reported that the effect on DPN protection was even more intense (OR = 0.28, 95% CI 0.1 - 0.7, P = 0.002) when the A allele of the -866G/A polymorphism occurs in a haplotype containing the T allele of -55C/T polymorphism in the *UCP3* gene. The same study did not report any association between the -866G/A polymorphism and DN or DR.

In addition, Rudofsky *et al*. (61) did not observe any association of the -866G/A polymorphism with DPN, DN or DR in DM2 patients from Germany. Lindholm *et al*. (57) reported that the Ins/Del polymorphism was not associated with micro- or macroalbuminuria in DM2 patients from Scandinavia. More recently, Crispim *et al*. (58) showed that the A Val Ins / A Val Ins haplotype (-866G/A, Ala55Val and Ins/Del polymorphisms) was associated with increased risk to proliferative DR, the most severe form of DR, in both DM2 patients (OR = 5.3, 95% CI 2.2 - 12.4, P < 0.00001) and type 1 DM patients (OR = 6.0, 95% CI 1.7 - 20.8, P = 0.005) from Rio Grande do Sul (Brazil).

CONCLUSIONS

DM2 and its chronic complications are multifactorial diseases associated with both genetic and environmental risk factors. The knowledge of factors associated with DM2 will allow us to better understand the disease and its chronic complications, and may provide us with more effective approaches to treatment and prevention. UCP2 plays important roles in decreasing ROS formation by mitochondria, negative regulation of insulin secretion by beta-cells, and regulation of FFAs metabolism. These mechanisms are associated with the pathogenesis of DM2 or its microvascular complications, and, in

fact, several studies strongly suggest that *UCP2* gene polymorphisms may contribute to the biological variation of insulin secretion and DM2 susceptibility.

ACKNOWLEDGMENTS

This study was partially supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnólogico (CNPq), and Fundo de Incentivo à Pesquisa e Eventos (FIFE) at Hospital de Clínicas de Porto Alegre. The authors thank Haroldo Paraguassú de Souza for the construction of Figure I of this paper.

Disclosure: The authors declare no conflict of interest.

REFERENCES

1. Malerbi D, Franco L. Multicenter study of the prevalence of diabetes mellitus and impaired glucose tolerance in the urban Brazilian population aged 30-69 yr. The Brazilian Cooperative Group on the Study of Diabetes Prevalence. Diabetes Care. 1992 Nov;15(11):1509-16.
2. American Diabetes Association. Diagnosis and classification of diabetes mellitus. Diabetes Care. 2010 Jan;33 Suppl 1:S62-9.
3. Fong D, Aiello L, Gardner T, King G, Blankenship G, Cavallerano J, et al. Retinopathy in diabetes. Diabetes Care. 2004 Jan;27 Suppl 1:S84-7.
4. Carpena M, Rados D, Sortica D, Souza B, Reis A, Canani L, et al. Genetics of diabetic nephropathy. Arq Bras Endocrinol Metabol. 2010 Mar;54(3):253-61.
5. Bloomgarden Z. Diabetic neuropathy. Diabetes Care. 2008 Mar;31(3):616-21.
6. Fisler J, Warden C. Uncoupling proteins, dietary fat and the metabolic syndrome. Nutr Metab (Lond). 2006;3:38.
7. Echtay K. Mitochondrial uncoupling proteins--what is their physiological role? Free Radic Biol Med. 2007 Nov;43(10):1351-71.
8. Dalgaard L, Pedersen O. Uncoupling proteins: functional characteristics and role in the pathogenesis of obesity and Type II diabetes. Diabetologia. 2001 Aug;44(8):946-65.
9. Papa S, Skulachev V. Reactive oxygen species, mitochondria, apoptosis and aging. Mol Cell Biochem. 1997 Sep;174(1-2):305-19.
10. Wang C, Wang C, Wei Y. Mitochondrial dysfunction in insulin insensitivity: implication of mitochondrial role in type 2 diabetes. Ann N Y Acad Sci. 2010 Jul;1201:157-65.
11. Mootha V, Lindgren C, Eriksson K, Subramanian A, Sihag S, Lehar J, et al. PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nat Genet. 2003 Jul;34(3):267-73.
12. Patti M, Butte A, Crunkhorn S, Cusi K, Berria R, Kashyap S, et al. Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1. Proc Natl Acad Sci U S A. 2003 Jul;100(14):8466-71.
13. Kelley D, He J, Menshikova E, Ritov V. Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. Diabetes. 2002 Oct;51(10):2944-50.
14. Petersen K, Dufour S, Shulman G. Decreased insulin-stimulated ATP synthesis and phosphate transport in muscle of insulin-resistant offspring of type 2 diabetic parents. PLoS Med. 2005 Sep;2(9):e233.
15. Petersen K, Dufour S, Befroy D, Garcia R, Shulman G. Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. N Engl J Med. 2004 Feb;350(7):664-71.
16. Lowell B, Shulman G. Mitochondrial dysfunction and type 2 diabetes. Science. 2005 Jan;307(5708):384-7.
17. Mulder H, Ling C. Mitochondrial dysfunction in pancreatic beta-cells in Type 2 diabetes. Mol Cell Endocrinol. 2009 Jan;297(1-2):34-40.
18. Nedergaard J, Ricquier D, Kozak L. Uncoupling proteins: current status and therapeutic prospects. EMBO Rep. 2005 Oct;6(10):917-21.
19. Cui Y, Xu X, Bi H, Zhu Q, Wu J, Xia X, et al. Expression modification of uncoupling proteins and MnSOD in retinal endothelial cells and pericytes induced by

high glucose: the role of reactive oxygen species in diabetic retinopathy. Exp Eye Res. 2006 Oct;83(4):807-16.
20. Sale M, Hsu F, Palmer N, Gordon C, Keene K, Borgerink H, et al. The uncoupling protein 1 gene, UCP1, is expressed in mammalian islet cells and associated with acute insulin response to glucose in African American families from the IRAS Family Study. BMC Endocr Disord. 2007;7:1.
21. Erlanson-Albertsson C. Uncoupling proteins--a new family of proteins with unknown function. Nutr Neurosci. 2002 Feb;5(1):1-11.
22. Chan C, Saleh M, Koshkin V, Wheeler M. Uncoupling protein 2 and islet function. Diabetes. 2004 Feb;53 Suppl 1:S136-42.
23. Fleury C, Neverova M, Collins S, Raimbault S, Champigny O, Levi-Meyrueis C, et al. Uncoupling protein-2: a novel gene linked to obesity and hyperinsulinemia. Nat Genet. 1997 Mar;15(3):269-72.
24. Krauss S, Zhang C, Lowell B. The mitochondrial uncoupling-protein homologues. Nat Rev Mol Cell Biol. 2005 Mar;6(3):248-61.
25. Barnstable C. Mitochondria and the regulation of free radical damage in the eye. J Ocul Biol Dis Infor. 2009 Sep;2(3):145-8.
26. Pecqueur C, Alves-Guerra M, Gelly C, Levi-Meyrueis C, Couplan E, Collins S, et al. Uncoupling protein 2, in vivo distribution, induction upon oxidative stress, and evidence for translational regulation. J Biol Chem. 2001 Mar;276(12):8705-12.
27. Affourtit C, Brand M. On the role of uncoupling protein-2 in pancreatic beta cells. Biochim Biophys Acta. 2008 2008 Jul-Aug;1777(7-8):973-9.
28. Azzu V, Jastroch M, Divakaruni AS, Brand MD. The regulation and turnover of mitochondrial uncoupling proteins. Biochim Biophys Acta. 2010 2010 Jun-Jul;1797(6-7):785-91.
29. Brand M, Affourtit C, Esteves T, Green K, Lambert A, Miwa S, et al. Mitochondrial superoxide: production, biological effects, and activation of uncoupling proteins. Free Radic Biol Med. 2004 Sep;37(6):755-67.
30. Esteves T, Brand M. The reactions catalysed by the mitochondrial uncoupling proteins UCP2 and UCP3. Biochim Biophys Acta. 2005 Aug;1709(1):35-44.
31. Thompson M, Kim D. Links between fatty acids and expression of UCP2 and UCP3 mRNAs. FEBS Lett. 2004 Jun;568(1-3):4-9.
32. Zhang C, Baffy G, Perret P, Krauss S, Peroni O, Grujic D, et al. Uncoupling protein-2 negatively regulates insulin secretion and is a major link between obesity, beta cell dysfunction, and type 2 diabetes. Cell. 2001 Jun;105(6):745-55.
33. Arsenijevic D, Onuma H, Pecqueur C, Raimbault S, Manning B, Miroux B, et al. Disruption of the uncoupling protein-2 gene in mice reveals a role in immunity and reactive oxygen species production. Nat Genet. 2000 Dec;26(4):435-9.
34. Krauss S, Zhang C, Scorrano L, Dalgaard L, St-Pierre J, Grey S, et al. Superoxide-mediated activation of uncoupling protein 2 causes pancreatic beta cell dysfunction. J Clin Invest. 2003 Dec;112(12):1831-42.
35. Li L, Skorpen F, Egeberg K, Jørgensen I, Grill V. Uncoupling protein-2 participates in cellular defense against oxidative stress in clonal beta-cells. Biochem Biophys Res Commun. 2001 Mar;282(1):273-7.
36. Chan C, MacDonald P, Saleh M, Johns D, Marbàn E, Wheeler M. Overexpression of uncoupling protein 2 inhibits glucose-stimulated insulin secretion from rat islets. Diabetes. 1999 Jul;48(7):1482-6.
37. Reilly J, Thompson M. Dietary fatty acids Up-regulate the expression of UCP2 in 3T3-L1 preadipocytes. Biochem Biophys Res Commun. 2000 Nov;277(3):541-5.

38. Azzu V, Brand M. The on-off switches of the mitochondrial uncoupling proteins. Trends Biochem Sci. 2010 May;35(5):298-307.
39. Sasahara M, Nishi M, Kawashima H, Ueda K, Sakagashira S, Furuta H, et al. Uncoupling protein 2 promoter polymorphism -866G/A affects its expression in beta-cells and modulates clinical profiles of Japanese type 2 diabetic patients. Diabetes. 2004 Feb;53(2):482-5.
40. Kubota T, Mori H, Tamori Y, Okazawa H, Fukuda T, Miki M, et al. Molecular screening of uncoupling protein 2 gene in patients with noninsulin-dependent diabetes mellitus or obesity. J Clin Endocrinol Metab. 1998 Aug;83(8):2800-4.
41. Cassell P, Neverova M, Janmohamed S, Uwakwe N, Qureshi A, McCarthy M, et al. An uncoupling protein 2 gene variant is associated with a raised body mass index but not Type II diabetes. Diabetologia. 1999 Jun;42(6):688-92.
42. Yu X, Jacobs DJ, Schreiner P, Gross M, Steffes M, Fornage M. The uncoupling protein 2 Ala55Val polymorphism is associated with diabetes mellitus: the CARDIA study. Clin Chem. 2005 Aug;51(8):1451-6.
43. D'Adamo M, Perego L, Cardellini M, Marini M, Frontoni S, Andreozzi F, et al. The -866A/A genotype in the promoter of the human uncoupling protein 2 gene is associated with insulin resistance and increased risk of type 2 diabetes. Diabetes. 2004 Jul;53(7):1905-10.
44. Bulotta A, Ludovico O, Coco A, Di Paola R, Quattrone A, Carella M, et al. The common -866G/A polymorphism in the promoter region of the UCP-2 gene is associated with reduced risk of type 2 diabetes in Caucasians from Italy. J Clin Endocrinol Metab. 2005 Feb;90(2):1176-80.
45. Sesti G, Cardellini M, Marini M, Frontoni S, D'Adamo M, Del Guerra S, et al. A common polymorphism in the promoter of UCP2 contributes to the variation in insulin secretion in glucose-tolerant subjects. Diabetes. 2003 May;52(5):1280-3.
46. Gable D, Stephens J, Cooper J, Miller G, Humphries S. Variation in the UCP2-UCP3 gene cluster predicts the development of type 2 diabetes in healthy middle-aged men. Diabetes. 2006 May;55(5):1504-11.
47. Xiu L, Weng J, Sui Y, Wang J, Yan J, Huang Z. [Common variants in beta 3-adrenergic-receptor and uncoupling protein-2 genes are associated with type 2 diabetes and obesity]. Zhonghua Yi Xue Za Zhi. 2004 Mar;84(5):375-9.
48. Krempler F, Esterbauer H, Weitgasser R, Ebenbichler C, Patsch J, Miller K, et al. A functional polymorphism in the promoter of UCP2 enhances obesity risk but reduces type 2 diabetes risk in obese middle-aged humans. Diabetes. 2002 Nov;51(11):3331-5.
49. Wang H, Chu W, Lu T, Hasstedt S, Kern P, Elbein S. Uncoupling protein-2 polymorphisms in type 2 diabetes, obesity, and insulin secretion. Am J Physiol Endocrinol Metab. 2004 Jan;286(1):E1-7.
50. Ochoa MC, Santos JL, Azcona C, Moreno-Aliaga MJ, Martínez-González MA, Martínez JA, et al. Association between obesity and insulin resistance with UCP2-UCP3 gene variants in Spanish children and adolescents. Mol Genet Metab. 2007 Dec;92(4):351-8.
51. Dalgaard L, Andersen G, Larsen L, Sørensen T, Andersen T, Drivsholm T, et al. Mutational analysis of the UCP2 core promoter and relationships of variants with obesity. Obes Res. 2003 Nov;11(11):1420-7.
52. Rosmond R, Bouchard C, Björntorp P. Lack of association between the uncoupling protein-2 Ala55Val gene polymorphism and phenotypic features of the Metabolic Syndrome. Biochim Biophys Acta. 2002 Nov;1588(2):103-5.

53. Hsu YH, Niu T, Song Y, Tinker L, Kuller LH, Liu S. Genetic variants in the UCP2-UCP3 gene cluster and risk of diabetes in the Women's Health Initiative Observational Study. Diabetes. 2008 Apr;57(4):1101-7.
54. Esterbauer H, Schneitler C, Oberkofler H, Ebenbichler C, Paulweber B, Sandhofer F, et al. A common polymorphism in the promoter of UCP2 is associated with decreased risk of obesity in middle-aged humans. Nat Genet. 2001 Jun;28(2):178-83.
55. Jia JJ, Zhang X, Ge CR, Jois M. The polymorphisms of UCP2 and UCP3 genes associated with fat metabolism, obesity and diabetes. Obes Rev. 2009 Sep;10(5):519-26.
56. Oberkofler H, Iglseder B, Klein K, Unger J, Haltmayer M, Krempler F, et al. Associations of the UCP2 gene locus with asymptomatic carotid atherosclerosis in middle-aged women. Arterioscler Thromb Vasc Biol. 2005 Mar;25(3):604-10.
57. Lindholm E, Klannemark M, Agardh E, Groop L, Agardh CD. Putative role of polymorphisms in UCP1-3 genes for diabetic nephropathy. J Diabetes Complications. 2004 2004 Mar-Apr;18(2):103-7.
58. Crispim D, Fagundes N, dos Santos K, Rheinheimer J, Bouças A, de Souza B, et al. Polymorphisms of the UCP2 gene are associated with proliferative diabetic retinopathy in patients with diabetes mellitus. Clin Endocrinol (Oxf). 2010 May;72(5):612-9.
59. Walder K, Norman RA, Hanson RL, Schrauwen P, Neverova M, Jenkinson CP, et al. Association between uncoupling protein polymorphisms (UCP2-UCP3) and energy metabolism/obesity in Pima indians. Hum Mol Genet. 1998 Sep;7(9):1431-5.
60. Rudofsky G, Schroedter A, Schlotterer A, Voron'ko OE, Schlimme M, Tafel J, et al. Functional polymorphisms of UCP2 and UCP3 are associated with a reduced prevalence of diabetic neuropathy in patients with type 1 diabetes. Diabetes Care. 2006 Jan;29(1):89-94.
61. Rudofsky G, Schrödter A, Voron'ko OE, Schlotterer A, Humpert PM, Tafel J, et al. Promoter polymorphisms of UCP1, UCP2, and UCP3 are not associated with diabetic microvascular complications in type 2 diabetes. Horm Metab Res. 2007 Apr;39(4):306-9.
62. Zee RY, Ridker PM, Chasman DI. Mitochondrial uncoupling protein gene cluster variation (UCP2-UCP3) and the risk of incident type 2 diabetes mellitus: the Women's Genome Health Study. Atherosclerosis. 2011 Jan;214(1):107-9.

Table 1. Association studies between -866G/A, Ala55Val and Ins/Del polymorphisms in the *UCP2* gene and type 2 diabetes mellitus.

Polymorphism	Population	Characteristics	Results	Refs.
-866G/A	Danish (749 obese and 816 non-obese).	BMI, body fat content and insulin levels.	No association.	(51)
-866G/A	Italian (483 DM2 patients and 565 controls). Non-diabetic offspring of DM2 patients.	DM2. Insulin sensitivity in non-diabetic offspring.	A/A genotype was associated with increased risk for DM2 in women (OR = 1.84, P = 0.037), but not in men. Association of the A/A genotype with decreased insulin sensitivity in non-diabetic offspring (P = 0.01).	(43)
-866G/A	301 healthy Italian Caucasians.	Insulin sensitivity.	Insulin secretion was higher in subjects carrying the A/A genotype than in G/G or A/G carriers (P <0.05).	(45)
-866G/A	Japanese (416 DM2 patients and 172 controls).	DM2 and insulin secretion levels.	No association with DM2. Association between the A allele and earlier DM2 onset (P = 0.042), increased frequency of insulin treatment (P = 0.0027), and lower insulin secretion levels (P = 0.016).	(39)
-866G/A	Italian (746 DM2 patients and 118 controls).	DM2.	The A/A genotype was associated with protection for DM2 (OR = 0.51, P = 0.003).	(44)
-866G/A	2936 healthy middle-aged English men.	DM2 incidence in a 15 years follow-up study.	A/A genotype was associated with increased risk of developing DM2 (HR = 1.94, P = 0.009).	(46)
-866G/A	Austrian (391 non-diabetic	DM2.	Genotype A/A associated with increased risk for DM2 (OR = 2.12,	(48)

	controls, 201 DM2 patients with obesity and 39 obese subjects).	Insulin sensitivity.	P = 0.035).	
Ala55Val	Chinese (173 DM2 patients, 119 obese subjects and 117 non-diabetics controls).	DM2 and obesity.	The Val/Val genotype was associated with increased risk for DM2 (OR = 4.62, P = 0.0001) and obesity (OR = 3.71, P = 0.001).	(47)
Ala55Val	3684 healthy American participants of the CARDIA Study.	DM2 incidence in a 15 years follow-up study.	The Val/Val genotype was associated with a higher incidence of DM2 (OR = 1.75, P = 0.020).	(42)
Ala55Val	Japanese (210 DM2 patients, 42 obese subjects and 218 non-diabetic controls).	DM2 and obesity.	No association.	(40)
Ala55Val	284 Swedish men.	Metabolic syndrome characteristics.	No association.	(52)
Ins/Del	Indians (76 DM2 patients and 335 controls).	DM2.	No association.	(41)
-866G/A and Ala55Val	Postmenopausal American women (1584 DM2 incident cases and 2198 controls).	DM2.	No association.	(53)

-866G/A and Ala55Val	American women (22.715 healthy Caucasian).	Risk of incident DM2 in a 13 years follow-up study.	No association.	(62)
-866G/A and Ins/Del	Spanish children (193 obese cases and 173 controls).	Obesity and IR.	Association of the -866G Del -55T / -866G Del -55T* haplotype with increased risk for obesity (OR = 1.95, 95% CI 1.43 - 2.65) and IR (OR = 9.54, 95% CI 2.28 - 39.9).	(50)
-866G/A, Ala55Val and Ins/Del	Caucasians from Northern Europe (131 DM2 patients and 118 controls).	DM2.	Association of the G Val Ins / A Val Del haplotype with higher BMI (P = 0.028), IR (P = 0.031), and triglycerides (P = 0.026) and plasma insulin levels (P = 0.029).	(49)

* -55 C/T polymorphism (rs1800849) in the *UCP3* gene. BMI = body mass index. DM2 = type 2 diabetes mellitus. HR = hazard ratio. IR = insulin resistance. OR = odds ratio. 95% CI = 95% confidence interval.

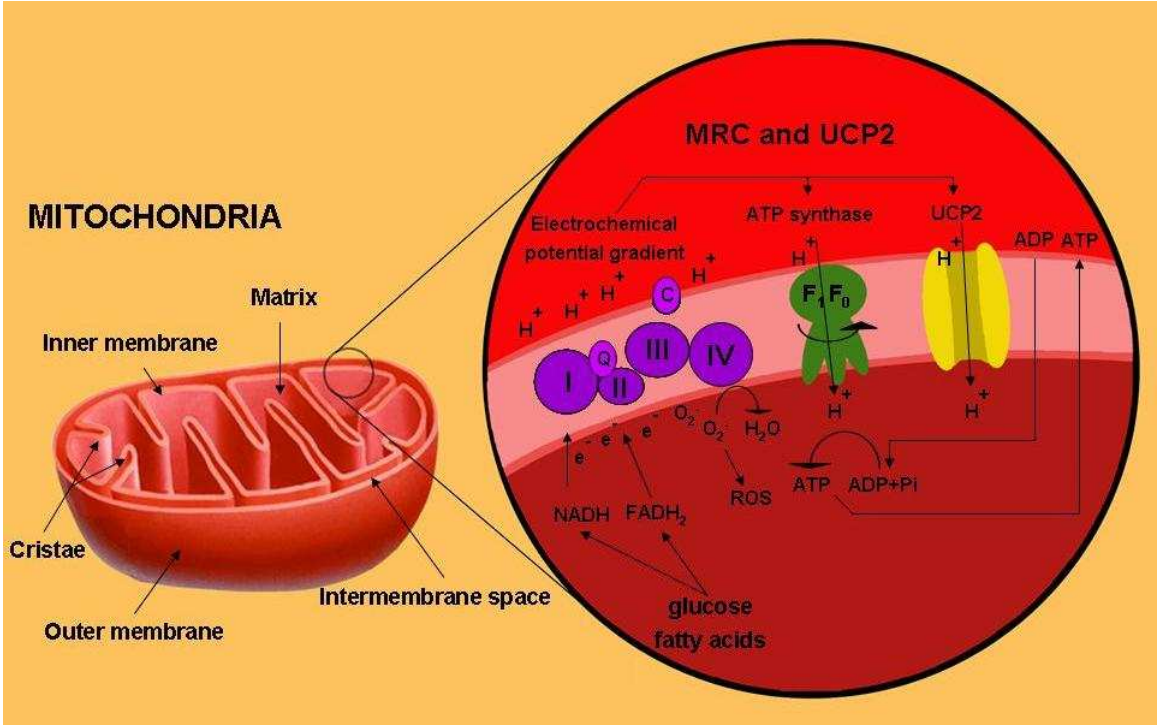


Figure 1. UCP2 localization and function in the mitochondrial respiratory chain (MRC). Numbers I, II, III and IV correspond to MRC complexes. F1Fo is the fifth MRC complex (ATP synthase complex). ROS = reactive oxygen species. Q = coenzyme Q; C = cytochrome-c. The cellular metabolism of substrates generates electrons (e-) in the form of reduced hydrogen carriers – NADH and $FADH_2$. NADH and $FADH_2$ donate electrons to the MRC, which comprises five multienzymatic complexes that are located in the mitochondrial inner membrane. Electrons are ultimately transported to molecular oxygen, which is reduced to water in the last step of the MRC. As electrons are transported along the MRC, a fixed number of protons (H+) are pumped from the mitochondrial matrix into the intermembrane space, producing a proton gradient across the mitochondrial inner membrane. The energy that is conserved in this proton gradient drives the synthesis of ATP from ADP and inorganic phosphate (P_i) by F1F0 complex as protons are transported back from the intermembrane space into the mitochondrial matrix. ATP is then made available to the cell for various

processes that require energy. Proton leak, which, in part, is mediated by UCP2, uncouples the processes of electron transport/proton-gradient generation, and consequently reduces the ATP synthesis.

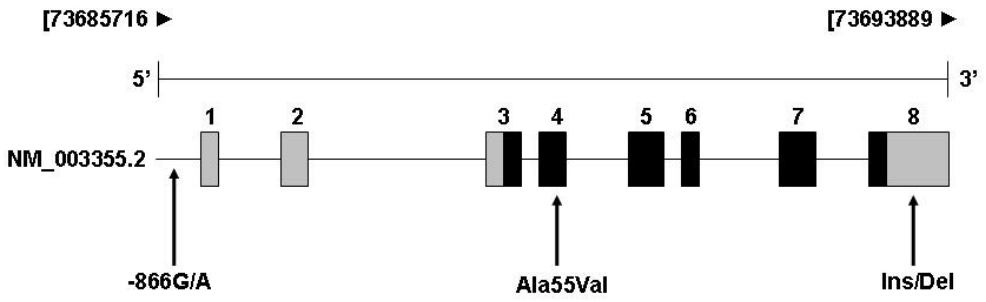


Figure 2. Map of the *UCP2* gene locus on chromosome 11 (region 11q13). The eight exons (boxes) are numbered from left to right according to transcription region. The black boxes represent the coding regions, and the light gray boxes represent the non-coding region, including the 3'UTR region of exon 8. The vertical arrows show the main common polymorphisms associated with DM2 or its microvascular chronic complications. Figure adapted from http://www.nbci.nlm.nih.gov/gene/7351.

Parte II

Artigo Original

The presence of the -866A / 55Val /Ins haplotype in the *uncoupling protein 2* (*UCP2*) gene is associated with decreased *UCP2* expression in human retinal tissue

The presence of the -866A / 55Val /Ins haplotype in the *uncoupling protein 2* (*UCP2*) gene is associated with decreased *UCP2* expression in human retinal tissue

Bianca M. de Souza[1,3], Taís S. Assmann[1], Lúcia M. Kliemann[2], Alexandre S. Marcon[4], Jorge L. Gross[1,3], Luiz 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.

Correspondence:

Dr. Daisy Crispim

Rua Ramiro Barcelos 2350; prédio 12; 4° andar. CEP 90035-003, Porto Alegre, RS, Brasil. E-mail: daisy_crispim@hotmail.com

Short title: *UCP2* expression in human retinal samples.

Word count: 4669

Summary

Background and objective Uncoupling protein 2 (UCP2) decreases the reactive oxygen species (ROS) formation by mitochondria. ROS overproduction is related to diabetic retinopathy (DR), a diabetes mellitus (DM) chronic complication. Recently, we reported that the -866A/55Val/Ins haplotype (-866G/A, Ala55Val and Ins/Del polymorphisms) of the *UCP2* gene was associated with increased risk for DR in DM patients. Here, we evaluated the effects of this haplotype and polymorphisms which constitute it on *UCP2* gene expression in retina. We also evaluated *MnSOD2* gene expression accordingly to *UCP2* haplotypes and individual polymorphisms.

Design and methods This cross-sectional study included 188 cadaveric cornea donors. In a subset of 91 retinal samples differentiated according to the presence of the mutated haplotype and risk alleles of the three polymorphisms, *UCP2* and *MnSOD2* mRNA concentrations were measured by real-time PCR technique.

Results Mutated haplotype carriers (homozygous + heterozygous) presented a lower *UCP2* mRNA expression than reference haplotype carriers (8.4 ± 7.6 *vs*. 18.8 ± 23.7 arbitrary units; P = 0.046). Accordingly, *UCP2* mRNA levels were lower in -866A and 55Val allele carriers when compared with other genotypes of these polymorphisms (P = 0.010 and P = 0.003, respectively). *UCP2* gene expression did not differ between Ins allele carriers and Del/Del carriers (P = 0.556). Subjects carrying the heterozygous haplotype showed increased *MnSOD2* mRNA levels (P = 0.025).

Conclusion The presence of the -866A/55Val/Ins haplotype is associated with decreased *UCP2* mRNA expression in human retina. Possibly, *MnSOD2* expression might influence the UCP2 effect on the pathogenesis of DR.

Keywords: *UCP2* gene expression, DNA polymorphisms, haplotype, human retina, diabetic retinopathy.

Introduction

Diabetic retinopathy (DR) is a common sight-threatening microvascular complication affecting patients with diabetes mellitus (DM) and it represents a major cause of new cases of blindness in adults aged 20-74 years (1). Proliferative DR (PDR), which is the most severe form of DR, might affect 10-20% of DM patients (1, 2). Although the risk of developing this complication increases with poor glycemic control, arterial hypertension and long-term duration of DM, the occurrence of PDR is also influenced by genetic factors (3). Recent studies indicate that one of the main mechanisms linking hyperglycemia to DR is the mitochondrial overproduction of reactive oxygen species (ROS) (1).

The uncoupling protein 2 (UCP2) is a member of an anion carrier protein family located in the mitochondrial inner membrane and is expressed in a wide range of tissues, including white adipose tissue, kidneys, liver, pancreatic islets and retinal cells (4-8). UCP2 mildly uncouples substrate oxidation in the mitochondria from ATP synthesis by mitochondrial respiratory chain. This is 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, and consequently decreasing ATP production (5, 8). The uncoupling thus leads to tissue-specific functions such as regulation of free fatty acids metabolism, inhibition of insulin secretion from pancreatic beta cells and decreasing ROS formation by mitochondria (5).

Overproduction of ROS or a failure in intracellular antioxidant defenses against ROS will result in the pathogenesis of some diseases, such as DM and its chronic diabetic complications (9). It is well established that there is a positive correlation between mitochondrial inner membrane potential and ROS production. At high

membrane potentials, even a small increase in membrane potential generates a large stimulation of hydrogen peroxide (H_2O_2) production. Therefore, "mild uncoupling", i.e., a small decrease in membrane potential, has been suggested to have a natural antioxidant effect (10). Since even a "mild uncoupling" has a large effect on reducing ROS production, the hypothesis that UCP2 protects against oxidative stress has strong support and is now generally accepted (8, 11). One important enzyme acting in the defense against mitochondrial ROS overproduction is the manganese superoxide dismutase 2 (MnSOD2), which converts superoxide anions (O_2^-), generated as byproducts of the mitochondrial oxidative phosphorylation, to H_2O_2. Afterward, H_2O_2 can be rapidly removed from mitochondria by the enzyme glutathione (GSH) peroxidase (12).

Based on the role of UCP2 in the protection against oxidative stress, we recently investigated whether three common *UCP2* gene polymorphisms (-866G/A, Ala55Val and Ins/Del), previously described in association with type 2 DM and/or obesity in other populations (13-15), were also associated with DR in DM patients from Rio Grande do Sul (Brazil) (16). Interestingly, our results showed that the -866A / 55Val / Ins haplotype, in homozygosis or heterozygosis, was associated with an increased risk for PDR in both type 2 DM and type 1 DM groups (16). Therefore, in the present study, we evaluated the possible effect of the mutated -866A / 55Val / Ins haplotype as well as the individual polymorphisms which constitute this haplotype, on *UCP2* gene expression in human retina obtained from cadaveric cornea donors. In addition, we also examined the *MnSOD2* gene expression in retina accordingly to *UCP2* haplotypes and individual polymorphisms.

Materials and methods

Samples

Three hundred and seventy-six human eyes were obtained from 188 cadaveric cornea donors identified through the *Central de Transplantes do Rio Grande do Sul* (a Brazilian organization that regulates cadaveric organs donation in Rio Grande do Sul, Brazil), and collected in two general hospitals of Porto Alegre (Rio Grande do Sul), namely Hospital de Clínicas de Porto Alegre and Hospital Santa Casa de Misericórdia. A standard questionnaire was used to retrospectively collect information in medical records about age, gender, presence of systemic arterial hypertension and DM, smoking, 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, snapped frozen in liquid nitrogen and stored at -80°C until assayed for mRNA expression analyses. Peripheral blood samples were also collected from each subject for DNA extraction and subsequent genotyping of the -866G/A, Ala55Val e Ins/Del polymorphisms. Following genotyping, subjects were divided in groups according to the presence of the mutated haplotype (-866A / 55Val / Ins). In addition, subjects were also classified according to the presence of different -866G/A, Ala55Val or Ins/Del genotypes.

To estimate the allele frequencies of *UCP2* gene polymorphisms in the general population of Rio Grande do Sul (Brazil), we also genotyped 439 healthy blood donors (mean age = 44.0 ± 7.8, male = 55%). The protocol of this study was approved by the Hospital ethical committees, and relatives of all donors gave their written informed consent authorizing the use of the material, which would otherwise be discarded.

Genotyping

DNA was extracted from peripheral blood leukocytes using a standardized salting-out procedure. The -866G/A polymorphism (rs659366) in the promoter region of the *UCP2* gene was determined by digesting polymerase chain reaction (PCR) products with the enzyme *Mlu*I (Invitrogen Life Technologies, San Diego, CA, USA) as previously described (17). Digestion fragments were resolved on 2% agarose gels containing GelRed™ Nucleic Acid Gel Stain (Biotium Inc., CA, USA) and visualized under ultraviolet illumination. Evaluation of the 45bp Ins/Del polymorphism in the 3'untranslated region (UTR) of exon 8 was done by PCR using primers described elsewhere (18). The primers amplified products of 457bp (insertion allele) or 412bp (deletion allele), which were resolved on 2% agarose gels stained with GelRed™ Nucleic Acid Gel Stain (Biotium Inc.) and visualized under ultraviolet light. Genotypes of both -866G/A and Ins/Del polymorphisms were recorded using the ImageMaster System VDS (GE HealthCare, London, UK).

Genotyping of the Ala55Val (C/T) polymorphism (rs660339) in exon 4 was performed using specific primers and probes contained in the Human Custom TaqMan Genotyping Assay 40x (Assays-By-Design Service; Applied Biosystems, Foster City, CA; USA). Primer and probe sequences used were: 5'-GTCTTGGCCTTGCAGATCCA-3' (forward primer), 5'-GTCAGAATGGTGCCCATCACA-3' (reverse primer), FAM 5'-TGGGCGCTGGCTGTA-3' and VIC 5'-TGGGCGCTGACTGTA-3'. The reactions were conducted in 96-well plates, in a total 5 μl reaction volume using 2 ng of genomic DNA, TaqMan Genotyping Master Mix 1x (Applied Biosystems), and Custom TaqMan Genotyping Assay 1x (Applied Biosystems). The plates were then positioned in a real-time PCR thermal cycler (7500 Fast Real Time PCR System; Applied Biosystems) and

heated for 10 minutes at 95ºC, followed by 50 cycles of 95ºC for 15 seconds and 63ºC for 1 minute. Fluorescence data files from each plate were analyzed using automated allele-calling software (System Sequence Detection v.1.4; Applied Biosystems).

RNA isolation

Retinal tissues (250mg) were homogenized in phenol-guanidine isothiocyanate (Trizol®; Invitrogen Life Technologies). Total 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 total RNA were assessed using NANODROP 2000 spectrophotometer (Thermo Scientific Inc., Wilmington, DE, USA).

Quantification of UCP2 and MnSOD2 gene expression by real-time reverse transcription PCR

Real-time reverse transcription-PCR (RT-PCR) was performed in two separate reactions: first, total RNA was reverse transcribed into cDNA, then the cDNA was amplified by real-time PCR. Reverse transcription of 1 μg of total 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 $(dT)_{12\text{-}18}$ method. cDNA samples were diluted to a final concentration of 0.25 μg/μl, and stored at -20°C until real-time PCR reactions.

Real-time PCR experiments were performed in a 7500 Fast Real-Time PCR System thermal cycler (Applied Biosystems) according to the manufacture's protocol. Real-time PCR was performed by monitoring in real time the increase in fluorescence of the SYBER® Green dye, as described elsewhere (19). Primers for *UCP2*, *MnSOD2*

and *β-actin* genes were designed using published human gene sequences and the Primer Express 3.0 Software for Real-Time PCR (Applied Biosytems), and obtained from Invitrogen™ Life Technologies. Forward and reverse primer sequences were projected to target two consecutive exons of a gene in order to prevent the amplification of any contaminating genomic DNA.

Real-time PCR reactions for both *UCP2* and *MnSOD2* genes 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 *UCP2*, *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 all analyzed genes were as follows: an initial cycle of 95°C for 20s, followed by 50 cycles of 95°C for 3s and 60°C for 30s. The specificity of the real-time PCR was determined using melting curve analyses, which showed a single sharp peak at a temperature of approximately 76.5°C for the *UCP2* gene, 78°C for the *β-actin* gene, and 75°C for *MnSOD2* gene.

Quantification of the *UCP2* cDNA (arbitrary units - AU) was performed using the relative standard curve method (20), whose data were analyzed using the System Sequence Detection v.1.4 Software (Applied Biosystems) and the human *β-actin* as an endogenous gene expression control.

Quantification of the *MnSOD2* cDNA was performed by relative quantification using the comparative C_T method, as previously described (21), and also using the human *β-actin* as an endogenous gene expression control (reference gene). Validation assays were done by amplification of the target (MnSOD2) and reference (*β-actin*) genes, separately, using serial dilutions of an mRNA sample. Both target and reference cDNAs presented equal efficiencies of amplification. The comparative C_T method

calculates changes in gene expression as relative fold difference (n fold change) between an experimental and an external calibrator sample, including a correction for non-ideal amplification efficiencies (21).

The following primer sequences were used for expression analyses: UCP2 5'-TTGGGTTCAAGGCCACAGAT-3' (forward primer), UCP2 5'-CCAGCCCCAAGAAACTTCAC-3' (reverse primer), MnSOD2 5'-AAATTGCTGCTTGTCCAAATCAG-3' (forward primer), MnSOD2 5'-ATCAATCCCCAGCAGTGGAAT -3' (reverse primer), β-actin 5'-GCGCGGCTACAGCTTCA-3' (forward primer) and β-actin 5'-CTTAATGTCACGCACGATTTCC-3' (reverse primer).

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. Between all pairs of biallelic loci, we examined widely used measures of linkage disequilibrium (LD), Lewontin's D' |D'| and r^2 (22). The haplotypes constructed from the combination of the three *UCP2* polymorphisms and their frequencies were inferred using the phase 2.1 program, which implements a Bayesian statistical method (23). We also used the Phase 2.1 program to compare the distributions of different *UCP2* haplotypes between cadaveric human retina donors and subjects from the general population of Rio Grande do Sul (Brazil) through permutation analyses of 10.000 random replicates (23).

Results are expressed as mean ± standard deviation (SD), mean ± standard error (SE) or percentage. Clinical characteristics and *UCP2* and *MnSOD2* gene expression were compared between genotypes and haplotypes by using unpaired Student's t-test,

One-Way ANOVA or χ^2, as appropriate. Variables with skewed distribution were logarithmically transformed before analyses. Pearson's correlation coefficient tests was used to assess the correlation between *UCP2* and *MnSOD2* gene expression or between *UCP2* or *MnSOD2* gene expression and age.

UCP2 and *MnSOD2* gene expressions were evaluated in the following groups: 1) diabetic patients (n = 15) *vs*. nondiabetic subjects (n = 24); 2) reference haplotype (-866G 55Ala Del / -866G 55Ala Del; n = 27) *vs*. heterozygous haplotype (n = 17) *vs*. mutated haplotype (-866A 55Val Ins / -866A 55Val Ins; n = 13); 3) -866G/G (n = 27) *vs*. A/G (n = 33) *vs*. A/A (n = 26) genotypes; 4) Ala/Ala (n = 27) *vs*. Ala/Val (n = 43) *vs*. Val/Val (n = 16) genotypes; and 5) Del/Del (n = 53) *vs*. Ins/Del (n = 24) *vs*. Ins/Ins (n = 14) genotypes. $P < 0.05$ was considered statistically significant. The Statistical Package for Social Science 18.0 Professional Software (SPSS, Chicago, IL) was used for statistical analyses.

Results

Sample description

One hundred and eighty-eight cadaveric cornea donors were genotyped for the -866A/G, Ala55Val and Ins/Del polymorphisms in the *UCP2* gene and had their *UCP2* haplotypes inferred. The main clinical characteristics of these subjects were as follows. Age varied from 13 to 79 years (mean 56.1 ± 15.1 years). Males comprised 57.4% (n = 108) of the sample, 39.4% (n = 74) of all patients had arterial hypertension, and 55.3% (n = 104) were smokers.

Type 2 DM was diagnosed in 7.9% (n = 15) of all patients. Diabetic patients differed from nondiabetic subjects regarding age (66.6 ± 6.8 *vs*. 55.5 ± 14.4,

respectively; P = 0.005) and presence of arterial hypertension (71.4% *vs*. 24.2%; P = 0.015). Both diabetic and nondiabetic groups were similar regarding gender and proportion of smokers (data not shown).

Genotypic and allelic distributions

Genotypic and allelic frequencies of the -866G/A, Ala55Val and Ins/Del polymorphisms in the *UCP2* gene did not differ statistically among retina donors and subjects from the general population of Rio Grande do Sul (**Table 1**), and all genotypes were in agreement with those predicted by the HWE (P > 0.05). The -866G/A polymorphism is in almost complete LD with the Ala55Val polymorphism (|D'| = 0.991; r^2 = 0.905), but only in moderate LD with the Ins/Del polymorphism (|D'| = 0.855; r^2 = 0.485). The Ala55Val polymorphism is also in partial LD with the Ins/Del polymorphism (|D'| = 0.878; r^2 = 0.471). It is worth mentioning that genotypic and allelic frequencies of the three analyzed *UCP2* polymorphisms were similar between diabetic and nondiabetic subjects from the retina donors group (P > 0.05; data not shown).

Haplotype distributions

We used a Bayesian statistical method to estimate the frequency of different haplotypes produced by the combination of the -866G/A, Ala55Val and Ins/Del polymorphisms in retina donors (**Table 2**). The first letter of the haplotypes refers to the -866G/A polymorphism, the second to the Ala55Val polymorphism and the third to Ins/Del polymorphism. Seven haplotypes were inferred in retina donors. Haplotypes Ht1 [-866G / 55Ala / Del], Ht4 [-866A / 55Val / Del] and Ht5 [-866A / 55Val / Ins] were inferred in frequencies higher than 5% and altogether accounted for 90.2% of the

observed haplotypes, with the remaining 9.8% being shared among haplotypes Ht2, Ht3, Ht6 and Ht7. Permutation analysis showed that the distributions of these 7 haplotypes were not statistically different between retina donors and subjects from the general population ($P > 0.05$; data not shown).

Taking into consideration the results of our previous study showing that the presence of Ht5 [-886A / 55Val / Ins] was significantly associated with an increased risk for PDR in DM patients (16), we only selected subjects with the Ht5 [-866A / 55Val / Ins] (in homozygosis or heterozygosis) or Ht1 [-866G / 55Ala / Del] (reference haplotype) for subsequent expression analyses in retina.

UCP2 gene expression in human retina

The mean ± SD *UCP2* mRNA level in the whole retinal tissue group was 15.9 ± 26.3 AU. No significant difference was observed when *UCP2* gene expression was analyzed by gender (men: 14.1 ± 26.2 *vs*. women: 18.2 ± 26.4 AU; $P = 0.192$) or hypertensive status (normotensives: 20.9 ± 35.5 *vs*. hypertensives: 17.6 ± 23.3 AU; $P = 0.523$). Retinal *UCP2* gene expression did not correlate with age ($r = 0.145$, $P = 0.167$), and it was not statistically different between diabetic and nondiabetic donors (25.9 ± 46.6 *vs*. 15.4 ± 23.7 AU, respectively; $P = 0.348$). However, due to the small number of retinal donors with DM ($n = 15$), we decided do not include these subjects in subsequent expression analyses.

UCP2 mRNA levels in human retinal samples stratified by the presence of the analyzed *UCP2* haplotypes or according to the individual genotypes of the -866G/A, Ala55Val and Ins/Del polymorphisms are depicted in **Figure 1**. *UCP2* gene expression did not differ among mutated haplotype (A Val Ins / A Val Ins), heterozygous haplotype (A Val Ins / G Ala Del) or reference haplotype (G Ala Del / G Ala Del) groups (P =

0.137; **Figure 1A**). However, when comparing subjects carrying one or two copies of the mutated haplotype (homozygous + heterozygous) with homozygous subjects for the reference haplotype, we observed that carriers of at least one copy of the mutated haplotype presented a lower *UCP2* gene expression than reference haplotype carriers (8.4 ± 7.6 *vs*. 18.8 ± 23.7 AU; P = 0.046).

Interestingly, when evaluating the retinal *UCP2* gene expression among individual genotypes of the -866G/A polymorphism, we observed that *UCP2* mRNA levels were significantly lower in both A/A and A/G genotype groups than in the G/G genotype group (P = 0.014; **Figure 1B**). Similarly, *UCP2* mRNA levels were lower in -866A allele carriers (A/A + A/G) when compared with G/G carriers (7.4 ± 6.1 *vs*. 18.8 ± 23.7 AU, respectively; P = 0.010).

Since the Ala55Val polymorphism is in almost complete LD with the -866G/A polymorphism, subjects carrying the Ala/Val or Val/Val genotypes also seem to present lower *UCP2* gene expression than subjects with the Ala/Ala genotype (P = 0.007; **Figure 1C**); although, after Tukey post hoc test, only the comparison between Ala/Val and Ala/Ala groups reached conventional statistical significance (P < 0.05). The mean *UCP2* mRNA expression was lower in Val allele carriers (Val/Val + Ala/Val) when compared with the Val/Val genotype group (7.3 ± 6.1 *vs*. 18.8 ± 23.7, respectively; P = 0.003).

Retinal *UCP2* mRNA levels were similar among different Ins/Del genotypes (P = 0.613; **Figure 1D**). Likewise, *UCP2* gene expression did not differ between Ins allele carriers (Ins/Ins + Ins/Del) and Del/Del carriers (9.2 ± 9.3 *vs*. 12.6 ± 15.5 AU, respectively; P = 0.556).

It is worth mentioning that the above results concerning the retinal *UCP2* gene expression in subjects stratified by the presence of *UCP2* haplotypes or individual

polymorphisms were not significantly different when including diabetic subjects in these analyses (data not shown).

MnSOD2 gene expression in human retina

The mean ± SD *MnSOD2* mRNA expression in the whole retinal tissue sample was -0.9 ± 1.2 n fold change. 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 change; P = 0.393), hypertensive status (normotensives: -0.7 ± 1.4 *vs*. hypertensives: -1.0 ± 1.1 n fold change; P = 0.652) or DM diagnosis (nondiabetic: -0.6 ± 0.9 *vs*. DM subjects: -0.9 ± 1.0; P = 0.594). *MnSOD2* gene expression did not correlate with age (r = -0.152, P = 0.142) neither with *UCP2* mRNA levels (r = 0.125, P = 0.232). Diabetic subjects (n = 15) were also excluded from subsequent expression analyses.

MnSOD2 mRNA levels in retinal samples stratified by the presence of the analyzed *UCP2* haplotypes or according to the individual genotypes of the -866G/A, Ala55Val and Ins/Del polymorphisms are depicted in **Figure 2**. Subjects carrying the heterozygous haplotype showed higher *MnSOD2* mRNA levels than homozygous for the mutated haplotype or homozygous for the reference haplotype (P = 0.025; **Figure 2A**). However, when comparing subjects carrying one or two copies of the mutated haplotype (homozygous + heterozygous) with homozygous for the reference haplotype, we did not observe any significant difference in *MnSOD2* gene expression between both groups (-0.9 ± 1.2 *vs*. -1.2 ± 1.5; P = 0.335).

Moreover, we observed that *MnSOD2* mRNA levels were higher in heterozygous for the -866A/G polymorphism than homozygous A/A or G/G (P = 0.022; **Figure 2B**). No significant difference on *MnSOD2* gene expression was observed when comparing A allele carriers (A/A + A/G) with G/G genotype carriers (-0.7 ± 1.0 *vs*. -1.2

± 1.5 n fold change, respectively; P = 0.099). Similarly, *MnSOD2* gene expression was higher in heterozygous for the Ala55Val polymorphism than in homozygous Val/Val or Ala/Ala (P = 0.003; **Figure 2C**), but it was not significantly different between Val allele carriers (Val/Val + Ala/Val) and Val/Val genotype carriers (-0.7 ± 1.0 *vs*.-1.2 ± 1.5, respectively; P = 0.092). Finally, *MnSOD2* mRNA levels were also higher in heterozygous for the Ins/Del polymorphism than in homozygous Ins/Ins or Del/Del (P = 0.004; **Figure 2D**). Nevertheless, no significant difference on *MnSOD2* gene expression was observed when comparing Ins allele carriers (Ins/Ins + Ins/Del) with Del/Del subjects (-0.75 ± 1.11 *vs*. -1.0 ± 1.2, respectively; P = 0.392).

Discussion

In the present study, we observed that retinal samples from nondiabetic cornea donors carrying the -866A / 55Val / Ins haplotype (homozygous + heterozygous) presented a lower *UCP2* gene expression than homozygous for the reference haplotype. It is well known that functional polymorphisms can influence gene expression regulating the final quantity of protein related to a given disease. The A allele of the -866G/A polymorphism has been reported to increase *UCP2* transcriptional activity in transfected INS-1E cells derived from rat beta-cells (24). However, data in human tissues have been conflicting, reporting either increased (18, 24-26) or decreased (27, 28) *UCP2* mRNA levels to be associated with the A allele. Computational analyzes demonstrated that this polymorphism is involved in putative binding sites for specific transcription factors (25). It was hypothesized that preferential binding of some transcription factors to the G or A allele in the promoter sequence may confer tissue-specific advantages to either allele (25). Reporter gene assay experiments conducted by Esterbauer *et al*. (25) showed

that the A allele has a 22% more effective transcriptional activity in differentiated adipocytes. On the other hand, Oberkofler *et al*. (28) reported that this same allele was implicated in decreased transcription rates in human endothelial cells and macrophages. This last study is in agreement with our present result showing that *UCP2* mRNA levels in retinal samples were decreased in -866A allele carriers in comparison with G/G genotype carriers.

The Ins/Del polymorphism is located in the 3' UTR region of the *UCP2* gene, and it also appears to be functional because mRNA transcribed from the insertion allele displayed a shorter half-life in a foetal myoblast cell line than mRNA transcribed from the deletion allele (29). Here, the Ins/Del polymorphism was not individually associated with changes in *UCP2* mRNA levels. Nevertheless, we cannot exclude the possibility that this polymorphism may be associated with mRNA stability in human retina.

The Ala55Val (C/T) polymorphism causes a conservative amino acid change, and until now, there has been no evidence that it generates a functional change in the protein. Our results showed that retinal samples from 55Val allele carriers also presented lower *UCP2* mRNA expression when compared with the Ala/Ala genotype group. Thus, considering that the Ala55Val (C/T) polymorphism is tightly linked to the -866G/A polymorphism, and that the -866G/A polymorphism causes changes in the *UCP2* mRNA expression, it is probable that the Ala55Val polymorphism is not a real disease-causing variant, but could be simply reflecting the effect of the -866G/A polymorphism.

As already mentioned, the “mild uncoupling” of mitochondrial respiratory chain due to UCP2 activity decreases ROS production (30). Accordingly, endothelial cells from bovine retinal cells incubated with high glucose levels increase the *UCP2* expression, which protects them from ROS damage derived from glucotoxicity, and also

suggests a protective role of this protein in the pathogenesis of DR (7). However, despite the recognized role of UCP2 in the protection against oxidative stress, and that hyperglycemia-induced oxidative stress is one of the major contributors to the accelerated loss of retinal capillary cells in DM (31), only a few studies evaluated the association between *UCP2* gene polymorphisms and the occurrence of this complication (16, 32, 33). Rudofsky *et al*. (32) did not report any association between the -866G/A polymorphism and DR in type 1 DM patients. In addition, Rudofsky *et al*. (33), studying type 2 DM patients from Germany, did not observe any association between the same polymorphism and DR. More recently, our group showed that the presence of the -866A / 55Val / Ins haplotype was associated with an increased risk for PDR in both type 2 and type 1 DM patients (16).

Retinal mitochondria become dysfunctional in DM and the production of superoxide radicals is increased (34, 35). In this context, the inhibition of oxidative stress through overexpression of *MnSOD2* prevents retinal endothelial cells from undergoing the accelerated apoptosis that precedes the onset of DR in diabetic mice (34). Thus, the protection of mtDNA from glucose-induced oxidative damage is one of the plausible mechanisms by which MnSOD2 ameliorates the development of DR (34). In the present study, *MnSOD2* mRNA levels were increased in retinal samples from heterozygous for the mutated haplotype when compared with both homozygous haplotypes. Besides, heterozygosis for individual -866A, Ala55Val and Ins/Del polymorphisms were also associated with increased *MnSOD2* mRNA levels.

Based on the data of the *UCP2* mRNA expression in retina from nondiabetic cornea donors, we therefore hypothesized that in retinal cells, the presence of the -866A / 55Val / Ins haplotype, mainly due to the effect of the -866A allele, leads to a decreased *UCP2* mRNA expression. Therefore, as a result of this decreased *UCP2* expression,

retinal cells of patients carrying the mutated haplotype might have higher ROS levels, and consequently, these patients will have an increased risk for PDR as compared with patients having other haplotypes. It is noteworthy that our previous study reported that the magnitude of the association between the -866A / 55Val / Ins haplotype and PDR was higher when considering the presence of this haplotype in two copies than in one copy (OR = 5.3 and $P < 0.00001$ and OR = 2.1 and $P < 0.006$, respectively, in type 2 DM patients), suggesting an additive inheritance model. However, here, the *UCP2* gene expression was similar between homozygous and heterozygous for the mutated haplotype, suggesting a dominant inheritance model and conflicting with our previous results. This could be explained by our data showing that heterozygous for the mutated *UCP2* haplotype and individual polymorphisms have an increased *MnSOD2* gene expression when compared with the homozygous haplotypes and genotypes, which might be compensating the decreased *UCP2* gene expression and consequent deficient ROS protection in heterozygous but not in homozygous patients.

In conclusion, the data presented here indicate that the presence of the -866A / 55Val / Ins haplotype is associated with a decreased *UCP2* mRNA expression in human retina. Possible interactions between UCP2 and MnSOD2 in retinal cells may influence the UCP2 effect on the pathogenesis of PDR. To our knowledge, these are the first data showing that *UCP2* gene polymorphisms might affect the expression of this protein in human retina. However, further functional studies will be necessary to confirm if changes on *UCP2* gene expression due to different haplotypes translate in changes on UCP2 protein levels.

Acknowledgements

This study was partially supported by grants from Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and 'Fundo de Incentivo à Pesquisa e Eventos' (FIPE) at Hospital de Clínicas de Porto Alegre. The authors thank Dr. Rosana R. Nothen and Fernando Pagnussato for their support with sample collection and preparation.

Competing interests / financial disclosure Nothing to declare.

References

1. Fong D, Aiello L, Gardner T, King G, Blankenship G, Cavallerano J, et al. Retinopathy in diabetes. Diabetes Care. 2004 Jan;27 Suppl 1:S84-7.
2. Kohner EM. Diabetic retinopathy. BMJ. 1993 Nov;307(6913):1195-9.
3. Warpeha KM, Chakravarthy U. Molecular genetics of microvascular disease in diabetic retinopathy. Eye (Lond). 2003 Apr;17(3):305-11.
4. Dalgaard L, Pedersen O. Uncoupling proteins: functional characteristics and role in the pathogenesis of obesity and Type II diabetes. Diabetologia. 2001 Aug;44(8):946-65.
5. Erlanson-Albertsson C. Uncoupling proteins--a new family of proteins with unknown function. Nutr Neurosci. 2002 Feb;5(1):1-11.
6. Fleury C, Neverova M, Collins S, Raimbault S, Champigny O, Levi-Meyrueis C, et al. Uncoupling protein-2: a novel gene linked to obesity and hyperinsulinemia. Nat Genet. 1997 Mar;15(3):269-72.
7. Cui Y, Xu X, Bi H, Zhu Q, Wu J, Xia X, et al. Expression modification of uncoupling proteins and MnSOD in retinal endothelial cells and pericytes induced by high glucose: the role of reactive oxygen species in diabetic retinopathy. Exp Eye Res. 2006 Oct;83(4):807-16.
8. Fisler J, Warden C. Uncoupling proteins, dietary fat and the metabolic syndrome. Nutr Metab (Lond). 2006;3:38.
9. Newsholme P, Haber EP, Hirabara SM, Rebelato EL, Procopio J, Morgan D, et al. Diabetes associated cell stress and dysfunction: role of mitochondrial and non-mitochondrial ROS production and activity. J Physiol. 2007 Aug;583(Pt 1):9-24.
10. Papa S, Skulachev V. Reactive oxygen species, mitochondria, apoptosis and aging. Mol Cell Biochem. 1997 Sep;174(1-2):305-19.
11. Brand M, Affourtit C, Esteves T, Green K, Lambert A, Miwa S, et al. Mitochondrial superoxide: production, biological effects, and activation of uncoupling proteins. Free Radic Biol Med. 2004 Sep;37(6):755-67.
12. Turrens JF. Mitochondrial formation of reactive oxygen species. J Physiol. 2003 Oct;552(Pt 2):335-44.
13. D'Adamo M, Perego L, Cardellini M, Marini M, Frontoni S, Andreozzi F, et al. The -866A/A genotype in the promoter of the human uncoupling protein 2 gene is associated with insulin resistance and increased risk of type 2 diabetes. Diabetes. 2004 Jul;53(7):1905-10.
14. Yu X, Jacobs DJ, Schreiner P, Gross M, Steffes M, Fornage M. The uncoupling protein 2 Ala55Val polymorphism is associated with diabetes mellitus: the CARDIA study. Clin Chem. 2005 Aug;51(8):1451-6.
15. Marti A, Corbalán MS, Forga L, Martinez-González MA, Martinez JA. Higher obesity risk associated with the exon-8 insertion of the UCP2 gene in a Spanish case-control study. Nutrition. 2004 Jun;20(6):498-501.
16. Crispim D, Fagundes N, dos Santos K, Rheinheimer J, Bouças A, de Souza B, et al. Polymorphisms of the UCP2 gene are associated with proliferative diabetic retinopathy in patients with diabetes mellitus. Clin Endocrinol (Oxf). 2010 May;72(5):612-9.
17. Sasahara M, Nishi M, Kawashima H, Ueda K, Sakagashira S, Furuta H, et al. Uncoupling protein 2 promoter polymorphism -866G/A affects its expression in beta-

cells and modulates clinical profiles of Japanese type 2 diabetic patients. Diabetes. 2004 Feb;53(2):482-5.
18. Walder K, Norman RA, Hanson RL, Schrauwen P, Neverova M, Jenkinson CP, et al. Association between uncoupling protein polymorphisms (UCP2-UCP3) and energy metabolism/obesity in Pima indians. Hum Mol Genet. 1998 Sep;7(9):1431-5.
19. Higuchi R, Fockler C, Dollinger G, Watson R. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Biotechnology (N Y). 1993 Sep;11(9):1026-30.
20. Biosystems A. Relative quantitation of gene expression experimental design and analysis: Relative standard curve method and comparative ct method (DeltaDelta-ct). Guide to performing relative quantitation of gene expression using real-time quantitative PCR. Foster City, California, USA: Applied Biosystems; 2004. p. 34-49.
21. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001 Dec;25(4):402-8.
22. Hedrick PW. Gametic disequilibrium measures: proceed with caution. Genetics. 1987 Oct;117(2):331-41.
23. Stephens M, Smith NJ, Donnelly P. A new statistical method for haplotype reconstruction from population data. Am J Hum Genet. 2001 Apr;68(4):978-89.
24. Cassell P, Neverova M, Janmohamed S, Uwakwe N, Qureshi A, McCarthy M, et al. An uncoupling protein 2 gene variant is associated with a raised body mass index but not Type II diabetes. Diabetologia. 1999 Jun;42(6):688-92.
25. Esterbauer H, Schneitler C, Oberkofler H, Ebenbichler C, Paulweber B, Sandhofer F, et al. A common polymorphism in the promoter of UCP2 is associated with decreased risk of obesity in middle-aged humans. Nat Genet. 2001 Jun;28(2):178-83.
26. Jia JJ, Zhang X, Ge CR, Jois M. The polymorphisms of UCP2 and UCP3 genes associated with fat metabolism, obesity and diabetes. Obes Rev. 2009 Sep;10(5):519-26.
27. Wang H, Chu W, Lu T, Hasstedt S, Kern P, Elbein S. Uncoupling protein-2 polymorphisms in type 2 diabetes, obesity, and insulin secretion. Am J Physiol Endocrinol Metab. 2004 Jan;286(1):E1-7.
28. Oberkofler H, Iglseder B, Klein K, Unger J, Haltmayer M, Krempler F, et al. Associations of the UCP2 gene locus with asymptomatic carotid atherosclerosis in middle-aged women. Arterioscler Thromb Vasc Biol. 2005 Mar;25(3):604-10.
29. Lindholm E, Klannemark M, Agardh E, Groop L, Agardh CD. Putative role of polymorphisms in UCP1-3 genes for diabetic nephropathy. J Diabetes Complications. 2004 2004 Mar-Apr;18(2):103-7.
30. Azzu V, Brand M. The on-off switches of the mitochondrial uncoupling proteins. Trends Biochem Sci. 2010 May;35(5):298-307.
31. Kowluru RA, Abbas SN. Diabetes-induced mitochondrial dysfunction in the retina. Invest Ophthalmol Vis Sci. 2003 Dec;44(12):5327-34.
32. Rudofsky G, Schroedter A, Schlotterer A, Voron'ko OE, Schlimme M, Tafel J, et al. Functional polymorphisms of UCP2 and UCP3 are associated with a reduced prevalence of diabetic neuropathy in patients with type 1 diabetes. Diabetes Care. 2006 Jan;29(1):89-94.
33. Rudofsky G, Schrödter A, Voron'ko OE, Schlotterer A, Humpert PM, Tafel J, et al. Promoter polymorphisms of UCP1, UCP2, and UCP3 are not associated with diabetic microvascular complications in type 2 diabetes. Horm Metab Res. 2007 Apr;39(4):306-9.

34. Madsen-Bouterse SA, Zhong Q, Mohammad G, Ho YS, Kowluru RA. Oxidative damage of mitochondrial DNA in diabetes and its protection by manganese superoxide dismutase. Free Radic Res. 2010 Mar;44(3):313-21.
35. Kanwar M, Chan PS, Kern TS, Kowluru RA. Oxidative damage in the retinal mitochondria of diabetic mice: possible protection by superoxide dismutase. Invest Ophthalmol Vis Sci. 2007 Aug;48(8):3805-11.

Table 1. Genotypic and allelic distributions of -866G/A, Ala55Val and Ins/Del polymorphisms in the *UCP2* gene in retina donors and general population.

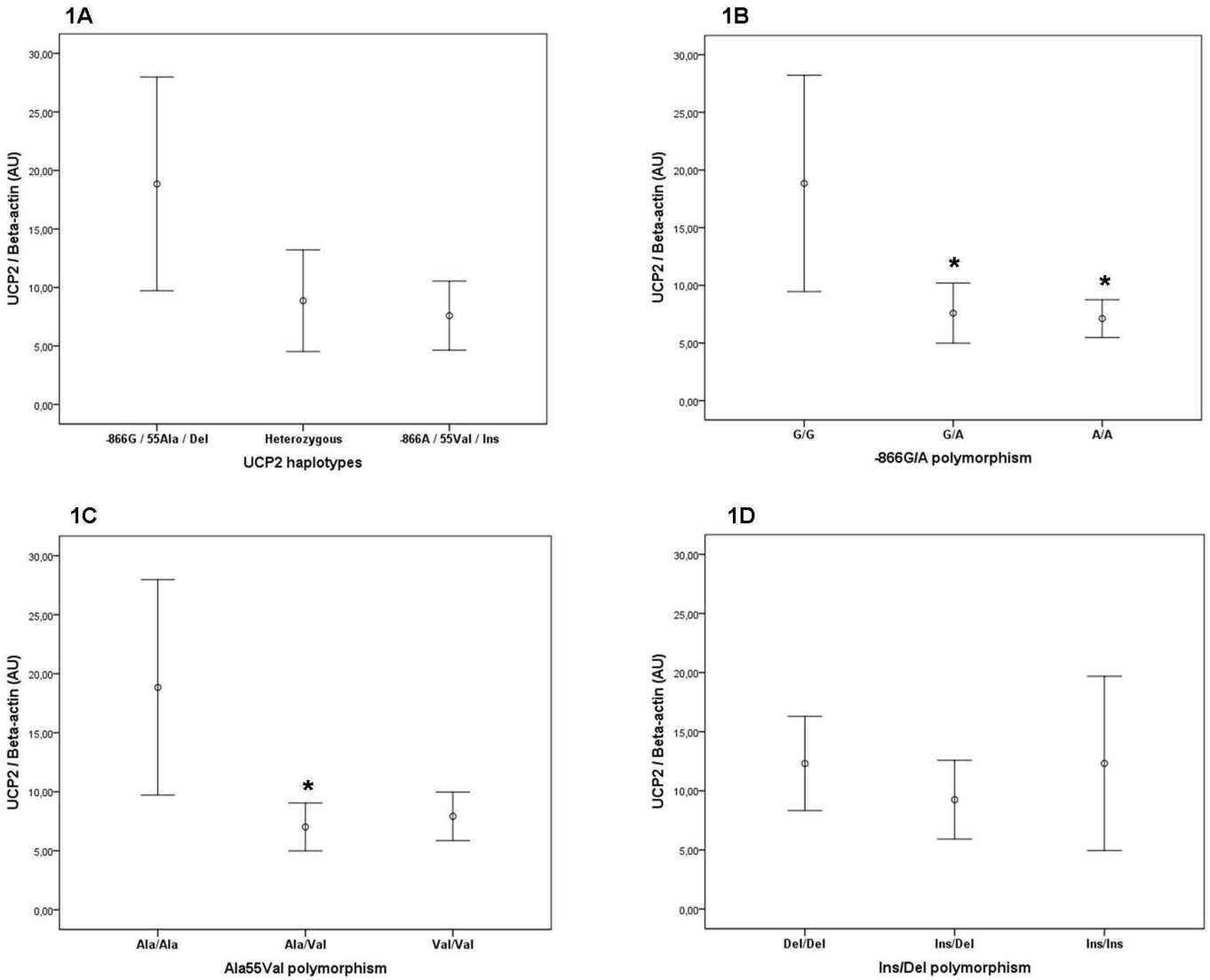
Polymorphisms	Retina donors	General population	P*
-866 G/A	n = 167	n = 440	
G/G	53 (31.7)	157 (35.7)	0.649
A/G	84 (50.3)	211 (47.9)	
A/A	30 (18.0)	72 (16.4)	
G	0.569	0.596	0.417
A	0.431	0.404	
Ala55Val	n = 186	n = 453	
Ala/Ala	50 (26.9)	142 (31.4)	0.436
Ala/Val	104 (55.9)	229 (50.5)	
Val/Val	32 (17.2)	82 (18.1)	
Ala	0.548	0.570	0.602
Val	0.452	0.430	
Ins/Del	n = 188	n = 458	
Del/Del	81 (43.1)	223 (48.7)	0.432
Ins/Del	87 (46.3)	191 (41.7)	
Ins/Ins	20 (10.6)	44 (9.6)	
Del	0.662	0.695	0.271
Ins	0.338	0.305	

Data are presented as number (%) or proportion. *P values were computed by the χ^2 test comparing human retina donors and subjects from the general population of Rio Grande do Sul (Brazil).

Table 2. *UCP2* gene haplotypes in cadaveric human retina donors.

Haplotypes	Retina donors
	n = 374
Ht1 [G Ala Del]	0.495
Ht2 [G Val Ins]	0.024
Ht3 [A Ala Del]	0.029
Ht4 [A Val Del]	0.128
Ht5 [A Val Ins]	0.279
Ht6 [G Ala Ins]	0.021
Ht7 [G Val Del]	0.024

n = number of chromosomes. The first letter of the haplotype refers to the -866G/A, the second to the Ala55Val, and the third to Ins/Del polymorphism.


1A
UCP2 / Beta-actin (AU)
30,00
25,00
20,00
15,00
10,00
5,00
0,00
-866G / 55Ala / Del
Heterozygous
-866A / 55Val / Ins
UCP2 haplotypes
1B
UCP2 / Beta-actin (AU)
*
*
G/G
G/A
A/A
-866G/A polymorphism
1C
UCP2 / Beta-actin (AU)
*
Ala/Ala
Ala/Val
Val/Val
Ala55Val polymorphism
1D
UCP2 / Beta-actin (AU)
Del/Del
Ins/Del
Ins/Ins
Ins/Del polymorphism

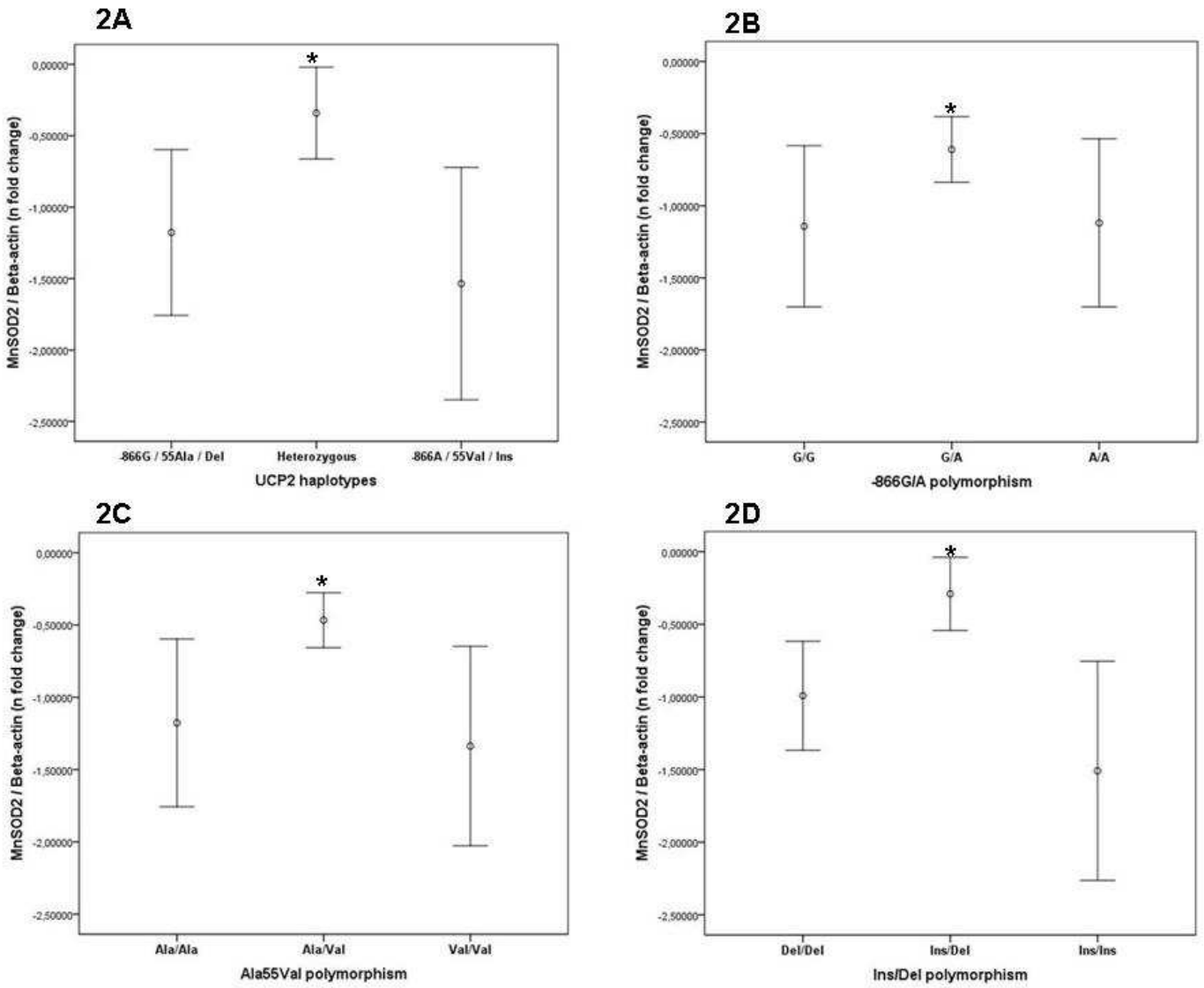

2A
MnSOD2 / Beta-actin (n fold change)
0,00000
-0,50000
-1,00000
-1,50000
-2,00000
-2,50000
*
-866G / 55Ala / Del
Heterozygous
-866A / 55Val / Ins
UCP2 haplotypes
2B
MnSOD2 / Beta-actin (n fold change)
*
G/G
G/A
A/A
-866G/A polymorphism
2C
MnSOD2 / Beta-actin (n fold change)
*
Ala/Ala
Ala/Val
Val/Val
Ala55Val polymorphism
2D
MnSOD2 / Beta-actin (n fold change)
*
Del/Del
Ins/Del
Ins/Ins
Ins/Del polymorphism

Figure 1. *UCP2* mRNA expression in human retinal samples. **A)** *UCP2* gene expression in samples stratified according to *UCP2* mutated (-866A / 55Val / Ins) and reference (-866G / 55Ala / Del) haplotypes. P = 0.137 (One-way ANOVA test). **B)** *UCP2* gene expression in samples stratified according to different genotypes of the -866A/G polymorphism. P = 0.014 (One-way ANOVA test). * P < 0.05 in relation to G/G genotype group (Tukey post hoc test). **C)** *UCP2* gene expression in samples stratified according to different genotypes of the Ala55Val polymorphism. P = 0.007 (One-way ANOVA test). * P < 0.05 in relation to Ala/Ala group (Tukey post hoc test). **D)** *UCP2* gene expression in samples stratified according to different genotypes of the Ins/Del polymorphism. P = 0.613 (One-way ANOVA test). Data are presented as mean ± 2 SE. AU = arbitrary units.

Figure 2. *MnSOD2* mRNA expression in human retinal samples. **A)** *MnSOD2* gene expression in samples stratified according to *UCP2* mutated (-866A / 55Val / Ins) and reference (-866G / 55Ala / Del) haplotypes. P = 0.025 (One-way ANOVA test). * P < 0.05 in relation to both homozygous haplotypes (Tukey post hoc test). **B)** *MnSOD2* gene expression in samples stratified according to different genotypes of the -866A/G polymorphism. P = 0.022 (One-way ANOVA test). * P < 0.05 in relation to G/G genotype group (Tukey post hoc test). **C)** *MnSOD2* gene expression in samples stratified according to different genotypes of the Ala55Val polymorphism. P = 0.003 (One-way ANOVA test). * P < 0.05 in relation to both Ala/Ala and Val/Val genotype groups (Tukey post hoc test). **D)** *MnSOD2* gene expression in samples stratified according to different genotypes of the Ins/Del polymorphism. P = 0.004 (One-way ANOVA). * P < 0.05 in relation to both Del/Del and Ins/Ins genotype groups (Tukey post hoc test). Values are represent as n fold change differences to the calibrator sample ($\Delta\Delta C_T$ method), and are presented as mean ± 2 SE.

S729e Souza, Bianca Marmontel de

Efeito do haplótipo -866A / 55VAL / INS constituído por três polimorfismos no gene da proteína desacopladora 2 (*UCP2*) na expressão deste gene na retina humana / Bianca Marmontel de Souza ; orient. Daisy Crispim Moreira ; co-orient. Luís Henrique Canani. – 2011.

70 f. : il.

Dissertação (mestrado) – Universidade Federal Rio Grande do Sul. Faculdade de Medicina. Programa de Pós-Graduação em Ciências Médicas: Endocrinologia. Porto Alegre, BR-RS, 2011.

1. Diabetes mellitus tipo 2 2. Retinopatia diabética 3. Polimorfismo genético 4. Haplotipos 5. Proteínas mitocondriais I. Moreira, Daisy Crispim II. Canani, Luís Henrique III. Título.

NLM: WK 835

Catalogação Biblioteca FAMED/HCPA