

**LOWER SOMATOSTATIN EXPRESSION IS AN EARLY EVENT IN DIABETIC  
RETINOPATHY AND IS ASSOCIATED WITH RETINAL NEURODEGENERATION**

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## ABSTRACT

**Objective:** To test hypothesis that a reduction of somatostatin (SST) in the retina exists in patients without clinically detectable diabetic retinopathy and that it is associated with retinal neurodegeneration.

**Research Design and Methods:** Human diabetic postmortem eyes (n=10) without clinically detectable retinopathy were compared with eyes (n=10) from non diabetic donors. SST mRNA (RT-PCR) and SST-28 immunoreactivity (confocal laser) were measured separately in neuroretina and retinal pigment epithelium (RPE). In addition, SST-28 (RIA) was measured in the vitreous fluid. Glial fibrillar acidic protein (GFAP) was assessed by immunofluorescence and Western-blot. Apoptotic cells were quantified using terminal dUTP nick-end labeling (TUNEL).

**Results:** A higher expression of SST was detected in RPE than in neuroretina in both groups. SST mRNA levels and SST-28 immunoreactivity were significantly lower in both RPE and the neuroretina from diabetic donors in comparison with non-diabetic donors. These results were in agreement with those obtained by measuring SST-28 in the vitreous fluid of the same donors. Increased GFAP and a higher degree of apoptosis were observed in diabetic retinas in comparison with non-diabetic retinas. These changes were most evident in those patients with the higher deficit of SST.

**Conclusions:** Underproduction of SST is an early event in the eyes of diabetic patients and is associated with glial activation and neural death. In addition, our results suggest that RPE is an important source of SST in the human eye. The possible role of the lower production of SST in the pathogenesis of diabetic retinopathy deserves to be investigated.

Diabetic retinopathy is the most common complication of diabetes and remains the leading cause of new blindness among working-age individuals in developed countries (1). Although diabetic retinopathy has been traditionally viewed as a disorder of retinal vasculature, retinal neurodegeneration may be a primary pathology that gives rise to microvascular changes (2, 3). The degenerative changes in the neuroretina include increased apoptosis, glial cell reactivity, microglial activation, and altered glutamate metabolism. When occurring together, these changes may explain some of the functional deficits in vision that first appear in diabetes even before vascular abnormalities can be appreciated. In fact, abnormal electroretinograms have been found in patients with type 1 diabetes previous to the development of clinically detectable vascular retinal pathology (4), as well as in rats at short time intervals after the onset of experimental diabetes (5).

Somatostatin (SST) is a widely distributed peptide and its diverse biological functions include neurotransmission, antisecretory and antiproliferative activities (6). SST and its receptors are found in the neuroretina of various species, including humans, and there is growing evidence that in the retina SST acts as both a neuromodulator and antiangiogenic factor (7). SST production in the human retina has been attributed to the neuroretina, predominantly in amacrine cells (8). However, it is unknown whether retinal pigment epithelium (RPE) is also able to produce SST.

In recent years we have demonstrated higher levels of SST in the vitreous fluid than in plasma in non-diabetic subjects, and a significantly lower intravitreal concentration of SST was detected in both PDR (9) and DME patients (10) than in non-diabetic control subjects, SST-28 being the main molecular variant accounting for this deficit (11). Although vitreous fluid analysis can be used as a

surrogate for the events that are taking place in the retina, studies to evaluate SST expression in the retina in diabetic patients are needed. Ocular globes from diabetic donors are a unique material that enables us not only to confirm that SST production is impaired in the diabetic retina, but also to explore whether this event occurs in the early stages of diabetic retinopathy.

The aim of the present study was to compare for the first time mRNA SST and SST immunoreactivity in the human retina from diabetic and nondiabetic donors. Retinal apoptosis and glial activation were also investigated. Since SST is produced in the neuroretina and retinal neurodegeneration is an early event in diabetic retinopathy, we tested the hypothesis that a reduction of SST associated with retinal neurodegeneration exists in patients without clinically detectable diabetic retinopathy

## RESEARCH DESIGN AND METHODS

Ten human postmortem eyes were obtained from five diabetic donors found to be free of fundoscopic abnormalities in ophthalmologic examinations performed during the preceding two years. Ten eye cups obtained from five non-diabetic donors matched by age were used as the control group. The eye cups were obtained between March 2005 and January 2006. During this period we obtained 10 eye cups from 5 consecutive diabetic donors and 42 from 21 non-diabetic donors. All diabetic donors were included in the study whereas only 5 non-diabetic donors (10 eyes), closely matched by age were selected. In the medical records of these donors there were no concomitant diseases that could influence circulating SST levels. The main features of both groups are shown in table 1 (online-appendix [available at <http://care.diabetesjournals.org>]). The time elapsed from death to eye enucleation was  $3.8 \pm 1.1$  h. and there were no differences

between diabetic and non-diabetic donors ( $3.4 \pm 1.1$  h. vs.  $4.2 \pm 0.9$  h.,  $p=n.s.$ ).

After enucleation, one eye of each donor was snap frozen at  $-80^{\circ}\text{C}$  and stored until assayed for mRNA and Western blot analyses. The other was fixed in 4% paraformaldehyde and embedded in paraffin for the immunohistochemical study.

All ocular tissues were used in accordance with applicable laws and with the Declaration of Helsinki for research involving human tissue, and were approved by the Ethics Committee of our hospital.

### Somatostatin measurements

#### *mRNA isolation and cDNA synthesis*

Neuroretina and RPE were harvested under the microscopic dissection of isolated right eye cups from donors. Poly A<sup>+</sup> mRNA from tissue samples was isolated using Dynabeads oligo (dT)<sub>25</sub> (Invitrogen, Oregon, USA). mRNA concentration was determined by spectrophotometric measures at 260 and 280 nm. Two hundred ng aliquots of poly A<sup>+</sup> mRNA were reverse transcribed using the Cloned AMV First-Strand cDNA synthesis Kit (Invitrogen, Oregon, USA) following the manufacturer's protocol for oligo (dT)<sub>20</sub> priming. The resulting cDNA was then used in both semi-quantitative and real-time PCR reactions.

#### *Semi-quantitative RT-PCR*

SST mRNA was evaluated by semi-quantitative RT-PCR using 2  $\mu\text{l}$  of the first strand cDNA as template and gene specific primers. Amplification of the cDNA samples with  $\beta$ -actin primers served as an endogenous control. In parallel with cDNA samples, the reactions were also performed without DNA template to exclude contamination of the PCR reaction mixtures. cDNA samples known to contain SST mRNA were amplified as a positive control. To insure that poly A<sup>+</sup> mRNA was not contaminated with genomic DNA, cDNA reactions were also performed without reverse transcriptase and amplified with each primer-pair. PCR was performed using Platinum Taq DNA polymerase (Invitrogen,

OR, USA). Sequences for primers used in semi-quantitative RT-PCR for SST and  $\beta$ -actin were designed using published gene sequences and Primer Select software (DNA Star INC, MA, USA). The sequences of the primer pairs were as follows: (1) SST (size: 270 bp): forward, 5'-GTCCTGGCCCTGGGCTGTGTAC-3'; reverse, 5'-TGCTGTCTCGGGGTGCCATAGC-3'. (2)  $\beta$ -Actin (size: 495 bp): forward, 5'-ATCGTGCGTGACATTAAGGAGAAGC; reverse, 5'-AGAAGCATTGCGGTGGACGAT. The reaction mixture contained: 5  $\mu\text{l}$  10X PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 1  $\mu\text{l}$  10 mM dNTP, 1.5  $\mu\text{l}$  50 mM MgCl<sub>2</sub>, 1  $\mu\text{l}$  of each 10  $\mu\text{M}$  forward and reverse primers, 1 unit Platinum Taq polymerase, and 2  $\mu\text{l}$  of cDNA from RT reaction. Identities of the products were confirmed by direct sequencing using an ABI Prism 3100 Genetic Analyzer (PE Applied Biosystems, Madrid, Spain).

#### *Quantitative RT Real-Time PCR (Q-RT-PCR)*

Quantitative real-time PCR (Q-RT-PCR) was performed using an ABI Prism 7000 Sequence Detection System (PE Applied Biosystems, Madrid, Spain) according to the manufacturer's protocol. The reactions were performed using TaqMan Universal PCR Master Mix (Applied Biosystems, Madrid, Spain), 2  $\mu\text{l}$  cDNA template and Assays-on-Demand<sup>TM</sup> Gene Expression Assay Mix (Hs00174949\_m1; Applied Biosystems, Madrid, Spain) which includes specific probe and primers, in a total volume of 20  $\mu\text{l}$ . Each sample was assayed in triplicate and control negative samples were included in each experiment. Human  $\beta$ -actin was used as an endogenous gene expression control (4333762T, Applied Biosystems, Madrid, Spain).

#### *SST-28 immunofluorescence*

For immunohistochemical study SST-28 rather than SST-14 was selected

because it is more abundant in the vitreous fluid and is the main molecular variant accounting for the deficit of intravitreal SST detected in diabetic patients (11).

Paraffinized eyes were serially cut at 7  $\mu\text{m}$  thickness. Sections were deparaffinized with xylene and rehydrated in ethanol. Sections were then fixed and placed in antigen-retrieval solution (Dako A/S, Glostrup, Denmark) for 20 minutes at 95°C. In order to eliminate the autofluorescence of RPE due to melanin and lipofuscin we used a method described elsewhere (12). Sections were then incubated for 1 h with 1% BSA in 0.3% Triton X-100 in PBS to block unspecific binding of the antibodies and then incubated overnight at 4 °C with a specific primary antibody to human SST-28 (abcam, Cambridge, UK). After washing, sections were incubated with Alexa Fluor® 633 (Molecular Probes; Eugene, OR, USA) secondary antibody at room temperature for 1 h. Slides were cover-slipped with a drop of mounting medium containing DAPI for visualization of cell nuclei (Vector laboratories, Burlingame, USA).

*Image Acquisition:* Images were acquired with a confocal laser scanning microscope (FV1000, Olympus, Hamburg, Germany), using a 633-nm laser line for SST-28 and a 405-nm laser for DAPI fluorophores. Each image was saved at a resolution of 1024 X 1024 pixel image size.

*Image analysis:* In order to quantify SST-28 immunofluorescence in RPE and the neuroretina the total fluorescence intensity values corresponding to ten field frame images (40X NA: 0.9) of each retina sample were measured. These results were then normalized taking into account the area analyzed. All these calculations were made using specific software (Fluoview ASW 1.4). We also measured the thickness of the entire neuroretina corresponding to five fields frame images (40X).

### ***SST-28 in vitreous fluid***

SST-28 levels were measured by RIA (Phoenix Pharmaceutical, Belmont, CA). The antibody used against SST-28 was

highly specific and recognized 100 % of SST-28 and did not have a cross-reaction with either SST-14 or cortistatin (11). The lower detection limit was 20 pg/ml.

Intravitreal proteins were determined by the Bradford method (13) using BSA as the standard.

### **Terminal Transferase dUTP Nick-End Labeling (TUNEL)**

Paraffin-embedded eye sections (7  $\mu\text{m}$  thickness) from diabetic and non-diabetic donors were processed with an apoptosis-detection kit (APO-BrdU TUNEL Assay kit; Molecular Probes; Eugene, OR, USA). The methodology used is described in detail in the online appendix. TUNEL staining in the diabetic retina was compared with that in the non-diabetic retina. For this purpose, each retina was visually scanned with a high power lens (60x) which covers 212x212. The total number of TUNEL positive cells was recorded for each retina in a masked fashion using the values corresponding to 15 fields frame images from 3 retinal sections (5 fields each). The mean value was then standardized (mathematically converted) to a 0.5  $\text{cm}^2$  area. In order to avoid false positive results (13) we have only considered as apoptotic those TUNEL positive cells in which nuclear fragmentation and/or condensation was present. For this purpose we used inter differential contrast (phase-contrast microscopy) and propidium-iodide immunofluorescence.

### **Glial fibrillar acidic protein (GFAP) assessment**

#### ***Western-blot***

Neuroretina samples were transferred into a lysis buffer [(1% triton X-100, 100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1.5 mM EDTA (pH 8.0), 25 mM NaF, 0.5 mM  $\text{Na}_3\text{VO}_4$ , 0.1 mM PMSF and Complete protease inhibitors (ROCHE, Basel, Switzerland)] and then homogenized by sonication. The protein concentration was determined using Bradford assay (Bio-Rad Laboratories AB, Sundbyberg, Sweden). A

total amount of 5 µg of protein from the neuroretina was resolved by 12% SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, USA). The membranes were incubated with a primary antibody against human GFAP (abcam, Cambridge, UK) diluted 1:200000 and further incubated with peroxidase-conjugated secondary antibody (Bio-Rad Laboratories AB, Sundbyberg, Sweden). Proteins were visualized using the enhanced chemiluminescence detection system (Supersignal CL-HRP Substrate System, Pierce, Rockford, IL, USA). The same blot was stripped and reblotted with an antibody specific to β-actin (Santa Cruz Biotechnology, Inc., California, USA) to normalize the GFAP levels. Densitometric analysis of the autoradiographs was performed with a GS-700 Imaging Densitometer (Bio-Rad Laboratories AB, Sundbyberg, Sweden) and analysed with Quantity One 4.6.2 software (Bio-Rad Laboratories AB, Sundbyberg, Sweden).

### **Immunofluorescence**

Tissue sections were incubated overnight at 4°C with the primary antibody chicken anti-human GFAP (1:1000; abcam, Cambridge, UK). After washing, sections were incubated with Alexa Fluor® 488 (Molecular Probes; Eugene, OR, USA) secondary antibody for 1 h. GFAP immunofluorescence in the neuroretina was quantified using a laser confocal scanning microscope. The procedure was the same as mentioned above for SST-28.

### **Retinal thickness**

Retinal thickness was measured in each retina from the apical edge of ONL to the internal limit of ganglion cell layer. Five fields frame image (40x confocal microscopy) were used for each retina and the mean value was calculated.

### **Statistical analysis**

Results are expressed as mean± SD. The comparisons between groups (diabetic

and non diabetic donors) or between tissues (RPE and neuroretina) were performed using the Student-t test. Correlation analyses were performed by using Spearman's correlation coefficient. Levels of statistical significance were set at  $p < 0.05$ .

## **RESULTS**

### **Expression of SST in the human retina. Comparison between diabetic and non-diabetic retinas.**

β-actin mRNA expression was similar in both the RPE and the neuroretina ( $p=n.s$ ). In addition no differences were observed in β-actin mRNA expression between diabetic and non-diabetic retinas ( $p=n.s$ ). Thus, we have calculated SST mRNA gene expression after normalizing with β-actin.

The comparative SST mRNA expression of diabetic and non diabetic retinas is shown in Fig. 1. SST mRNA expression was detected in the retinas from both non-diabetic and diabetic donors, being higher in the RPE than in the neuroretina in all cases. SST mRNA levels obtained in the neuroretina and in the RPE from diabetic donors were lower than those obtained from non-diabetic donors. To evaluate the quantitative expression of SST mRNA a Q-RT-PCR was performed (Fig.1). We detected a significantly higher expression of SST mRNA in the RPE than in the neuroretina in retinas from both non-diabetic donors ( $0.98 \pm 0.02$  vs.  $0.38 \pm 0.24$ ;  $p < 0.001$ ) and diabetic donors ( $0.44 \pm 0.30$  vs.  $0.11 \pm 0.09$ ;  $p = 0.018$ ). SST mRNA levels were lower in both the RPE and neuroretina from diabetic donors in comparison with non-diabetic donors ( $0.44 \pm 0.30$  vs.  $0.98 \pm 0.02$ ;  $p = 0.001$  and  $0.11 \pm 0.09$  vs.  $0.38 \pm 0.24$ ;  $p = 0.018$ , respectively).

### **SST-28 in the retina and in the vitreous fluid. Comparison of diabetic and non-diabetic retinas.**

Laser scanning confocal images of SST-28 immunofluorescence are displayed in Fig.1. SST-28 fluorescence intensity was

significantly higher in the RPE than in the neuroretina in both non-diabetic and diabetic donors (Fig.1). In addition, SST-28 was more abundant in both the RPE and neuroretina from non-diabetic donors in comparison with diabetic donors, but the differences were only at significant levels for the neuroretina.

No differences in intravitreal protein levels between diabetic and non diabetic donors were detected ( $1.24\pm 0.42$  mg/ml vs.  $1.06\pm 0.28$  mg/ml;  $p=0.40$ ). Intravitreal levels of SST-28 were lower, but not statistically significant, in diabetic donors than in non-diabetic donors in absolute values ( $75\pm 16$  pg/ml vs.  $281\pm 144$  pg/ml;  $p=0.20$ ) and also after adjusting by intravitreal proteins ( $56.3 \pm 7.2$  pg/mg vs.  $240\pm 115$  pg/mg;  $p=0.14$ ).

#### **Effect of diabetes in retinal neurodegeneration: apoptosis and glial activation.**

Diabetic retinas had a significantly higher percentage of apoptotic cells than the age-matched non-diabetic retinas both in the RPE ( $0.96\pm 0.31\%$  vs.  $0.16\pm 0.11\%$ ;  $p<0.0001$ ) and in the neuroretina ( $0.36\pm 0.14\%$  vs.  $0.07\pm 0.06\%$ ;  $p<0.001$ ). Furthermore, the number of apoptotic cells /cm<sup>2</sup> was also higher in diabetic donors in comparison with non diabetic donors in both RPE ( $184\pm 78$  vs.  $42\pm 28$ ;  $p<0.0001$ ) and in the neuroretina ( $180\pm 94$  vs.  $40\pm 37$ ;  $p<0.0001$ ).

In the neuroretinas apoptosis was highest in the ganglion cell layer and the lowest in the outer nuclear layer (Fig.3 online-appendix). This finding was observed in both diabetic donors and non-diabetic donors. The total thickness of the neuroretina was significantly lower in diabetic retinas in comparison with non-diabetic retinas ( $103.8 \pm 26.1$   $\mu$ m vs.  $127.1 \pm 26.2$   $\mu$ m;  $p=0.001$ ).

A higher ratio of apoptosis was found in RPE than in the neuroretina. This was true for both diabetic ( $0.96\pm 0.31\%$  vs.  $0.36\pm 0.14\%$ ;  $p= 0.01$ ) and non-diabetic retinas ( $0.16\pm 0.11\%$  vs.  $0.07\pm 0.06\%$ ;  $p<0.01$ ).

Neuroglial activation was demonstrated in diabetic eyes by an increase of GFAP production in the neuroretina. Densitometric analysis of immunoblots showed higher GFAP in the neuroretinas from diabetic donors in comparison with non-diabetic donors (Fig.2). GFAP immunofluorescence was also significantly higher in diabetic than in non-diabetic neuroretinas ( $6131\pm 1857$  vs.  $3038\pm 1590$ ;  $p<0.001$ ) (Fig.2). As previously described, Müller cells acquire prominent GFAP immunofluorescence throughout the extension of their processes in the diabetic retina, thus replacing the normal pattern of GFAP immunostaining (Fig.2).

A significant inverse correlation was observed between SST-28 immunofluorescence and the percentage of apoptotic cells ( $r= -0.87$ ;  $p= 0.002$ ). Similarly, lower SST-28 immunoreactivity was detected in those retinas with higher GFAP expression ( $r= -0.46$ ;  $p=n.s$ ). Finally, a significant direct correlation was found between the percentage of apoptotic cells and GFAP immunofluorescence ( $r= 0.75$ ;  $p= 0.02$ ). Taken together, these findings strongly suggest that the higher the degree of diabetic retinal neurodegeneration, the lower the rate of SST production.

#### **DISCUSSION**

In the present study we provide the first evidence that a significant reduction of both SST mRNA and SST-28 immunofluorescence exists in the diabetic retina and that it is associated with glial activation and neural death. It must be emphasized that the diabetic donors were found to be free of microvascular abnormalities in the ophthalmologic examinations performed in the two years preceding death, thus suggesting that a reduction of SST expression is an early event in the eyes of diabetic patients. In addition, it is worthy of note that we found RPE to be an important source of SST. In fact, both SST mRNA and SST-28 immunofluorescence were significantly higher in the RPE than in the

neuroretina in both non-diabetic donors and diabetic donors.

The precise reason by which diabetic patients have a lower SST mRNA expression remains to be elucidated. The design of our study does not permit us to determine whether the lower expression of SST is a cause or a consequence of retinal apoptosis. However, it should be noted that SST has proapoptotic effects (14). Therefore, in the case that SST decrease was the primary mechanism, a lower rather than higher apoptosis would be expected in diabetic patients. In consequence, our results point to retinal neurodegeneration, and in particular, retinal apoptosis as one of the mechanisms involved in the lower production of SST in diabetic patients. In fact, the higher the number of apoptotic cells, the lower the degree of SST-28 immunoreactivity. Nevertheless, given that SST has neuroprotective effects (15), the lower levels existing in diabetic patients could also contribute to retinal neurodegeneration.

Apoptotic cells have been observed in the neuroretina of diabetic experimental models (2, 16, 17) as well as in human diabetic neuroretinas (2, 18). In the present study, neuronal apoptosis accounted for an 18% reduction in the thickness of the neuroretina, which is similar to that described by Barber et al. in rats after 7.5 months of diabetes (2). Apart from an elevated rate of apoptosis in diabetic neuroretinas we have found that it was even higher in RPE. Apoptosis of RPE is an important event in the pathogenesis of age-related macular degeneration, but it has not been previously reported in diabetic patients. Therefore, future studies are required to investigate the significance and the potential role of apoptosis of RPE in the development of diabetic retinopathy.

As previously reported in experimental (19, 20) and in human studies (17, 21), we have detected an increased GFAP production by Müller cells in diabetic retinas. There is experimental evidence to show that reactive changes in Müller cells

such as up-regulation of GFAP occur early in the course of the disease and precede the onset of overt vascular abnormalities (3). In the present study we have confirmed this finding in human diabetic retinas. The mechanisms and significance of the increased GFAP content in diabetic retinas have not been well established. However, it has been reported that the upregulation of GFAP in Müller cells in the retinas of diabetic rats is inhibited by aldose reductase inhibitors (22, 23), as well as by melatonin (24), suggesting that a hyperactive polyol pathway and oxidative stress contribute to the progression of Müller cell gliosis. In addition, Müller cell gliosis is characterized by responses to the inflammatory milieu present in the diabetic retina (25). It is worth mentioning that we have found a clear relationship between the number of apoptotic cells and GFAP immunoreactivity. This finding supports the concept that diabetes (and its related metabolic pathways) is a common mediator in these two processes.

The role of SST in retinal physiology and in the development of diabetic retinopathy is far from being understood. Apart from its antiangiogenic properties, SST is a neuroactive peptide that influences retinal physiology (26). Recently, it has been demonstrated that SST modulates the NO/cGMP system, a signal transduction system that is of major importance in retinal physiology (27), and dopamine release (28). Furthermore, Yoshida et al (29) in a microarray analysis of genes differently expressed in young and elderly human retinas suggested that SST is essential in regulating retinal function. However, further studies are required to determine the precise relevance of SST deficit in the development of diabetic retinopathy.

Our findings may also have therapeutic consequences. Until now, the rationale for using SST analogues in the treatment of diabetic retinopathy has been based on their capacity to lower circulating IGF-1 levels. However, the role of serum IGF-1 in the development of diabetic

retinopathy is controversial (30), and it is possible that a direct neurotrophic and antiangiogenic effect in the retina rather than an indirect effect caused by a reduction in circulating IGF-1 could be the main mechanism accounting for the effectiveness of SST analogues in diabetic retinopathy (12, 31-34). Recently, we have shown lower levels of SST in DME (10). In the retina, various ion/water transport systems are located at the apical side of RPE adjacent to the subretinal space and indeed a high expression of SSTR-2 has been shown on this apical membrane of the RPE (8). Therefore, SST could modulate the balance of fluid and ion transport through the retina. In addition, SST and SST analogues have anti-inflammatory effects (35). Given that SST has anti-edema and anti-inflammatory properties, it could be postulated that its deficit might be involved in the pathogenic events that lead to DME. Local delivery of SST analogues bypasses the blood-retinal barrier allowing higher SST levels than could be achieved by systemic administration. The lower retinal expression of SST in diabetic donors observed in the present study and our previous observation

that intravitreal SST was also lower in PDR and DME patients lead us to propose intravitreal delivery of SST as a replacement treatment for arresting or preventing both PDR and DME. Obviously, specific interventional studies using intravitreal injection of SST analogues or subretinal injections of viral vector encoding for SST gene therapy will be needed to confirm this hypothesis.

In conclusion, we provide evidence that SST is not only expressed in the neuroretina but also in the RPE. In addition, a reduction of SST expression and production is an early event in the diabetic retina and is associated with apoptosis and glial activation. Our findings suggest that a reduction in SST production could be involved in the development of diabetic retinopathy and thus open up new therapeutic strategies.

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## Figure Legends

**Figure 1.** **A)** Expression of SST mRNA in the retina (RPE and neuroretina) of diabetic and nondiabetic donors. M: size marker. C: positive control (human brain). RPE: retinal pigmented epithelium. N: neuroretina. Human  $\beta$ -actin was used as internal control and, as can be seen, its signal intensity was similar in the retina of diabetic and nondiabetic donors. **B)** Real-time quantitative RT-PCR analysis of SS mRNA in human retinas. Bars represent the mean $\pm$ SD of the values obtained in the five diabetic and the five nondiabetic donors studied. SST mRNA gene expression was calculated after normalizing with  $\beta$ -actin. \*p:  $p < 0.01$ . **C)** Comparison of SST-28 immunofluorescence (red) in the human retina between representative samples from a diabetic donor (a) and non-diabetic donor (b). RPE, retinal pigment epithelium; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. The bar represents 20  $\mu$ m. **D)** Quantification of SST-28 immunofluorescence in non-diabetic and diabetic retinas. \*p:  $p < 0.01$

**Figure 2.** **A)** Western blot analysis of neuroretina corresponding to four representative diabetic and non-diabetic retinas. **B)** Densitometric quantification showing higher GFAP in the retinas from five diabetic donors in comparison with retinas from five non-diabetic donors ( $0.24 \pm 0.19$  vs.  $0.10 \pm 0.05$  arbitrary units/ $\mu$ g total protein,  $p = 0.16$ ). Results are expressed as the mean  $\pm$  SD. The relative intensities of the GFAP bands were normalized by those of the  $\beta$ -actin bands. **C)** Comparison of GFAP immunofluorescence (green) in the human retina between representative samples from a non-diabetic donor (a) and a diabetic donor (b). ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. In the diabetic retina, the Müller cells' endfeet show abundant GFAP immunofluorescence and the radial processes stain intensely throughout both the inner and outer retina. The bar represents 20  $\mu$ m. **D)** Quantification of GFAP immunofluorescence in non-diabetic and diabetic retinas.

**Figure 1**

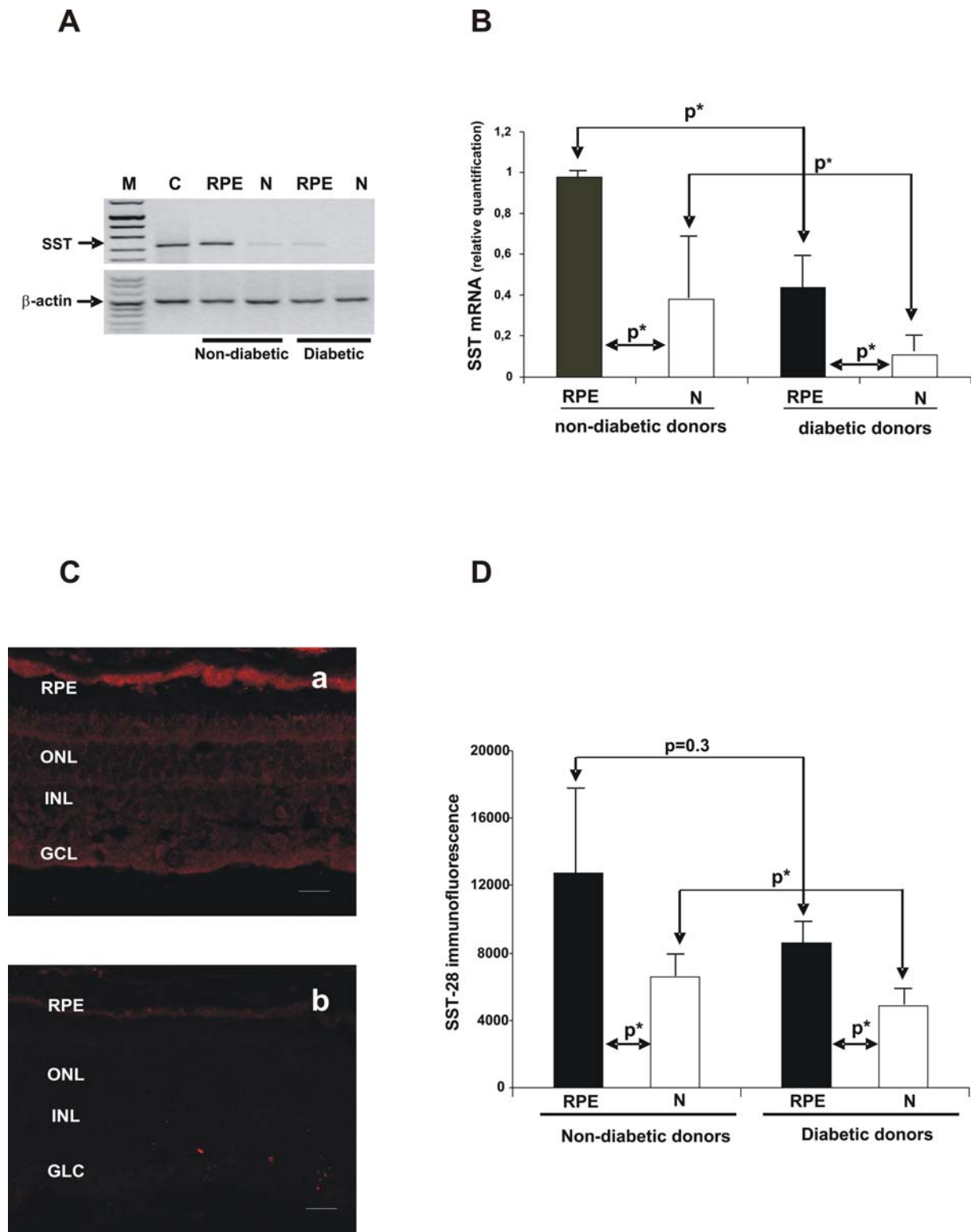


Figure 2

