In vivo Evaluation of Retinal and Choroidal Structure in a Mouse Model of Long-Lasting Diabetes. Effect of Topical Treatment with Citicoline

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Abstract: Recent evidences indicate early, diabetes-driven, retinal neurodegeneration as the origin of diabetic retinopathy. To verify the possibility to prevent the disease, we investigated in a mouse model of type 1 diabetes the effect of long-lasting hyperglycemia on retinal and choroidal structures and, in parallel, we tested the effect of topical treatment with the neuroprotective agent citicoline. Forty wild-type C57B6 mice were included in this study. Diabetes was induced by a single intravenous injection of alloxan. Five animals were considered as sham-treated controls, 15 animals as sham-treated diabetic mice, 5 animals as citicoline-treated controls and 15 animals as citicoline-treated diabetic mice. After eight months of diabetes *in vivo* analysis of the retina was performed using the Spectralis HRA (Heidelberg Retinal Angiography) + OCT. Neuroretinal abnormalities, in particular a significant narrowing of Retinal Nerve Fiber Layer (19.3 ± 2.2 vs 23.3 ± 2.4, μm ± SD, p=0.01), Ganglion Cells/Inner Plexiform Layer (54.3 ± 5.1 vs 62.6 ± 4.0, p=0.03), Ganglion Cells Complex (73.9 ± 4.8 vs 83.8 ± 3.4, p=0.003) and Retinal thickness (223.8 ± 3.9 vs 236.7 ± 5.8, p=0.0004) were detected in the diabetic mouse that showed also a significant reduction of Choroidal thickness (67.4 ± 3.3 vs 84.7 ± 1.9, p=0.0001). In line with the hypothesis that neuroprotection might help preventing diabetic retinopathy, neuroretinal but not choroidal (choroid lacks a neuronal component) dysfunctions were prevented by citicoline. Altogether these findings demonstrate that diabetes-driven neuroretinal dysfunctions can be monitored *in vivo* by OCT in the mouse. Retinal neuroprotection as obtained by topical citicoline protects from these abnormalities suggesting this approach as a possible way to prevent diabetic retinopathy.

Keywords: Diabetes, diabetic retinopathy, neuroprotection, citicoline.

1. INTRODUCTION

Diabetic retinopathy (DR) is a common and specific ocular complication of diabetes mellitus that still represents the leading cause of preventable blindness in working-aged people [1].

Although the retinal microvasculature represents the final target of DR, recent studies suggest that the neuroretina is also affected in early stages of DR and that this phenomenon probably precedes and contributes to the subsequent development of vascular abnormalities [2-4]. Neural dysfunction involves different cells inside the neurovascular unit and typical findings of neurodegeneration, such as glial activation and apoptosis, have been identified ex vivo in retinas of diabetic patients before any biomicroscopic signs of classic DR [5-7].

Altogether these findings suggest that retinal neuroprotection started soon after the onset of diabetes might, at least in principle, have a role in preventing the development of DR. On this regard, citicoline (cytidine 5'-diphosphocholine), an endogenous psychostimulant/ nootropic substance, was shown to have neuroprotective effects through the preservation and restoration of phosphatidylcholine levels and the reduction of phospholipase A_2 activity [8, 9]. Accordingly, citicoline has been successfully used for several years in case of retinal neurodegeneration [10] and, in association with hypotensive therapy, for the treatment of glaucoma [11-13]. To test citicoline as a protective agent against the onset of DR in diabetic patients would reasonably take several years of treatment before reaching a substantial conclusion. To circumvent the problem, we planned to verify the protective effect of citicoline against DR in a mouse model of diabetes. In this case we choose a model of type 1 diabetes to avoid possible interferences of citicoline on insulin secretion (and, consequently, on the onset/progression of DR), as suggested in the literature [14].

Several studies showed that, despite diabetic mice are for some reasons protected from advanced stages of DR, they nonetheless develop early retinal structural lesions commonly seen in diabetic patients [15, 16]. Diabetes-driven ocular alterations have been so far studied only on isolated retinas, after the animals sacrifice. This approach has many limits: the retinal

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status can be evaluated only at the end of the experimental study, it may induce tissue damages and, finally, it offers only a marginal evaluation of the pathophysiology of the disease. The use of *in vivo* approaches like fluorescein angiography (FA) and optical coherence tomography (OCT) could permit to overcome these problems. FA is useful to search *in vivo* for the presence of vascular abnormalities, while OCT procures instead high detailed images of the thickness of the different retinal layers [17].

Aim of our study was therefore to verify the possibility to detect and quantify *in vivo* early retinal abnormalities in a mouse model of long-standing type 1diabetes by means of FA and OCT and to investigate the role of neuroprotection on these dysfunctions.

2. MATERIALS AND METHODS

The experimental study was approved by the Institutional Animal Care and Use Committee (IACUC) of the San Raffaele Scientific Institute in Milan, according to the National Legislation (D.L. 116/1992) and the European Directive (2010/63/EU) about the use of laboratory animals, and with the licence of the Italian Board of Health.

2.1. Animal Model and Experimental Groups

C57Bl/6 female wild-type mice were involved in this study (Charles River Laboratories, Calco, Italy). In particular, 8 weeks old mice (n = 40) were divided into four groups: 5 animals were considered as sham-treated controls (group 1), 15 animals as shamtreated diabetic mice (group 2), 5 animals as citicolinetreated controls (group 3) and 15 animals as citicolinetreated diabetic mice (group 4). For the whole duration of the study, that lasted 8 months, the animals had free access to water and to regular chow. Mice were made diabetic through an intravenous injection of alloxan (72mg/kg, Sigma, Munich, Germany), to selectively destroy the pancreatic *β*-cells [18]. Animals' weight and glycemia were strictly controlled during the entire study; the glycemic level was measured weekly by Glucomonitor (Free Style, Abbott, Rome, Italy) on blood samples (10μL) taken from the tail. In the diabetic group blood glucose level was maintained in a range between 200-400mg/dl by subcutaneous positioning of pellets with slow release of insulin (LinBit, LinShin, Toronto, Canada). This procedure was performed to avoid excessive weight loss and dehydration. The mice were treated with an eye drop/day of citicoline formulation (2%, final concentration of citicoline = 39,19mM, Omikron Italia, Roma, Italy) or buffered saline solution five days at week, during the entire duration (8 months) of the study.

2.2. Spectralis HRA + OCT Examination

In vivo analysis of the retina was performed using the Spectralis HRA (Heidelberg Retinal Angiography, Heidelberg, Germany) + OCT, an instrument that allows to obtain both detailed OCT scans and clear FA images. For the OCT scan capture, the Spectralis represents an integration of two important analytical technique, the cSLO (Confocal Scanning Laser Ophthalmoscopy) and the SD-OCT (Spectral Domain OCT); the result is the acquisition of high quality scans in which the retinal profile and the retinal layers are clearly visible.

On the day of the experiment, anesthesia was induced by a single intraperitoneal injection of 80mg/ml Ketamine, 12mg/ml Xylazine (Sigma-Adrich). To obtain mydriasis, a single drop of tropicamide 0.5% (Visumidriatic, Tubilux Pharma, Pomezia, Italy) was instilled. The mouse was then placed on a stage in front of the lens of the Spectralis system, with a cover slip laid on the cornea previously hydrated by an ophthalmic solution of hydroxyethylcellulose (Gel 4000 2%; Bruschettini, Genova, Italy). For all animals, only the right eye was examined.

The OCT pictures were acquired using a bidimensional scan (B-scan), through an automatic real time (ART) procedure and with a field of view slumped of 30 degrees and focused on the optic nerve head. The scans of the mouse retina were also acquired in EDI (Enhanced Depth Imaging), a method developed by the OCT-Spectralis and useful for a precise study of the choroidal vasculature. In detail, for each animal, a scan of the entire retina was obtained by a reticular pattern of 49 horizontal-equidistant (120μm each) frames; finally, a circular scan around the optic nerve head was also performed to study the retinal nerve fiber layer (RNFL).

OCT analysis was followed by the FA study. 1% fluorescein (5mL/Kg Monico S.p.A., Venezia, Italy) was administered by a single intraperitoneal injection (100μL). For each animal we acquired the images of central and peripheral retinal vasculature.

2.3. Data Analysis

OCT and FA images were compared between the four groups of animal under evaluation.

Manual measurements of retinal and choroidal structures were performed by dropping perpendiculars at intervals of 900μm on both sides of the optic nerve head and averaging their lengths [17]. Figure **1A** shows an OCT performed in a control mouse. Three consecutive retinal scans for every eye were considered to get the averaged measurement of each layer. The different retinal layers evaluated in this study are described in full detail in this figure. Figures **1B**, **1C** and **1D** show OCTs performed respectively in a diabetic mouse, in a control mouse treated with citicoline and in a diabetic mouse treated with citicoline. Image analysis was performed by a single investigator masked with respect to treatment group [19]. To verify the reproducibility of the results, retinal layers were then measured by a second, independent investigator. When compared by linear regression, the analyses performed by the two investigators gave rise to coefficients of correlation (r) between 0.96 and 0.98.

FA images were analyzed at the end of the OCT scan processing. In particular, images of central vasculature (Figure **1F**) were utilized to compare the caliber of vessels between control and diabetic animals, while images of peripheral vasculature were investigated to identify vascular abnormalities related to DR (Figure **1G**). In each animal the diameter of all

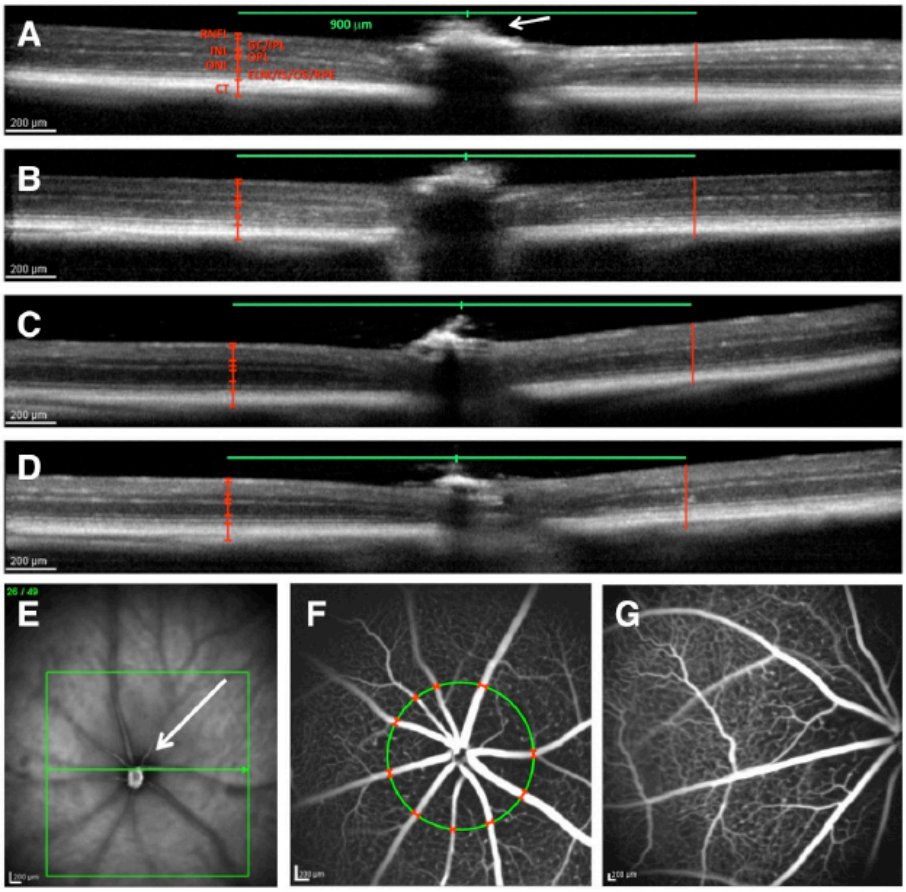

Figure 1: OCT and FA in the mouse. (**A**) OCT acquisition of a mouse eye (control animal), in this panel an example of a manual evaluation of retinal layers is also shown: 1) Retinal Nerve Fiber Layer (RNFL); 2) Ganglion Cells/Inner Plexiform Layer (GC/IPL); 3) Inner Nuclear Layer (INL); 4) Outer Plexiform Layer (OPL); 5) Outer Nuclear Layer (ONL); 6) External Limiting Membrane/Inner-Segment of photoreceptor/Outer-Segment of photoreceptor/Retinal Pigmented Epithelium (ELM/IS/OS/RPE) 7) Choroidal Thickness (CT). In our study we also considered the Ganglion Cells Complex (GCC) constituted by RNFL plus GC/IPL and the Retinal Thickness (RT) consisting of all the retinal layers (from 1 to 6) with the only exception of the choroids (layer 7). The white arrow points to the optic nerve. (**B**) OCT acquisition of a mouse eye (diabetic animal) (**C**) OCT acquisition of a mouse eye (control animal treated with citicoline). (**D**) OCT acquisition of a mouse eye (diabetic animal treated with citicoline). (**E**) Fundus map, the green arrow indicates the area where the scan is performed, the white arrow points to the optic nerve. (**F**) FA image of mouse retinal vasculature (control animal) centered on the head of the optic nerve, the circle indicates the distance from the optical nerve head (1000μm) where the diameter of retinal vessels were measured and (**G**) same as in F, but focused on the peripheral areas of the retina (Scale Bar 200μm).

vessels emerging from the optical nerve head were measured, at a distance of 1000µm from it (Figure **1F**).

2.4. Statistical Analysis

The mean value of all examined parameters was calculated for each animal. Data were analyzed by ANOVA. One-way ANOVA, with Bonferroni's correction, was used to compare the mean values of the different measurements between the four groups of animals. P-value <0.05% was considered to be significant. Linear regression analysis was used to examine association between two variables and to calculate Pearson correlation coefficient. All the analyses were done with the statistical program SAS JMP™.

3. RESULTS

At the end of the study the number of animals in the four groups was slightly different from the beginning. In the group 1 (sham-treated controls; final n=4) one mouse died because of infection, in the group 3 (citicoline-treated controls; final n=4) one mouse died because of anaesthesia just before OCT examination, in the group 2 (sham-treated diabetic mice; final n=10) and 4 (citicoline- treated diabetic mice; final n=12) no mice died but we excluded respectively 5 and 3 mice because they did not develop hyperglycemia despite alloxan treatment.

Data obtained from weekly measurements of weight show that the mean weight during the study did not differ in the four groups considered (Figure **2A**). As expected, diabetic mice both treated or not treated with

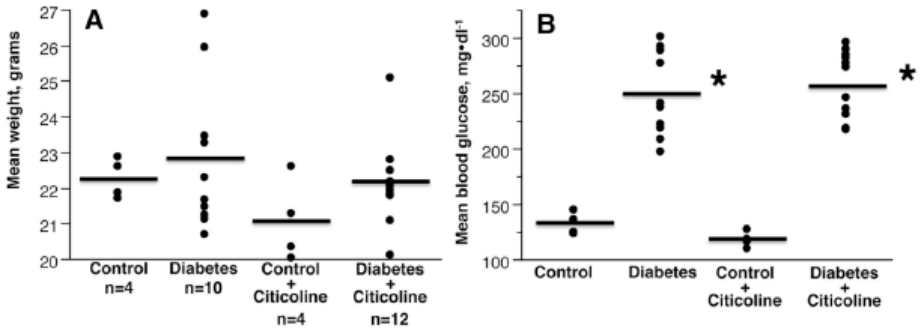

Figure 2: Weight and glucose control. Mean weight (**A**) and blood glucose level (**B**) during the entire study in the four groups of mice considered (*P=0.001).

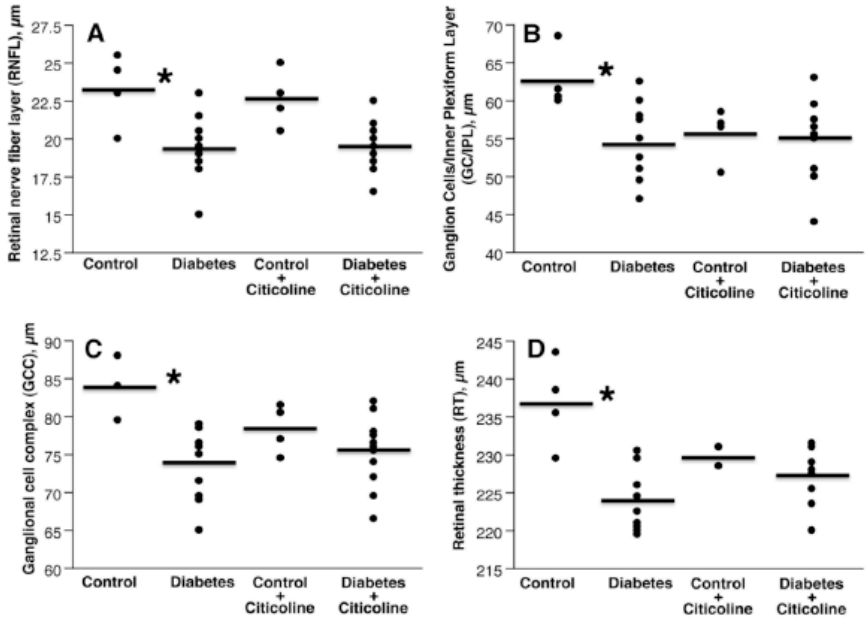

Figure 3: Graphical representation of the thickness of retinal layers performed by OCT at the end of the study. The measurements were performed manually. (**A**) RNFL (*P= 0.02 vs diabetes and 0.01 vs diabetes + citicoline); (**B**) GC/IPL (*P=0.03 vs diabetes and 0.04 vs diabetes + citicoline); (**C**) GCC (*P= 0.003 vs diabetes and 0.01 vs diabetes + citicoline); (**D**) RT (*P= 0.0004 vs diabetes and 0.01 vs diabetes + citicoline).

citicoline showed glycemic levels significantly higher compared to the respective control groups (Figure **2B**).

3.1. *In Vivo* Analysis–Data From OCT

At the end of the 8 months of treatment the retinal status of the animals was studied with an *in vivo* combined imaging examination of the retina, consisting in OCT (Figure **1A-D**) and FA (Figure **1F, G**) analyses.

The results obtained by the manual measurement of retinal layers (see Figure **1A** for full description of the different layers considered) showed a significant reduction of the thickness of RNFL (Figure **3A**), GC/IPL (Figure **3B**), GCC (Figure **3C**) and RT (Figure **3D**) in sham-treated diabetic mice when compared to shamtreated control mice, suggesting that prolonged hyperglycemia directly affects these retinal layers (see Table **1** for details).

Citicoline-treated control mice showed a small, not significant reduction of the thickness of the same layers described above when compared to sham-treated control mice (Figure **3A-D**). Interestingly this phenomenon was not paralleled by a similar reduction of layers' thickness in citicoline-treated diabetic mice. As a result, no difference of layer's thickness (Figure **3A-D**) could be demonstrated between citicolinetreated diabetic mice and citicoline-treated control mice, suggesting a protective effect of citicoline against the toxic effect of prolonged hyperglycemia (see Table **1** for details).

This same conclusion could also be drawn from the evidence that an inverse correlation between mean blood glucose levels measured during the study and RNFL thickness could be demonstrated in shamtreated diabetic mice (Figure **4A**) and that the correlation was lost after treatment with citicoline (Figure **4B**).

Table 1: Thickness of the Retinal Layers Performed by OCT at the End of the Study

Layers (µm)	Control	Diabetes	Control + citicoline	Diabetes + citicoline	P
RNFL	23.3±2.4	19.3±2.2	22.6±1.9	19.5±2.0	0.02 Control vs Diabetes 0.01 Control vs Diabetes+ citicoline
GC/IPL	62.6±4.0	54.3±5.1	55.6±3.5	55.0±4.9	0.03 Control vs Diabetes 0.04 Control vs Diabetes+ citicoline
INL	19.6±2.2	19.6±3.0	19.5±2.2	21.3±3.9	ns
OPL	15.2±0.6	15.4±0.9	16.0±2.1	14.7±0.7	ns
ONL	47.1±3.8	48.2±2.8	48.3±1.0	49.3±1.7	ns
ELM/IS/OS/RPE	67.9±2.0	65.9±2.6	66.4±2.7	66.2±2.6	ns
CT	84.7±1.9	67.4±3.3	81.4±0.7	68.0±7.2	0.0001 Control vs Diabetes, 0.0001 Control vs Diabetes+ citicoline, 0.0005 Control+ citicoline vs Diabetes , 0.0006 Control+ citicoline vs Diabetes. + citicoline

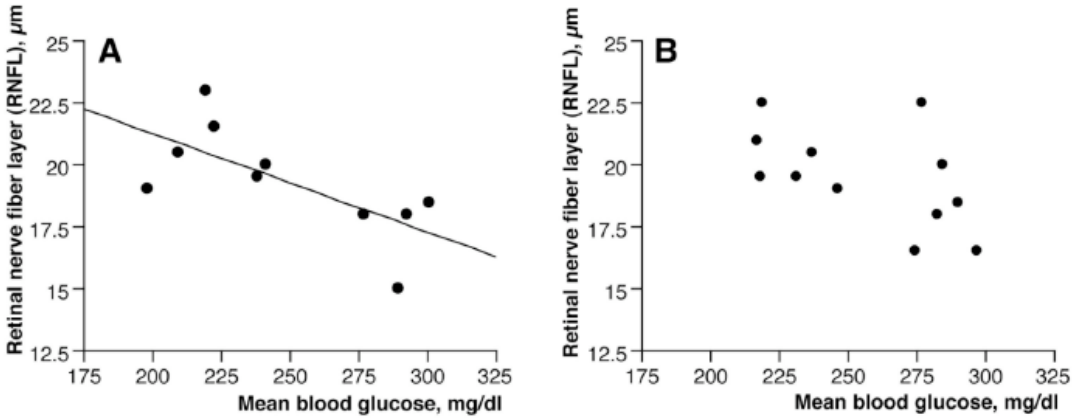

Figure 4: Mean blood glucose and RNFL. (**A**) Inverse correlation between mean blood glucose and RNFL thickness measured in sham-treated diabetic mice (P=0.02, r=0.68). (**B**) The correlation is lost when the same analysis is performed in citicoline-treated diabetic mice (P=0.08, r=0.52).

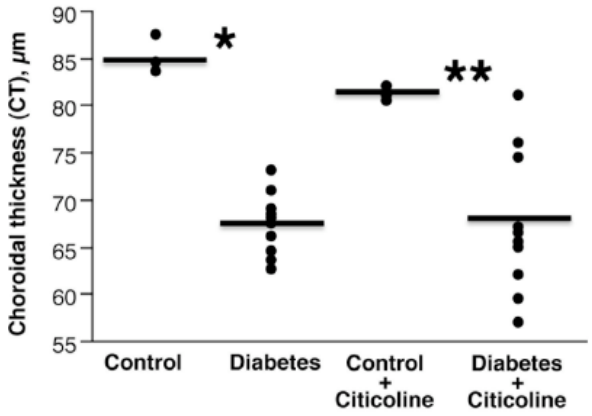

Figure 5: Graphical representation of the choroidal thickness (CT) performed by OCT at the end of the study. *P=0.0001 vs diabetes and 0.0001 vs diabetes + citicoline. **P= 0.0005 vs diabetes and 0.0006 vs diabetes + citicoline.

Of interest, no effect of diabetes (and no effect of citicoline) could be demonstrated in the other retinal layers investigated in this study (INL, OPL, ONL and ELM/IS/OS/RPE, as shown in Table **1**).

From the FA analysis we could not find any vascular sign specific for DR and also the diameter of retinal vessels did not differ among the groups of mice considered in the study (not shown).

Finally, also choroidal thickness (CT) was significantly reduced in sham-treated diabetic mice when compared to their respective controls (Figure **5** and Table **1**). In this case, however, no protective effect of citicoline treatment could be demonstrated (Figure **5** and Table **1**), in line with the hypothesis that the protective effect of citicoline is restricted to the neural component of the retina.

4. DISCUSSION

The results of this study show that OCT is able to detect neuroretinal abnormalities specific to diabetic animals that can therefore be attributed to the hyperglycemic state. Up to now, a number of clinical studies have demonstrated that in early phases of DR there is a pathologic involvement of the neuroretinal structure, observed by OCT in diabetic patients as a reduction of retinal thicknesses [5, 7] and confirmed on histological sections of animal models as a reduction in number of ganglion cells [15, 16]. The innovative component of our study consists in the demonstration that, taking advantage of the OCT, it is possible to observe and to measure *in vivo* in a mouse model of diabetes these glucose-induced retinal abnormalities, a technique that, when applied sequentially, could allow to monitor along time the progression of the disease.

The results obtained from the manual measurement of retinal layers on OCT scans showed a significant narrowing of RNFL, GC/IPL, GCC, and RT layers in the group of sham-treated diabetic animals when compared to the sham-treated control ones. On the contrary, thicknesses of INL, OPL, ONL and ELM/IS/OS/RPE layers were not affected by hyperglycemia. From these data it is therefore possible to conclude that diabetic animals present a reduction in retinal thickness and that this reduction is mostly explained by the involvement of the inner retinal layers. In particular, the narrowing of RNFL and GC/IPL is in line with a possible loss of ganglion cells.

We also verified the effect of the neuroprotective agent citicoline in preventing the early loss of neuronal retinal cells. Two parallel findings seem to confirm the efficacy of this treatment: a) the significant narrowing of RNFL, GC/IPL, GCC and RT layers induced by hyperglycemia is no more detectable in citicolinetreated mice, and b) the inverse correlation between glycemia and RNFL thickness present in sham-treated diabetic mice is lost in their citicoline-treated counterpart.

As shown in Figure **3A-D**, RNFL, GC/IPL, GCC and RT layers tended to be thinner in control animals treated with citicoline. This phenomenon, that did not reach statistical significance, was reasonably explained by a physiologic adaptation of the retina to chronic treatment with citicoline.

Our study also revealed for the first time in the mouse the existence of a specific, diabetes- driven, choroidal dysfunction. In particular, sham-treated diabetic mice showed a significant narrowing of CT when compared to sham-treated control animals, suggesting that hyperglycemia might directly affect the choroidal vascular structure. This finding has an equivalent in the clinical practice: recent studies, in fact, demonstrated a reduction of CT in subfoveal region of diabetic patients [20, 21]. The observation that the same abnormality also characterizes diabetic mice could certainly open new possibilities for future intervention studies in this model. Finally, the finding that citicoline has no protective effect against the diabetes-driven CT thinning is in line with the evidence that choroid, at difference with the retina, lacks the neuronal component.

As described in the results section, the FA did not reveal any difference of retinal structure and vascular diameters between diabetic and control animals. On

this regard, there is increasing evidence in the literature that mice with pharmacologically induced diabetes exhibit a number of features observed in human DR, including alterations in electroretinogram measurements, neural apoptosis and Muller cell activation, although lacking symptoms of vascular pathology [22].

In conclusion, this study demonstrates that diabetic mice are characterized by a narrowing of different retinal structures related to a direct impact of hyperglycemia on the neural component of the retina, in particular on RNFL and GC/IPL. An effect of citicoline in reducing the impact of hyperglycemia on the onset of neuroretinal alterations was also demonstrated.

Further studies are now needed to verify whether the narrowing of neuroretinal layers induced by diabetes are mechanistically linked to the subsequent development of the classic dysfunctions of retinal microvasculature that characterize DR and, as a consequence, if early citicoline treatment might be able to prevent also the "vascular" stage of the complication.

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The authors declare to have no competing interests.

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