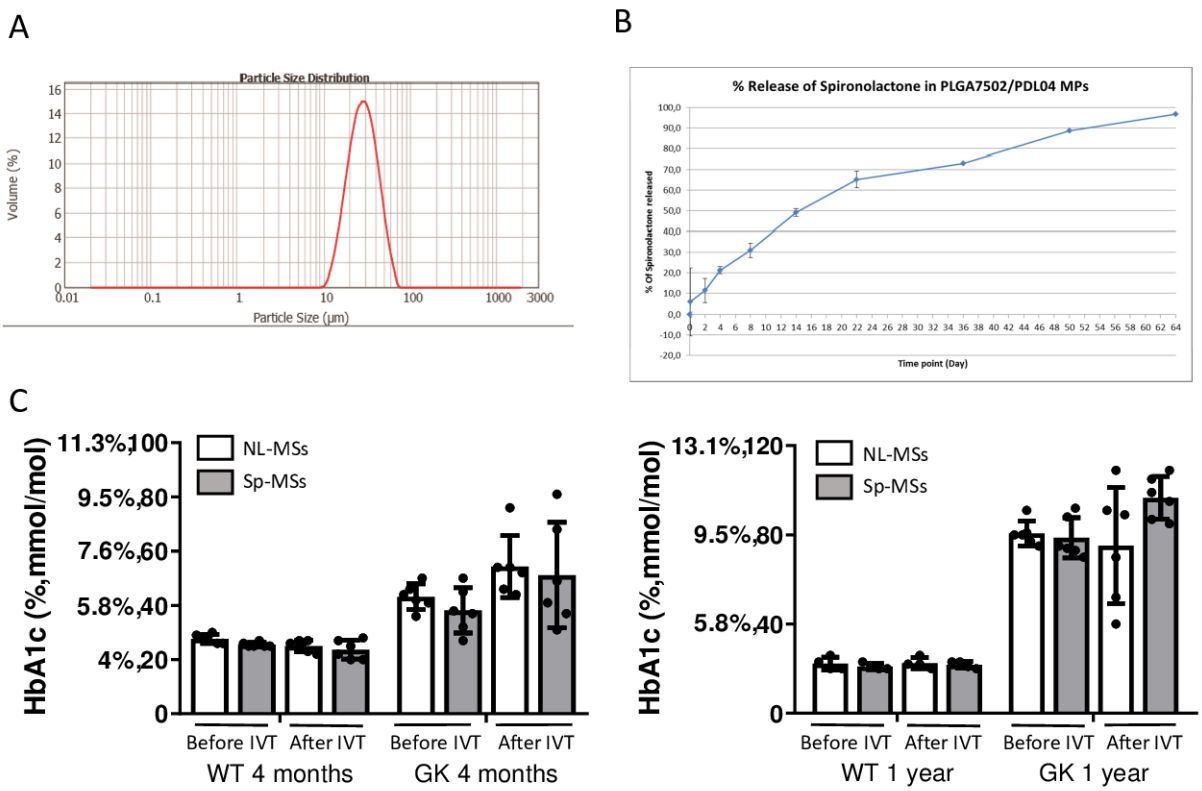
Supplemental Material and data

Supplementary Table 1. QPCR primer sequences.

Gene	Sequences	
Hprt1	Forward	5'- GCG AAA GTG GAA AAG CCA AGT-3'
	Reverse	5'- GCC ACA TCA ACA GGA CTC TTG TAG-3'
18S	Forward	5'- TGC AAT TAT TCC CCA TGA ACG -3'
	Reverse	5'- GCT TAT GAC CCG CAC TTA CTG G -3'
Nr3c2	Forward	5'- TAA GTT TCC CCA CGT GGT TC -3'
	Reverse	5'- ATC CAC GTC TCA TGG CTT TC -3'
Lcn2	Forward	5'- TCACCCTGTACGGAAGAACC -3'
	Reverse	5'- GGTGGGAACAGAGAAAACGA -3'
Plgf	Forward	5'- GTCCTTCTGAGTCGCTGTAG -3'
	Reverse	5'- TTCCTCCTTTCTGCCTTTGT -3'
Vegfα	Forward	5'- TGTGCGGGCTGCTGCAATGAT -3'
	Reverse	5'- TGTGCTGGCTTTGGTGAGGTTTGA -3'
Il18	Forward	5'- GCC TGA TAT CGA CCG AAC A -3'
	Reverse	5'- CCT TCC ATC CTT CAC AGA TAG G -3'
Ccl2	Forward	5'- GAA GCT GTA GTA TTT GTC ACC -3'
	Reverse	5'- TTC TAA TGT ACT TCT GGA CCC -3'
Icam1	Forward	5'- CAA ACG GGA GAT GAA TGG -3'
	Reverse	5'- TGG CGG TAA TAG GTG TAA AT -3'
Lgal3	Forward	5'- GCA ACA CGA AGC AGG ACA AT -3'
	Reverse	5'- CCC TGA GGT TCT TCA TCC GA -3'
Vldlr	Forward	5'- AAG TAT GTG ACC AGG AAC AG -3'
	Reverse	5'- GCA TTC ATC AAT ATC TCC ACA G -3'
Hey1	Forward	5'- CAG GAG GGA AAG GTT ATT TTG -3'
	Reverse	5'- TAG TTG TTG AGA TGG GAG AC -3'
Slc7a1	Forward	5'- AGG TAG ACC AGA ATG ACA TG -3'
	Reverse	5'- ATG ATA AGA ACG GCT AGG AG -3'
Tjp1	Forward	5'- CAC TCT TCC AGA ACC AAA AC -3'
	Reverse	5'- ACC CAC ACT ATC TCC TTT TC -3'
Sesn2	Forward	5'-ATC CTA TGC TTT GTA GAG GAC-3'
	Reverse	5'-TGT TAT AGG TGA GGC TGT AG-3'



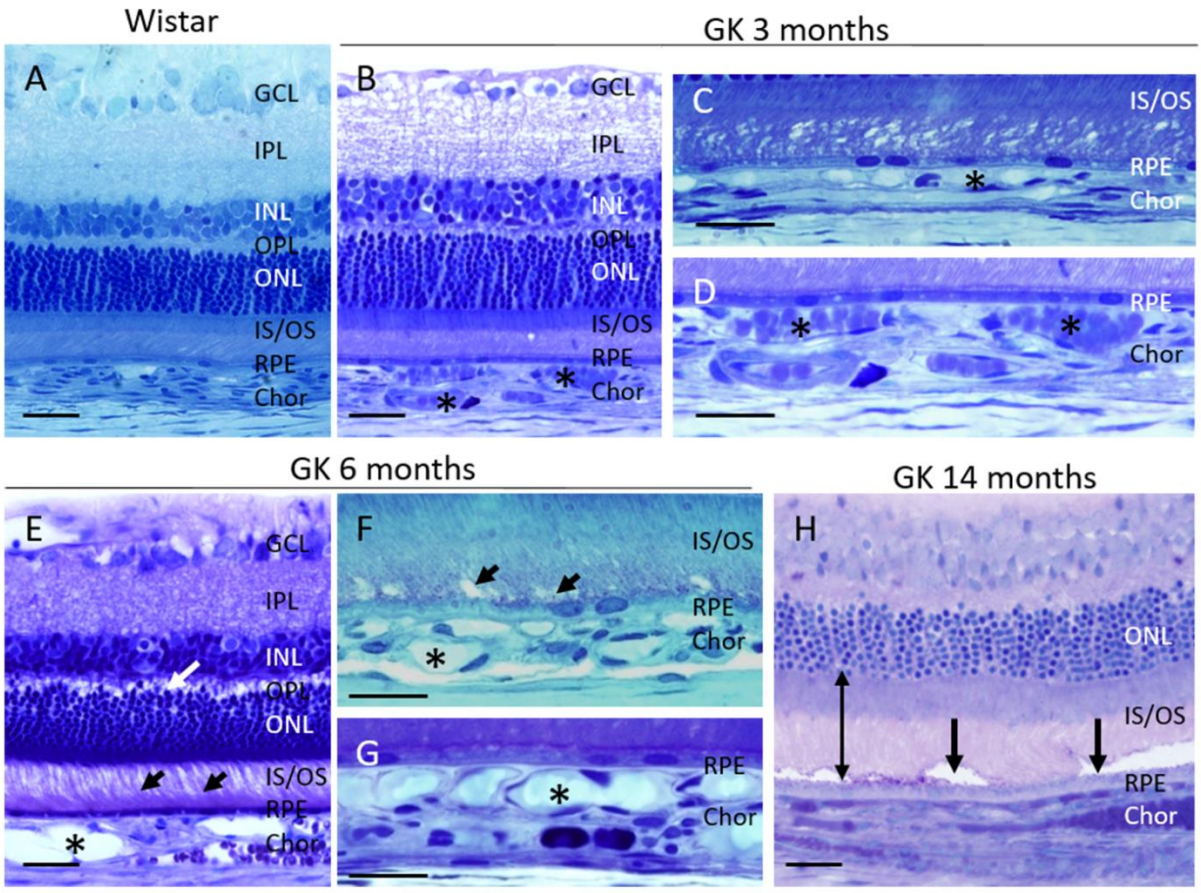
Supplementary Figure 1.

Characteristics of spironolactone-loaded PLGA microspheres (Sp-MSs).

A. Particle size distribution of Sp-MSs.

B. *In vitro* spironolactone release profile shows sustained drug release for over 64 days.

C. MSs and spironolactone released from MSs do not change the HbA1c level in young (left) and old (right) diabetic GK rats.



Supplementary Figure 2.

Morphological alterations in the retina of diabetic Goto-Kakizaki (GK) rats with aging.

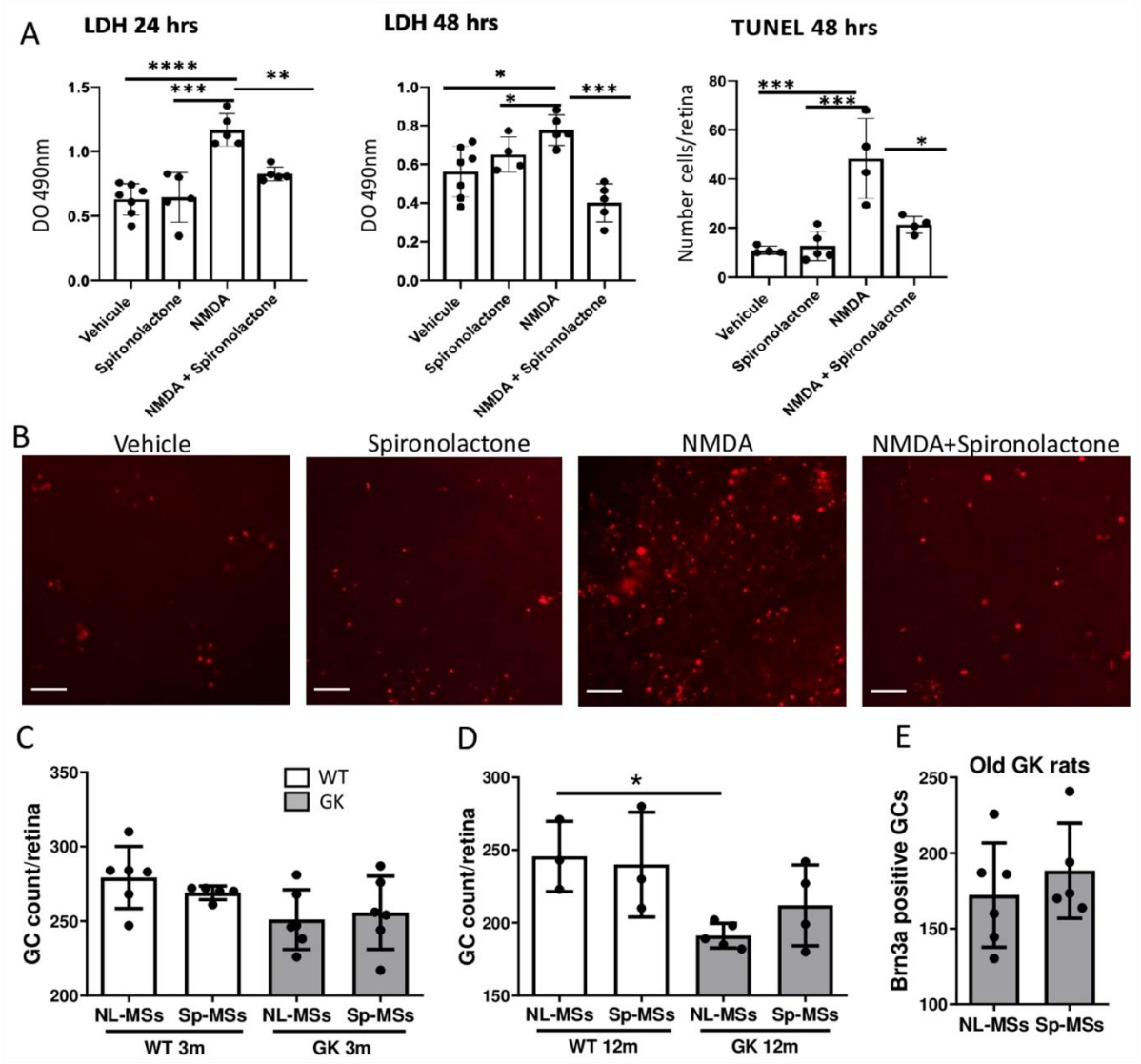
A. In non-diabetic Wistar rats, the retina shows organized layered structure.

B-D. In the retinas of 3 months old diabetic GK rats, the choroidal (chor) vessels are dilated (asterisks in B-D). Fluid is found focally in the photoreceptor inner/outer segments (IS/OS, in C).

E-G. In the retinas of 6 months old GK rats, small cysts (white arrow in E) present in the outer plexiform layer (OPL). Fluid is also accumulated frequently in the photoreceptor IS/OS (black arrows in E and F). Choroidal vessels including choriocapillaris are extensively dilated (asterisks in E-G).

H. In 14 months old GK rats, photoreceptor IS/OS is elongated (double-headed arrow), neuro-retina is separated focally (arrows) from the retinal pigment epithelium (RPE) that witnesses subretinal fluid accumulation.

Retinal sections were stained with 1% toluidine blue. n=3-4 rats per age group. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer. Bars: 50 µm in A, B, E and H; 20 µm in C, D, F and G.



Supplementary Figure 3.

Spironolactone prevents NMDA-induced ganglion cell death in rat neuroretinal explants. One-month Sp-MSs treatment has no effect on ganglion cell survival in GK rats.

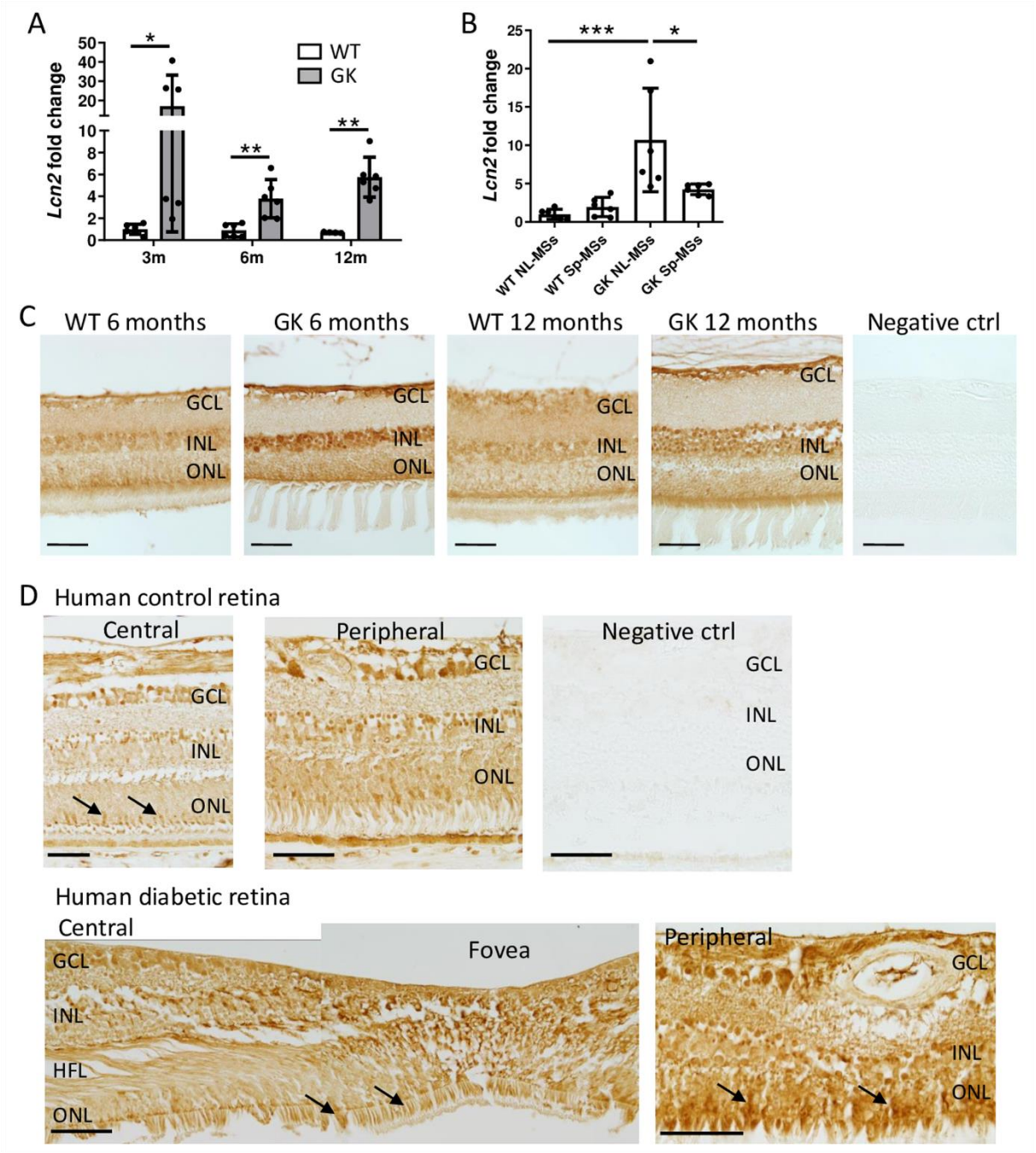
A. Spironolactone reduces the NMDA-induced necrosis in rat retinal explants measured by LDH release at 24 (left) and 48 hrs (middle). n=5-7 retinal explants per group. Quantification of TUNEL positive cells in rat retinal explants at 48 hrs after NMDA exposure shows decreased apoptotic cells in spironolactone-treated group. n=4-5 retinal explants per group. NMDA, exposure to NMDA 100µg/ml then vehicle treatment (0.1% ethanol in medium); NMDA+spironolactone, exposure to NMDA 100µg/ml then spironolactone 10 µM in medium. Control explants were incubated with 0.1% ethanol or spironolactone 10 µM in medium without NMDA exposure. Data are expressed as mean ± SD. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$ (Kruskal-Wallis test).

B. Representative microphotographs of rat retinal explants stained for TUNEL positive cells at 48 hrs after NMDA ± spironolactone. Bar: 50 µm.

C. Quantification of total ganglion cells (GCs) on retinal histological sections shows no difference in the number of GCs between young GK and Wister rats (WT) injected with non-loaded (NL-MSs) or spironolactone-loaded microspheres (Sp-MSs). n=5-6 rats per group. Data are expressed as mean ± SD. Kruskal-Wallis test was used.

D. Quantification of total GC on retinal histological sections shows a decrease in GCs in the retinas of old GK rats compared to WT rats. One month treatment with Sp-MSs cannot increase GC survival. n=3-5 rats per group. Data are expressed as mean ± SD. *, $p<0.05$ using Kruskal-Wallis test.

E. The number of Brn3a positive GCs does not differ between old GK rats treated with Sp-MSs and NL-MSs. n=6 in NL-MSs group and 5 in Sp-MSs group. Data are expressed as mean ± SD. Mann-Whitney test was used.



Supplementary Figure 4.

Increase in neutrophil gelatinase-associated lipocalin (NGAL/LCN2) in diabetic retinas from Goto-Kakizaki (GK) rats and human.

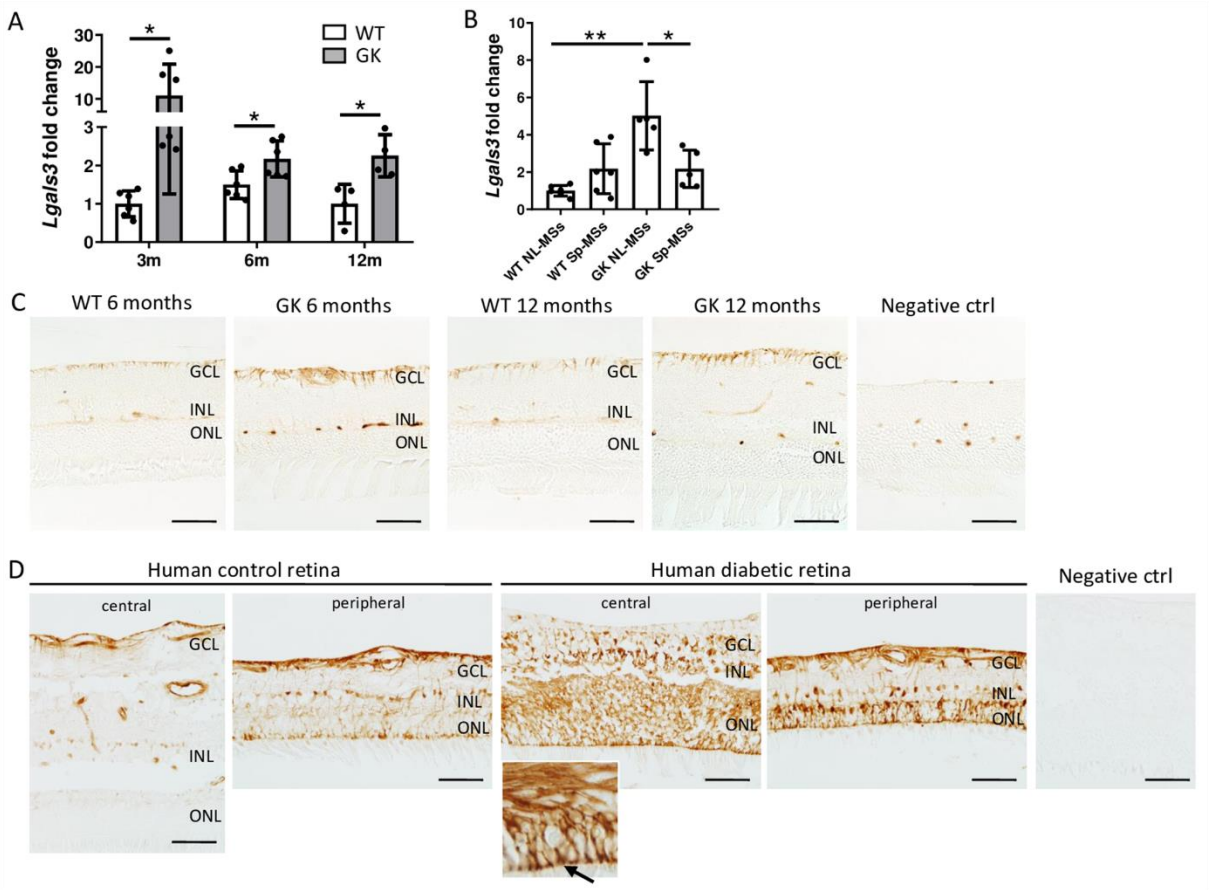
A. *Lcn2* gene expression is up-regulated in the retinas of diabetic GK rats as compared to age-matched Wister (WT) rats. *Hrpt1* and *18S* were used as housekeeping genes. n=5-6 rats per group. Data are expressed as mean ± SD. *, $p<0.05$, **, $p<0.01$ (Multiple t tests).

B. In 4 months old GK rats, intravitreous injection of spironolactone-loaded microspheres (Sp-MSs) down-regulates *Lcn2* expression in the retinas as compared to non-loaded microspheres

(NL-MSs). n=5-6 rats per group. Data are expressed as mean ± SD. *, $p<0.05$, ***, $p<0.001$ (Kruskal-Wallis test).

C. Immunohistochemistry shows enhancement of NGAL/LCN2 expression in the retinas of 6 months and 12 months diabetic GK rats as compared to age-matched WT rats (n=3-4 rats per group). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Bar: 100 µm.

D. In human non-diabetic retinas (n=4), NGAL/LCN2 distributes in all retinal layers in both central and peripheral retina. Allows indicate the cone cells. In the diabetic retinas (n=2), NGAL immunostaining is enhanced all over the retina, particularly in the photoreceptors (arrows). HFL, Henle's fiber layer. Bar: 100 µm.



Supplementary Figure 5.

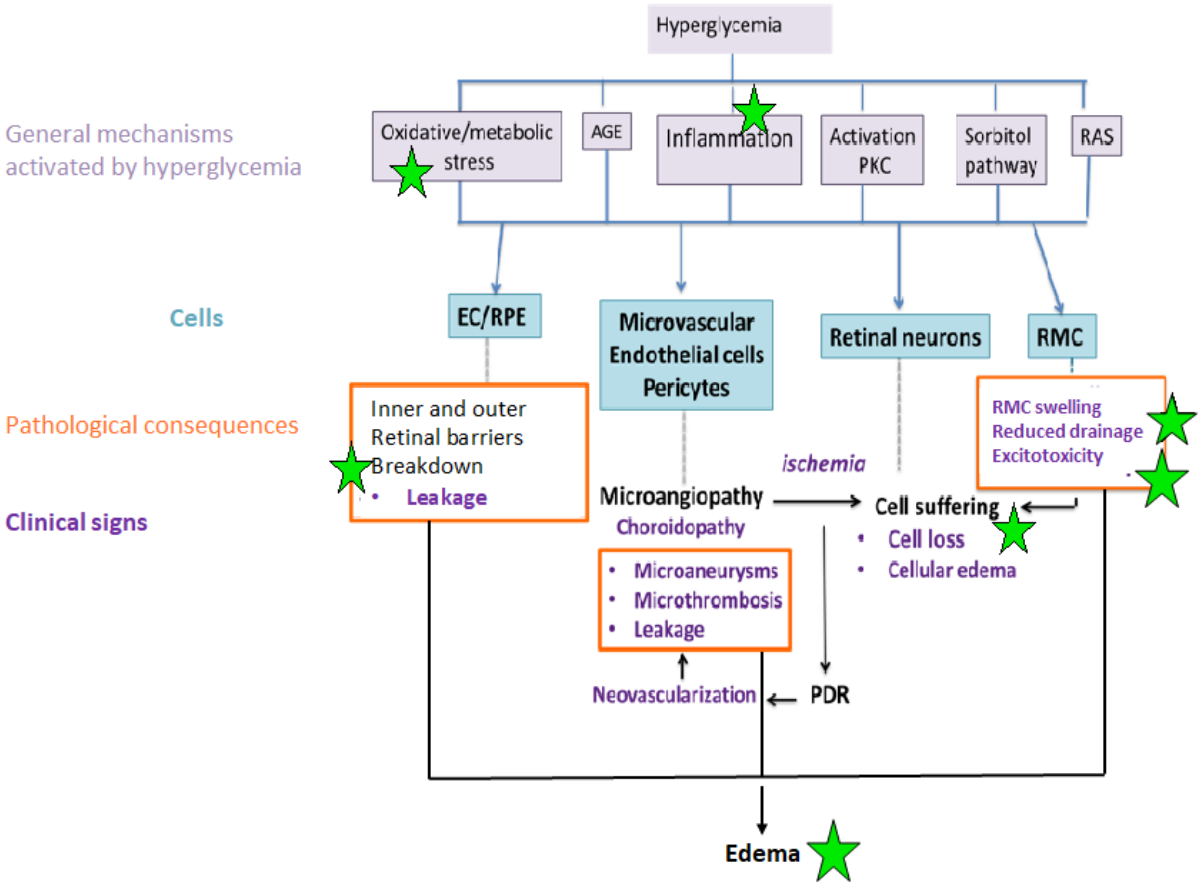
Increase in Galectin-3 in the diabetic retinas from Goto-Kakizaki (GK) rats and human.

A. *Lgals3* gene (encoding galectin-3) expression is up-regulated in the retinas of diabetic GK rats as compared to age-matched Wister (WT) rats. *Hrpt1* and *18S* were used as housekeeping genes. n=4-6 rats per group. Data are expressed as mean ± SD. *, $p<0.05$ (Multiple t tests).

B. Spironolactone released from microspheres (Sp-MSs) down-regulates *Lgals3* expression in the retinas of 4 months old GK rats as compared to non-loaded microspheres (NL-MSs). n=5-6 rats per group. Data are expressed as mean ± SD. *, $p<0.05$, ***, $p<0.001$ (Kruskal-Wallis test).

C. Galectin-3 is restricted to the endfeet of the retinal Müller glial cells in the retinas of Wister rats (WT). It is moderately increased in the diabetic retinas of GK rats at 6 and 12 months (n=3-4 rats per group). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Bar: 100 µm.

D. In human non-diabetic retinas (n=4), galectin-3 localizes in the Müller glial cells, particularly in the endfeet and perivascular processes. Its expression is highly enhanced in all retinal layers all over the diabetic retinas (n=2). Galectin-3 is observed also at the apices of the cells in the outer retina (insert, arrow). Bar: 100 µm.



Supplementary Figure 6.

Potential mechanisms of action of spironolactone on the diabetic retina.

Hyperglycemia causes oxidative and metabolic stress, advanced glycation end products (AGE), inflammation, activation of PKC, the sorbitol pathway and renin-angiotensin-system (RAS) in the retina, which affects different cell types: endothelial cells (EC), retinal pigment epithelial cells (RPE), pericytes, retinal cells, and retinal Müller glial cells (RMC). The pathological consequences are rupture of the inner and outer retinal barriers, microangiopathy, cell suffering and ischemia, RMC swelling, reduced water and potassium drainage and excitotoxicity. This leads to vascular leakage, microvascular abnormalities and thrombosis causing ischemia and proliferative diabetic retinopathy (PDR) with neovascularization and macular edema.

Spironolactone acts on cell that express the receptor (endothelial cells, RPE cells, RMC, retinal neurons and inflammatory cells). It reduces inflammation and oxidative stress, restores ion and water channels and thus drainage mechanisms, it reduces barriers breakdown and the excitotoxic cell death and thus act on retinal inflammation and edema.