PRECLINICAL REPORT

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IRIS PHARMA Study Number: R30F1217-1A

PHYSIOGENEX Study Number: RDFB044

Non-GLP Study

EVALUATION OF DIABETIC RETINOPATHY SYMPTOMS IN SDT FATTY RATS WITH UNILATERAL NEPHRECTOMY

IRIS PHARMA for PHYSIOGENEX

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Report version 1 draft 1 – 18 pages

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1. SUMMARY

The Spontaneously Diabetic Torii (SDT) fatty rat is a new model for obese type 2 diabetes. The aim of the present study was to investigate the effect of an uni-nephrectomy on ocular diabetic complication in SDT fatty rats. Male SDT fatty rats underwent nephrectomy or a sham operation. Sprague Dawley rats with or without nephrectomy were used as control. Animals were subjected to fluorescein angiography to assess vasculature in vivo, to electroretinography to assess neurologic function and to histology to assess glial activity and a breakdown of retinal barrier. Unfortunately in the study, most of the sham operated SDT fatty rat died during the shipment and only comparison between uni-nephrectomized SDT fatty and control SD rat was performed.

Compared with controls, uni-nephrectomized SDT rat showed a neurologic dysfunction and a reactive gliosis in retina. There was no evidence of vascular permeability. Electroretinographic dysfunction began at 12 weeks, manifested by decreased A-wave and B-wave amplitudes, increased implicit time of oscillatory potentials compared with controls. Activation of Müller cells, characterized by GFAP marker, was increased at 17 weeks in uni-nephrectomy SDT fatty in comparison to control SD. Retinal flat mounts of uni-nephrectomized SDT fatty after Evans blue dye perfusion showed retinal vessels dilatation and tortuosity with no evidence of permeability. No significant difference in the incidence of hyperfluorescence area was observed between groups although the center of the optic disc seemed to be larger in SDT fatty rat.

Features of diabetic retinopathy occurred in uni-nephrectomized SDT fatty rat by 12 weeks, establishing a model for assessing novel interventions to treat eye disease. However, these features and the onset of the disease could not be related to the nephrectomy. Additional studies will be required to confirm these preliminary results.

2. OBJECTIVE

The aim of the study was to investigate the effect of an uni-nephrectomy on ocular diabetic complication in SDT fatty rats.

3. ASSAY SYSTEM

All animals were treated according to the Directive 2010/63/UE European Convention for the Protection of Vertebrate Animals used for Experimental [**1**] and Other Scientific Purposes and to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research [**2**].

3.1 Animals

3.1.1 Animals and justification

Species: Rat.
Strain: Sprague Dawley (SD), Spontaneous Diabetic Toriii fatty (SDT fatty).
Age: 9 weeks at the arrival.
Number/sex: 39 males
Breeder: Clea Japan, Inc. Tokyo 153-8533, JAPAN.

3.1.2 Identification

All animals were tagged using a permanent ink marker, following the inclusion examination.

3.1.3 Clinical examination and health status

At the arrival, some rats were found dead: #33 to #40 from the sham operated SDT fatty group and #25, #27 to #30 from the uni-nephrectomized SDT fatty group.

3.2 Housing

3.2.1 Conventional animal husbandry

Animals were housed by two to three in standard cages except the SDT fatty that were housed individually.

All animals were housed under identical environmental conditions. The temperature was held at 22 ± 2°C and the relative humidity at 55 ± 10%.

Rooms were continuously ventilated (15 air volumes per hour). Temperature and relative humidity were continuously controlled and recorded.

Animals were routinely exposed (in-cage) to 10-200 lx light in a 12-hour light (from 7:00 a.m. to 7:00 p.m.) and darkness controlled cycle.

Animal had controlled enrichment as described in internal procedure.

3.2.2 Food and water

Throughout the study, animals had free access to food and water. They were fed a PURINA 5008 dry pellet diet.

Normal tap water, regularly analyzed by specific request of Iris Pharma, was available *ad libitum* from plastic bottles. For some groups, normal tap water was supplemented with 0.3% salt.

4. DESIGN AND PROCEDURE

4.1 Study design

Table 1: Study groups

Group n°	*Strain*	*Condition*	*Animal identification*
1	SD	Uni-nephrectomy and 0.3% salt supplemented water	#1, #3, #4, #5, #6, #7, #8, #8, #10, #11
2		Sham operated	#12 to #20
3	SDT fatty	Uni-nephrectomy and 0.3% salt supplemented water	#21 to #30
4		Sham operated	#31 to #40

Table 2: Schedule table

Age	Endpoints
4-week old	Arrival at Physiogenex
6-week old	Uni-nephrectomy or shame operation
7-week old	Fed blood glucose Beginning of the diet period (0.3% salt supplemented water)
9-week old	Arrival at IRIS PHARMA
12-week old	Body weight, Blood glucose Slit lamp examination Angiography, OCT Electroretinography (ERG)
17-wekk old	Body weight, Slit lamp examination Angiography, OCT Euthanasia Retinal flat mount

4.2 Experimental procedures: In-life part

4.2.1 Anesthesia

Animals were anesthetized by an intramuscular-intravenous injection of a mix Rompun® (xylazine)/ Imalgene® (5 mg/kg; 25 mg/kg).

4.2.2 General clinical signs

4.2.2.1 Body weights

The body weight of all animals was recorded as described in section **4.1**.

4.2.2.2 General appearance

Each day, the general clinical signs and the appearance of all animals were observed.

4.2.3 Ocular examination with a slit-lamp

Both eyes of each rat were examined using a slit lamp as described in section **4.1**. Only abnormal findings were recorded.

4.2.4 Angiography and OCT

Imaging of the eye was performed by using the combined confocal scanning laser ophthalmoscope and spectral domain optical coherence tomography (OCT) imaging device (Spectralis Heidelberg retinal angiography and OCT; Heidelberg Engineering, Heidelberg, Germany) with a 25-diopter lens fitted on a 30-degree angle lens. The pupils of anesthetized rat were dilated with tropicamide eye drops (Mydriaticum®) before image acquisition. Rats were injected sub-cutaneously with a solution of 10% sodium fluorescein (250 µL/100 g body weight).

For fluorescein angiography (FA), the Spectralis HRA/OCT was operated in the fluorescence mode with the excitation light provided by the 488-nm Argon laser. All images were acquired and analyzed on the Heidelberg Eye Explorer Software 1.5.12 with the Spectralis viewing module 3.1.0.

4.2.5 Electroretinography

Electroretinogram recordings were performed on anesthetized animals. Scotopic and photopic ERG were recorded on both eyes obtained using a single flash intensity of 3 cd.s/m^2 on dilated eye (system RETI-animal® from Roland Consult). The amplitude of a- and b-wave were measured. Oscillatory potentials (OPs) were isolated from the averaged recording traces using a 75-300 Hz digital filter. Amplitude of the oscillatory potentials was measured and the sum of the 4 OPs was calculated.

4.2.6 Retinal permeability quantitation

Vascular permeability in the retina was quantitated using an Evans blue quantitation technique. Briefly rats were anesthetized as described in section **4.2.1** and injected by intracardiac injection with a solution of Evans Blue dye (Sigma) at a concentration of 30 mg/Kg. Thirty min after the Evans blue dye injection, rats were euthanized.

4.2.7 In-life period termination

At the end of the measurement period (see section **4.1**), animals were euthanized by an intraperitoneal injection of overdosed pentobarbital following an anesthesia (see section **4.2.1**). This method is one of the recommended methods for euthanasia by the European authorities [**1**].

4.2.8 Sampling

Both eyes were sampled and fixed in 4% paraformaldehyde for 1h. The retina was dissected and flat-mounted. The retina was examined using an Apotom® Zeiss microscope.

4.3 Experimental procedures: histological part

In parallel to this experimental study, eyeballs from control Sprague Dawley and from uni-nephrectomized SDT fatty rats were shipped to Iris pharma and analyzed by histology.

The right eyes were fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. 7 to 10-µm thick sections were stained with hematoxylin and eosin (HE) and used for morphological analysis.

The left eyes were frozen in OCT tissue-tek with liquid nitrogen. 5 to 7-µm thick cryosections were subjected to immunohistochemical detection of vimentin (OMA 106001; Life Technologies) and GFAP (13-0300; Invitrogen). The sections were counterstained with DAPI dye to label all nuclei. The retina was examined by fluorescence using an Apotom® Zeiss microscope.

5. RESULTS

5.1 Animal behaviors

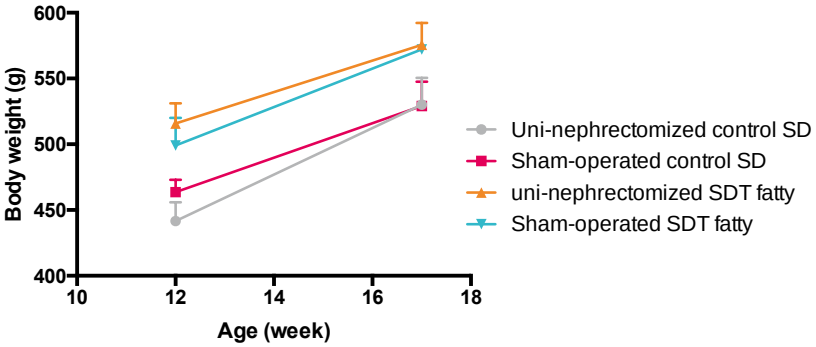
At the arrival, 8 out of 10 rats from the sham operated SDT fatty group and 5 out 10 rats from the uni-nephrectomized SDT fatty group were found dead.

The rat #23 from the uni-nephrectomized SDT fatty group and #32 from the sham operated SDT fatty group were found dead 48 and 24 hours after the systemic anesthesia, respectively.

5.2 Animal body weight

The **Figure 1** illustrates the changes in body weight of the control SD groups and of SDT fatty groups.

Figure 1: Animal body weight (mean ± SEM)



SDT fatty rats were heavier than their SD counterparts at 12-week old. No difference of body weight gain was observed between uni-nephrectomized SDT fatty and uni-nephrectomized control SD was observed. Throughout the 3-week study period, the increase was 12 ± 4% and 20 ± 6%, respectively.

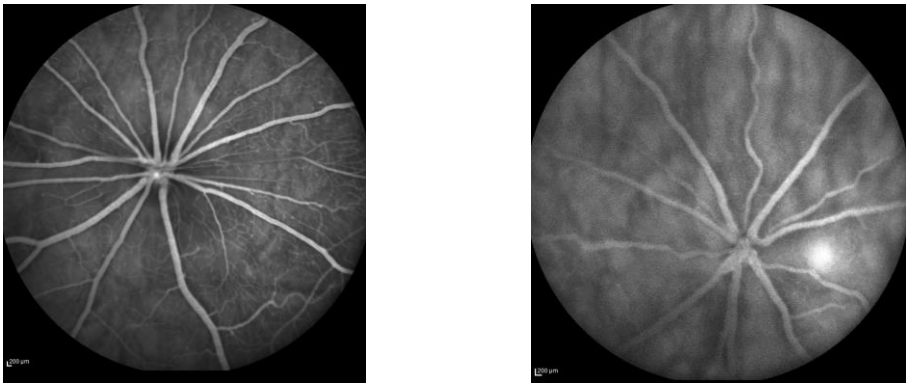
5.3 Ocular examination with a slit-lamp

Ophthalmic examination of the uni-nephrectomized or sham-operated SDT fatty rats revealed no corneal abnormalities at 12 weeks of age as well as 17 weeks of age. Although all lenses in the control groups, sham-operated or uni-nephrectomized SD, appeared to be normal and free of lens opacities, 100% of uni-nephrectomized or sham-operated SDT fatty rats developed mild opacity at 12 weeks. At the end of 17 weeks, the severity of the cataract was increased and no fundus examination could be performed.

5.4 Angiography and OCT

Owing to the rapid development of cataract and the absence of RPE pigmentation (albino strain), the mapping of the retinal vasculature with standard fluorescence angiography was very difficult. No angiogram or OCT could be performed with the rat #26 from the uni-nephrectomized SDT fatty group. Some differences were observed between SDT fatty and control SD rats. The **Figure 2** illustrates the angiograms. In the control SD rat uniform filling of fluorescence was seen and the capillaries were clear. In the SDT fatty rat, the capillaries were not defined properly. An hyperfluorescence dot was observed for the rat#24 (LE). Nevertheless according to the lens opacity observed in the SDT fatty rats it is not possible to related these abnormalities to a retinal permeability. No differences between groups were observed in OCT examination.

Figure 2: Fundus angiogram of FFA examination

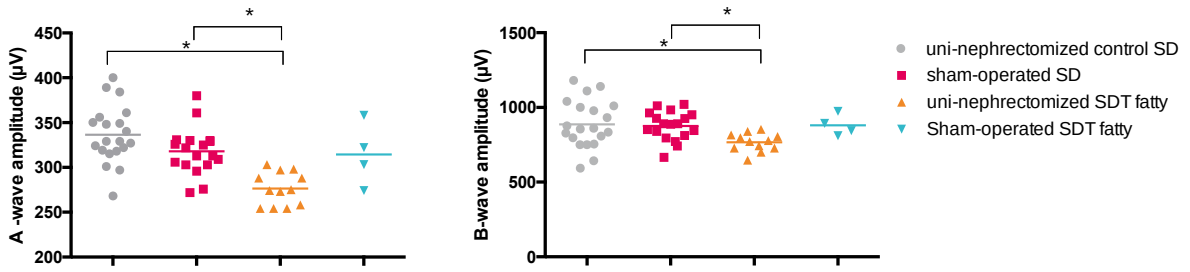


On the left side, rat#3 LE from control uni-nephrectomized control SD rat, on the right side, rat #22 LE from the uni-nephrectomized SDT fatty rat.

5.5 Electroretinography

Results of the standard scotopic ERG recording to a bright white flash (0 log cd s/m2) are presented in **Figure 3** and individually reported in **Table 4**. The A-wave amplitude represents the photoreceptor response and the B-wave the inner retinal cells activity.

Figure 3: Scotopic ERG recording

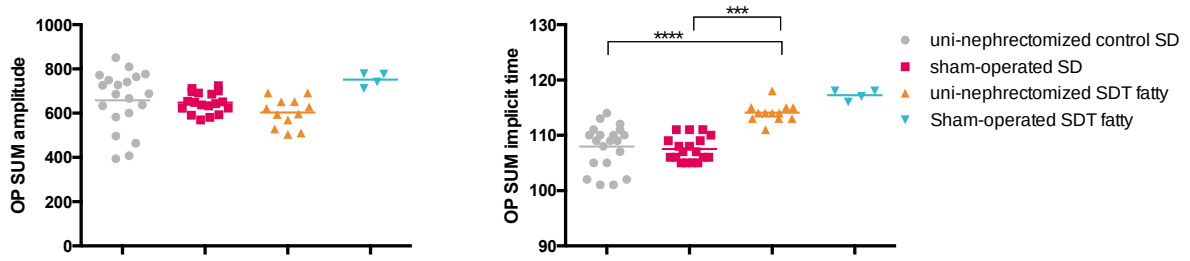


** p<0.05, Dun's multiple comparison test*

The uni-nephrectomized SDT fatty rat displayed significant reduction in A- and B-wave amplitudes in comparison to the uni-nephrectomized control SD rat. Nothing could be noted from the sham-operated SDT fatty rat according to the evaluated number of animals.

Oscillatory potentials (OPs) are high-frequency wavelets observed on the ascending slope of the B-wave and are regarded as reflecting inner retinal function, Therefore, we compared amplitudes and implicit times of the OPs extracted from scotopic responses. The mean overall OP amplitude was defined as the average sum of amplitudes of all four measured wavelets (OP1, OP2, OP3, and OP4) and the mean overall OP implicit time, defined as the average sum of implicit times of all four wavelets. Results of amplitude and implicit time are presented in **Figure 4** and reported in **Table 5**.

Figure 4: Oscillatory Potentials recording



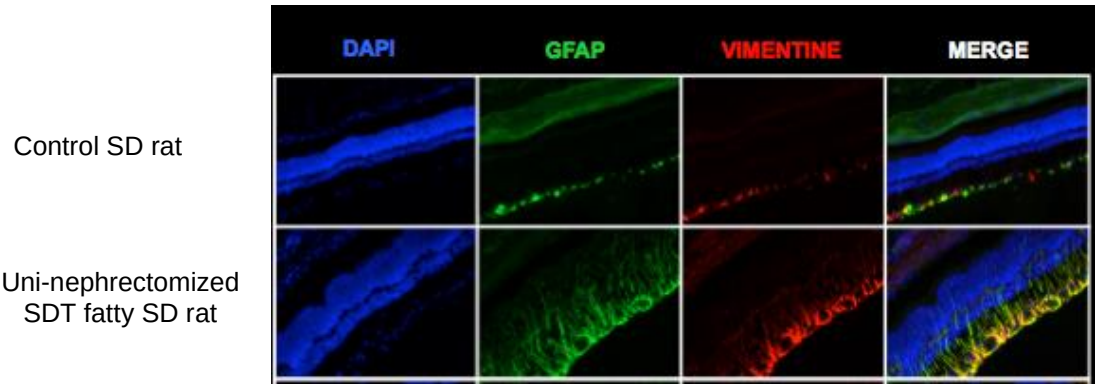
****p<0.005, Dun's multiple comparison test*

The OPs amplitudes were similar between the uni-nephrectomized SDT fatty and the uni-nephrectomized control SD rat. Nevertheless the peaks were significantly delayed.

5.6 Immunohistochemistry: GFAP and vimentin immunoreactivity

Previous published studies have shown that diabetes increases the total content of GFAP in retina. To further examine the effects of diabetes in uni-nephractomized SDT fatty rat, GFAP and vimentin were assessed in sagittal retinal sections. The results are illustrated in **Figure 5**.

Figure 5: GFAP and vimentin immunoreactivity in retinal sections



GFAP is the most widely used indicator of changes in Müller cells. In normal control retinas, GFAP immunostaining was mainly confined to the ganglion cell layer (GCL) where astrocytes and end feet of Müller cells are located. In uninephrectomized SDT fatty rat, GFAP expression increased, with most GFAP-positive processes vertically spanning the entire retina. A similar pattern was also observed for vimentin immunostaining, another marker of Müller cells.

5.7 Retinal vasculature on retinal flat-mount

The results are illustrated in section **8.3** page **14**.

At 17-week old, Evans blue retinal flat-mount of uni-nephrectomized SDT fatty rat showed vessel dilatation and tortuosity. The retinal vessels were larger and an extensive capillary network was visible between branches in comparison to uni-nephrectomized control SD rat. No significant difference in the incidence of hyperfluorescence area was observed between groups although the center of the optic disc seemed to be larger in SDT fatty rat.

6. CONCLUSION

Features of diabetic retinopathy such as retinal function deficit and gliosis of Müller cells were noted and occurred in uni-nephrectomized SDT fatty rat by 12 weeks. By 17 weeks there was no evidence of retinal vascular defect. Retinal vessels dilatation and tortuosity were observed without any evidence of retinal leakage. According to the number of sham-operated SDT fatty rat, these features and the onset could not be related to the nephrectomy. Additional studies will be required. Some additional assessment such as quantitation of inflammatory markers, level of intraocular VEGF level and PKC activity could be considered.

7. REFERENCES

1. French Decree n° 2013-118, dated February 01, 2013 publishing the European directive 2010/63/UE. J. Offic. Rep. Fr. 2013; Text 24 out of 130.

2. ASSOCIATION FOR RESEARCH IN VISION AND OPHTHALMOLOGY (ARVO). Statement for the Use of Animals in Ophthalmic and Vision Research.

3. EUROPEAN AGENCY FOR THE EVALUATION OF MEDICINAL PRODUCTS (EMEA) – COMMITEE FOR PROPRIETARY MEDICINAL PRODUCTS (CPMP). Note for guidance on non-clinical local tolerance testing of medicinal products. Adopted February 2001, CPMP/SWP/2145/00.

8. APPENDICES

8.1 Individual animal body weight

Table 3: Individual animal body weight

Group	Rat identification	12-week old	17-week old
uni-nephrectomized control SD rats	1	474	594
	11	424	498
	3	346	402
	4	430	510
	5	422	520
	6	444	542
	7	504	496
	8	418	636
	9	494	584
	10	460	518
Sham-operated control SD	12	446	566
	13	448	536
	14	486	520
	15	508	500
	16	416	620
	17	444	592
	18	476	482
	19	461	444
	20	488	502
uni-nephrectomized SDT fatty rats	21	570	624
	22	524	530
	23	492	dead
	24	472	562
	26	490	602
	27	546	560
Sham-operated SDT fatty rats	31	520	572
	32	478	dead

8.2 Individual ERG recording

Table 4: Individual A-wave and B-wave recording from 12-week old rats

			Implicit time (ms)		Amplitude (µV)	
Group	**Rat identification**	**Eye**	**a-wave**	**b-wave**	**a-wave**	**b-wave**
uni-nephrectomized control SD rats	1	RE	21	79	329	797
		LE	22	79	340	856
	11	RE	21	78	297	643
		LE	21	80	268	593
	3	RE	22	83	301	750
		LE	22	83	322	808
	4	RE	21	81	324	751
		LE	21	79	329	754
	5	RE	21	83	348	932
		LE	21	84	315	834
	6	RE	21	86	327	860
		LE	21	86	318	828
	7	RE	22	88	389	1000
		LE	21	87	349	877
	8	RE	22	85	400	1010
		LE	21	79	384	978
	9	RE	21	76	356	1140
		LE	20	76	361	1180
	10	RE	20	74	319	1040
		LE	20	76	350	1110
Sham-operated control SD	12	RE	21	82	303	742
		LE	21	83	380	927
	13	RE	21	86	322	926
		LE	21	86	330	983
	14	RE	21	82	306	890
		LE	21	79	272	795
	15	RE	20	72	276	665
		LE	21	72	330	890
	16	RE	20	78	313	812
		LE	20	79	326	845
	17	RE	21	83	331	1020
		LE	21	76	313	950
	18	RE	21	79	303	772
		LE	20	83	309	852
	19	RE	21	83	325	887
		LE	21	81	361	1010
	20	RE	21	79	296	839
		LE	21	77	329	963
uni-nephrectomized SDT fatty rats	21	RE	23	75	288	728
		LE	23	80	254	700
	22	RE	22	72	258	778
		LE	23	72	273	839
	23	RE	23	75	297	816
		LE	23	72	274	743
	24	RE	23	71	288	806
		LE	22	69	275	772
	26	RE	22	71	303	853
		LE	21	70	254	728
	27	RE	22	68	298	797
		LE	22	68	254	647
Sham-operated SDT fatty rats	31	RE	25	73	274	810
		LE	24	73	303	844
	32	RE	24	75	322	893
		LE	24	74	358	974

Table 5: Individual OPs recording from 12-week old rats

			Implicit time (ms)				Amplitude (µV)			
Group	**Rat identification**	**Eye**	**OP1**	**OP2**	**OP3**	**OP4**	**OP1**	**OP2**	**OP3**	**OP4**
uni-nephrectomized control SD rats	1	RE	15	21	30	42	127	102	161	106
		LE	15	22	32	43	110	86	129	82
	11	RE	15	21	31	43	129	94	149	92
		LE	16	22	32	43	110	70	129	85
	3	RE	15	22	30	42	161	139	259	168
		LE	15	22	30	42	155	138	243	149
	4	RE	14	22	31	42	163	128	270	164
		LE	14	21	30	42	158	125	258	147
	5	RE	14	22	31	43	155	127	190	110
		LE	15	22	31	42	149	133	205	113
	6	RE	15	22	31	43	153	149	202	129
		LE	15	22	31	42	142	154	210	131
	7	RE	17	23	31	43	174	131	253	108
		LE	15	21	29	40	179	164	297	169
	8	RE	16	22	31	42	173	164	291	143
		LE	15	21	29	40	173	179	338	160
	9	RE	14	20	28	39	159	144	332	141
		LE	14	20	28	40	168	151	258	163
	10	RE	14	20	28	39	175	146	265	163
		LE	14	20	29	39	188	136	255	185
Sham-operated control SD	12	RE	14	21	29	41	149	115	186	119
		LE	15	21	30	40	146	147	250	181
	13	RE	14	21	35	41	158	132	219	139
		LE	14	21	30	41	159	136	215	113
	14	RE	14	22	30	41	152	153	239	156
		LE	14	21	30	40	138	132	232	148
	15	RE	14	20	29	42	156	153	222	101
		LE	14	21	30	43	126	165	240	112
	16	RE	14	21	29	42	151	159	222	105
		LE	14	21	30	43	146	180	256	114
	17	RE	15	21	30	43	152	123	201	114
		LE	15	21	29	41	125	130	227	140
	18	RE	14	21	30	42	153	128	226	146
		LE	14	21	29	41	161	140	253	157
	19	RE	14	21	32	44	143	149	200	100
		LE	14	21	32	43	156	171	242	121
	20	RE	14	21	32	44	131	148	207	94
		LE	14	21	31	43	142	173	249	121
uni-nephrectomized SDT fatty rats	21	RE	14	23	32	44	105	116	267	139
		LE	15	23	32	43	94	97	245	160
	22	RE	14	23	32	45	112	104	271	134
		LE	15	24	32	43	120	104	280	148
	23	RE	16	25	33	44	110	71	222	125
		LE	16	23	32	43	95	80	207	122
	24	RE	14	24	33	44	112	73	245	139
		LE	15	24	33	43	107	80	224	99
	26	RE	15	23	32	43	135	87	287	182
		LE	14	23	32	42	126	62	259	147
	27	RE	15	24	32	43	129	82	272	208
		LE	14	25	33	43	112	89	257	193
Sham-operated SDT fatty rats	31	RE	16	25	33	43	121	92	304	223
		LE	17	25	33	43	125	100	284	202
	32	RE	17	25	33	43	140	95	329	214
		LE	16	24	33	43	161	105	331	179

8.3 Individual retinal flat-mount

Figure 6: Individual retinal flatmount from uni-nephrectomized SDT fatty rat

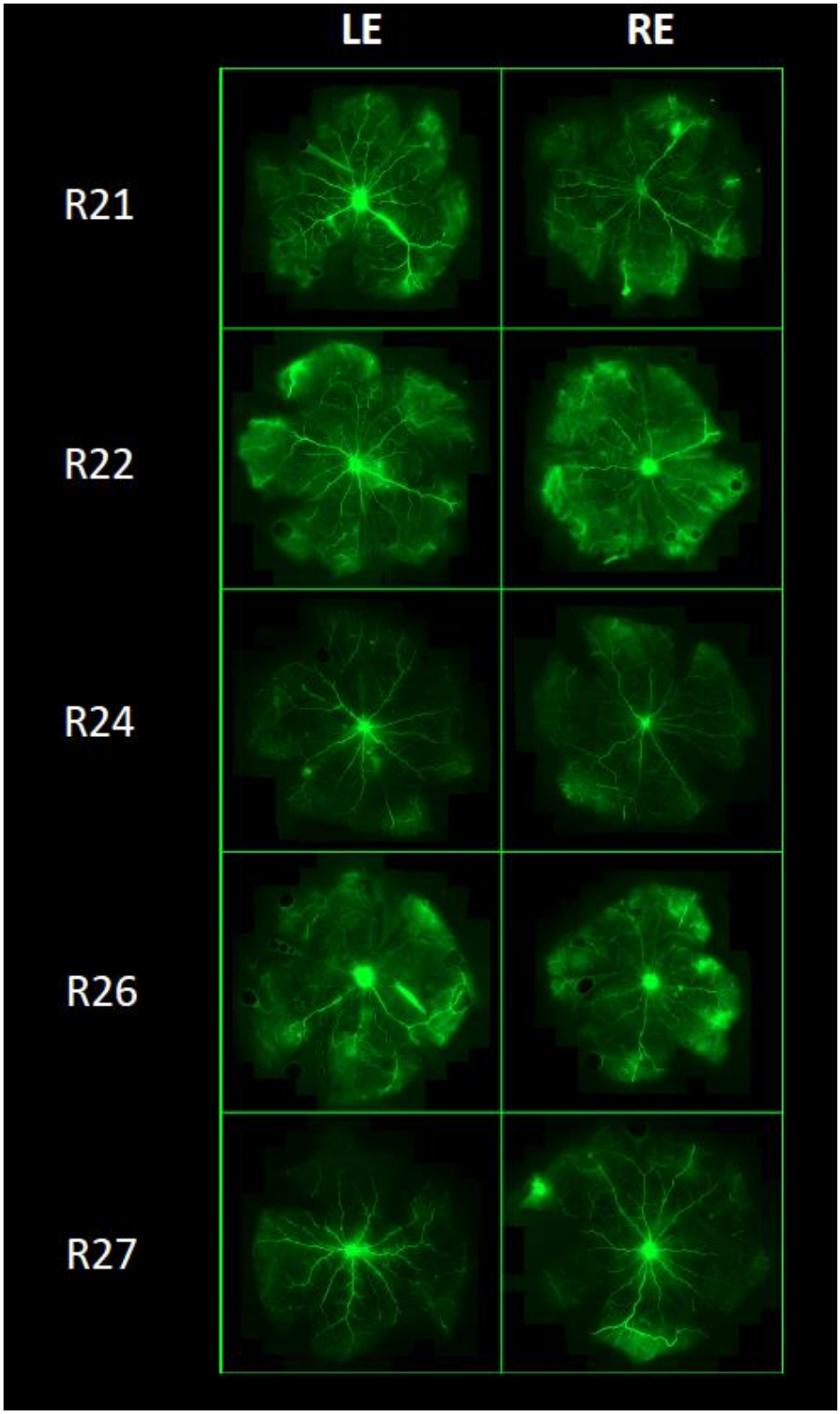


Figure 7: Individual retinal flatmount from sham operated SDT fatty rat

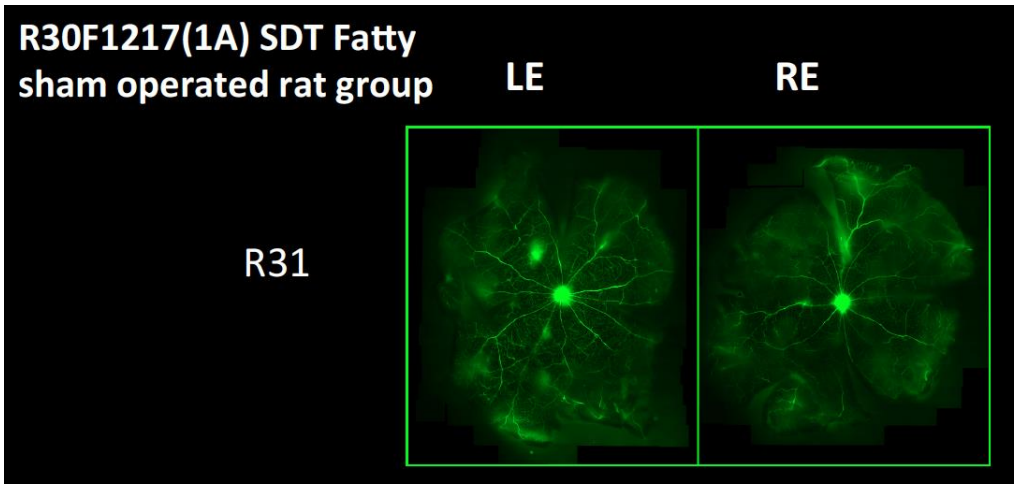
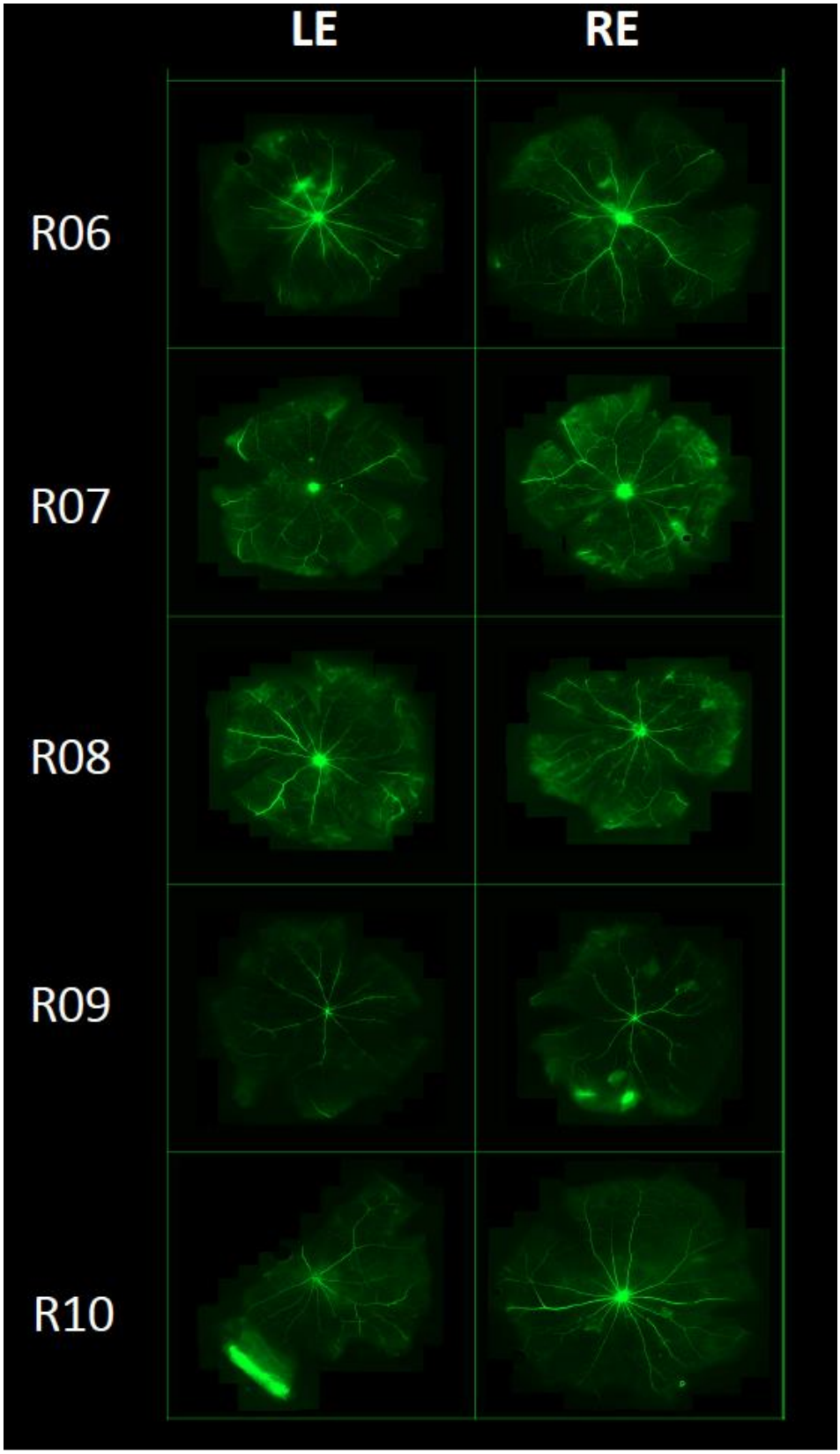


Figure 8: Individual retinal flatmount from uni-nephrectomized control SD rat



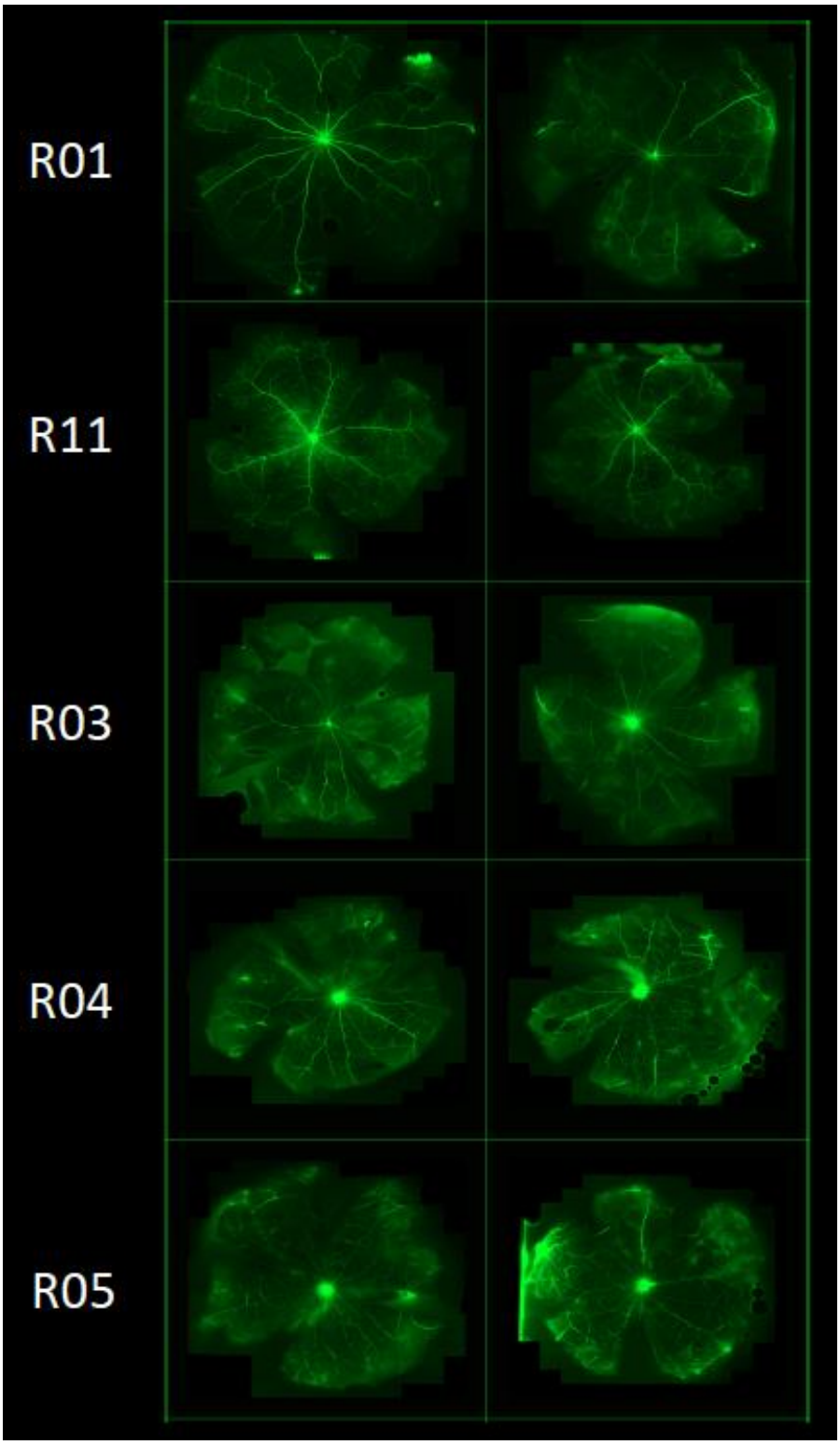

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Figure 9: Individual retinal flatmount from sham operated control SD rat