

Article

Metabolic Syndrome Predisposes Ossabaw Minipig Retina to an Early Neurodegenerative Milieu

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Abstract

The miniature (mini) Ossabaw pigs are proposed as a translational preclinical model for testing and developing novel therapeutics for human diseases, including cystic fibrosis, cancer, and metabolic syndrome (MetS). In recent years, pigs have gained similar attention for studying retinal abnormalities and disorders owing to their close resemblance in size, anatomy, vasculature, and pathology to the human eye compared with their rodent counterparts. In our previous study, Ossabaw minipigs fed a Western diet for 10 weeks and followed for 3.5 months exhibited early signs of retinal degeneration and vascular abnormalities, mimicking the early stages of diabetic retinopathy (DR). To further evaluate pathomorphological alterations across neuronal and non-neuronal cell types, the present study comprehensively investigated individual retinal layers using cell-type-specific immunostaining. We found that the Western diet-fed mini pigs had reduced rhodopsin and blue opsins, changes in bipolar and ganglion cells, and reduced density of pre- and post-synaptic connections. Moreover, the retinas of obese mini pigs showed evidence of gliosis and microglial activation. Our findings suggest that a Western diet-induced metabolic disorder exhibits an early neurodegenerative milieu and further demonstrate the suitability of Ossabaw mini pigs as a model for human retinal diseases associated with MetS, such as DR and diabetic macular edema (DME).

Keywords: retina; metabolic syndrome; ossabaw minipigs; diabetic retinopathy; diabetic macula edema; neurodegeneration



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1. Introduction

Metabolic Syndrome (MetS) is a condition characterized by a cluster of risk factors, including obesity (measured by waist circumference), hyperglycemia, hypertension, elevated triglycerides, and low HDL cholesterol [1,2]. MetS is a major public health concern, affecting one-third of the U.S. population [3]. People diagnosed with MetS are at a high risk of developing cardiovascular diseases, stroke, and type 2 diabetes mellitus (T2DM) [1,4]. MetS has a detrimental impact on multiple organ systems, including the eyes. Various eye diseases, including dry eye syndrome [5], cataracts [6], diabetic retinopathy (DR) [7], diabetic macular edema (DME) [8], central retinal artery occlusion (CRAO) [9], glaucoma [10],

and age-related macular degeneration (AMD) [11], are associated with vision loss and a poor quality of life in MetS patients. Pathological changes in the retina related to MetS may result from systemic factors, such as chronic inflammation [12] and increased oxidative stress [13]. Other significant effects of MetS, such as retinal microvascular injury, retinal microangiopathy [14], and the increased risk of DR, all contribute to retinal disorders.

In recent years, the One Health concept has set the stage for global initiatives that foster interdisciplinary collaboration to accelerate biomedical discoveries advancing human, animal, and environmental health (Centers for Disease Control, Atlanta, GA, USA). For decades, researchers and pharmaceutical companies have predominantly relied on rodent models, which often fail to translate into human outcomes [15–18]. The “One Health” approach has led to the development of various nonhuman primate models, including novel pig models [19–22]. While pigs are more expensive to maintain than rodents, their genetic and anatomical similarities, as well as their physiological and pathological responses, have contributed to a better understanding of human disease progression and mechanisms [21–23]. Pig models have been used as translational pre-clinical models to test new drugs and repurpose FDA-approved drugs for numerous human diseases [24]. In 2022, a pig heart was successfully transplanted into a human in a landmark case [25]. Hence, there is a strong emphasis on developing pig models that mimic human eye disorders.

Ossabaw minipigs are a breed of wild boar that adopted a “thrifty genotype” to survive in the harsh conditions of Ossabaw Island. When fed a Western diet, these pigs develop classical features of MetS, including hypertension, insulin resistance, glucose intolerance, and dyslipidemia [26]. Consequently, they serve as a translational model for studying cardiovascular dysfunction [26,27]. In our previous study, we observed ultrastructural neuronal and microvascular changes in the retina that mimicked early signs of DR pathogenesis [28] and compromised corneal collagens [29] in the Ossabaw minipig after 10 weeks on a Western diet. The present study advances our investigation by examining DR- and DME-related changes in the neuronal retina to establish the Western diet-fed Ossabaw pigs as suitable models for mimicking human retinal disease.

2. Materials and Methods

2.1. Ossabaw Minipigs

Pigs were maintained and tissues collected as described earlier [28]. In brief, Ossabaw minipigs (3.5 months) housed at the Animal Science Research Center at the University of Missouri, Columbia, were fed a Western diet (KT324) containing high-fat/high-fructose corn syrup and a high-calorie content for 10 weeks [30]. Lean pigs consumed a standard commercially available chow 5L80 diet (Lab Diet; 3.03 kcal/kg^{−1}, 71% carbohydrate, 18.5% protein, and 10.5% fat). At six months of age, following a 20 h overnight fast, pigs were anesthetized and euthanized for eye globe collection. To isolate the posterior eye cup, the pig eye was hemi-dissected on ice in a Petri dish with cold PBS to remove the anterior ocular tissues. The vitreous edge of the vitreous body was gently teased away from the retina using forceps until a portion of vitreous detachment was visible. Blunt forceps were then reached into the space between the retina and the vitreous to pull the vitreous body away from the eyecup. An intact vitreous body was extracted from the eyes and dissected within an hour of enucleation.

2.2. Embedding and Immunohistochemistry

The posterior eyecup was submerged in fresh 4% PFA and incubated at room temperature for 24 h. The eyecup was removed and cryopreserved by placing it in 15% sucrose solution for 24 h, followed by 30% sucrose solution for an additional 24 h. To embed, the eyecup was blotted dry, then submerged in Poly Vial (Electron Microscopy Sciences,

Washington, PA, USA) containing fresh OCT for 10 min to equilibrate. Markings were made on the OCT mold to ensure accurate orientation during sectioning. Eyes in OCT were placed in 2-methylbutane and kept over liquid nitrogen to ensure even freezing. Eyes were stored at -80°C freezer for at least 24 h before sectioning. Due to the horizontal band of dense retinal ganglion cells dorsal to the optic disk [31], the eye cup was halved in sagittal to ensure an accurate representation of the entire retina. The 15 μm -thick sections were collected onto coated glass slides. Immunohistochemistry was performed according to the standard procedure described earlier [28]. In brief, slides were washed in PBS for 5 min and blocked with 5% donkey serum. Antibodies (Table 1) were diluted in 5% donkey serum and incubated on tissue sections at 4°C overnight. Following three PBS washes, retinal sections were stained with Alexa Fluor secondary antibody (Thermo Fisher Scientific, Waltham, MA, USA) for 1 h at room temperature and mounted with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA, USA).

Table 1. Primary antibody used for retinal cells and subcellular structures in immunostaining.

Antibody	Marker	Company	Host	Primary Antibody	Secondary Antibody
NeuN	Ganglion cells	Thermo Fisher Scientific (MA5-33103)	MC Mouse IgG2b	1:100	1:200
GFAP	Astrocytes/ Muller gliosis	Novus Biologicals (NB300-141)	PC Rabbit IgG	1:100	1:500
GS	Muller cells	MilliporeSigma (G2781)	PC Rabbit IgG	1:100	1:200
Iba-1	Microglia	Wako (091-19741)	PC Rabbit IgG	1:100	1:200
Parvalbumin	Amacrine cells	MilliporeSigma (MAB1572)	MC-Mouse IgG	1:100	1:200
Calbindin	Horizontal cells	MilliporeSigma (C9848)	MC-Mouse IgG1	1:100	1:200
Protein Kinase C alpha	Rod bipolar cells	MilliporeSigma (P5704)	MC-Mouse IgG2a	1:50	1:50
CRALBP	Retinoid binding protein	Abcam (ab1503)	MC-Mouse IgG1	1:100	1:200
Postsynaptic density protein (PSD95)	Post Synapses	Thermo Scientific (MA1046)	MC-Mouse IgG1 kappa	1:100	1:200
Synaptophysin	Presynaptic Vesicle	Sigma (S5768)	MC-Mouse IgG1	1:100	1:200
Recoverin	Phototransduction	MilliporeSigma (AB5585)	PC Rabbit IgG	1:100	1:200
Blue-opsin	Cones	MilliporeSigma (AB5407)	PC-Rabbit IgG	1:50	1:200
Red/Green-opsin	Cones	MilliporeSigma (AB5405)	PC-Rabbit IgG	1:100	1:500
Rhodopsin	Rods	ChemiconSigma (MAB5356)	MC-Mouse IgG1	1:20	1:200
Ezrin	RPE	Abcam (ab41672)	PC-Rabbit IgG	1:100	1:1000

2.3. Imaging and Quantitative Analysis

The retinal sections were imaged using a Keyence BZ-X810, all-in-one Fluorescence microscope, using BZX Analyzer Software (version 10.6.1) BZ-H4Xe (Keyence Corporation of America, Itasca, IL, USA). Imaging settings were kept identical for Lean and MetS retinal sections, and similar areas within the posterior eyecup, peripheral to the visual streak, were imaged to ensure similar neuronal cell distribution. Image quantification was performed

using ImageJ macros (NIH, Bethesda, MD, USA). Background subtraction was performed for images, followed by threshold adjustment. The region of interest (ROI) was selected using the rectangular tool, and similar dimensions were applied to both experimental groups. Watershed was enabled, followed by particle analysis. The Percentage Area was obtained from the output [32], and the integrated density was calculated by multiplying the average area by the mean threshold value [33]. For Iba-1-stained images, amoeboid microglia were identified and manually counted to determine the total number of amoeboid microglia (cells/mm²), as described earlier [34].

2.4. Statistical Analysis

Data analyses were performed and plotted in GraphPad Prism 10.6.1 software (GraphPad Software, Inc., La Jolla, CA, USA). A comparison between the two groups was performed using Student's *t*-test, with *p* < 0.05 considered statistically significant. Individual data points are plotted and presented as the mean ± SD.

3. Results

Ossabaw minipigs fed a Western diet for ten weeks exhibit pathomorphological and neurodegenerative damage throughout the MetS retina.

3.1. Retinal Pigment Epithelium (RPE)

To understand the integrity and structure of retinal pigment epithelium in the MetS group, cellular retinaldehyde-binding protein (CRALBP, Figure 1a,b) and ezrin (Figure 1e,e',f,f') antibodies were used for immunostaining (Figure 1). CRALBP, a retinoid-binding protein expressed in the RPE and Müller glia [35], showed no differences in the RPE but had reduced intensity in the inner retina. Although there was a downward trend, the overall percentage of stained area and intensity in pigs fed a Western diet did not achieve statistical significance. (Figure 1c,d). Ezrin, a critical polarization marker in vertebrate intraocular neuroepithelial cells [36], was localized to the RPE apical process, which was seen to be noticeably denser in the Western diet-fed pigs (Figure 1f'). However, no significant differences in area percentage or integrated density were observed (Figure 1g,h).

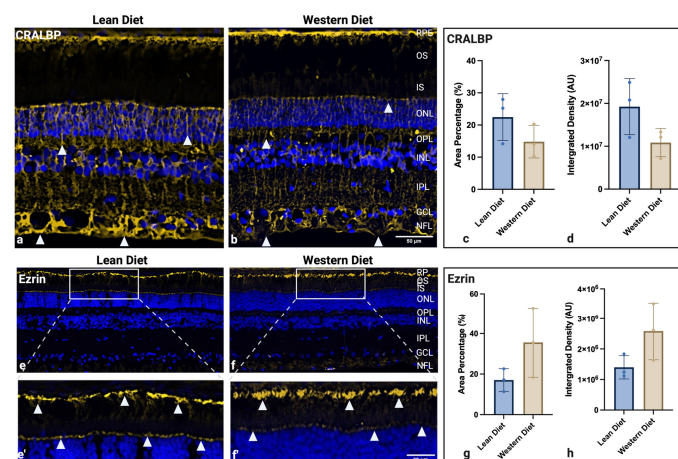


Figure 1. Retinas of Ossabaw minipigs fed a Lean or Western diet were immunostained for CRALBP and Ezrin, a marker of RPE. (a): CRALBP in Lean diet displays healthy morphology of retinoid-binding proteins (white arrowhead). (b): The Western diet revealed similar staining in RPE, but reduced retinoid-binding proteins from the outer nuclear layer (ONL) through the nerve fiber layer (NFL) (white arrowhead). (c,d): No significant differences in area percentage or integrated density. (e,f): Ezrin is visible in both groups, as shown by RPE staining. (e',f'): Thickened RPE apical processes seen in the MetS Western diet group (arrows). (g,h): Increased staining in the Western diet-fed pigs did not reach significance. Data represented as Mean ± SD (n = 3).

3.2. Retinal Photoreceptors: Rods and Cones

To visualize photoreceptors, the retina was stained for rhodopsin [37], blue opsin [38], red/green opsin [39], and recoverin [40] (Figure 2). Rhodopsin, a rod-specific pigment in the outer segment (OS) (Figure 2a,a',b,b'), showed reduced area percentage ($p = 0.0410$; Figure 2c) and integrated density ($p = 0.0155$; Figure 2d) in the retinas of minipigs fed on a Western diet, suggesting Rod degeneration. Notably, increased rhodopsin stain was seen in the nerve fiber layer (NFL) of the MetS pigs (Figure 2b). Next, we investigated various cone opsins, including blue (Figure 2e,e',f,f') and red/green opsins (Figure 2i,i',j,j'). In blue opsin, a reduced expression was observed in area percentage ($p = 0.0051$; Figure 2g) and integrated density ($p = 0.0050$; Figure 2h). Likewise, red/green opsins were shown to be less dense in the Western diet-fed pigs, but no significant differences were observed (Figure 2k,l). Finally, recoverin, a calcium-binding protein marker involved in phototransduction, was used to visualize cones and rods from the OS to the outer plexiform layer (OPL). Similar staining patterns were seen in both pigs (Figure 2m,m',n,n'), though a non-significant increase in percentage and integrated density was seen in the Western diet-fed pigs (Figure 2o,p).

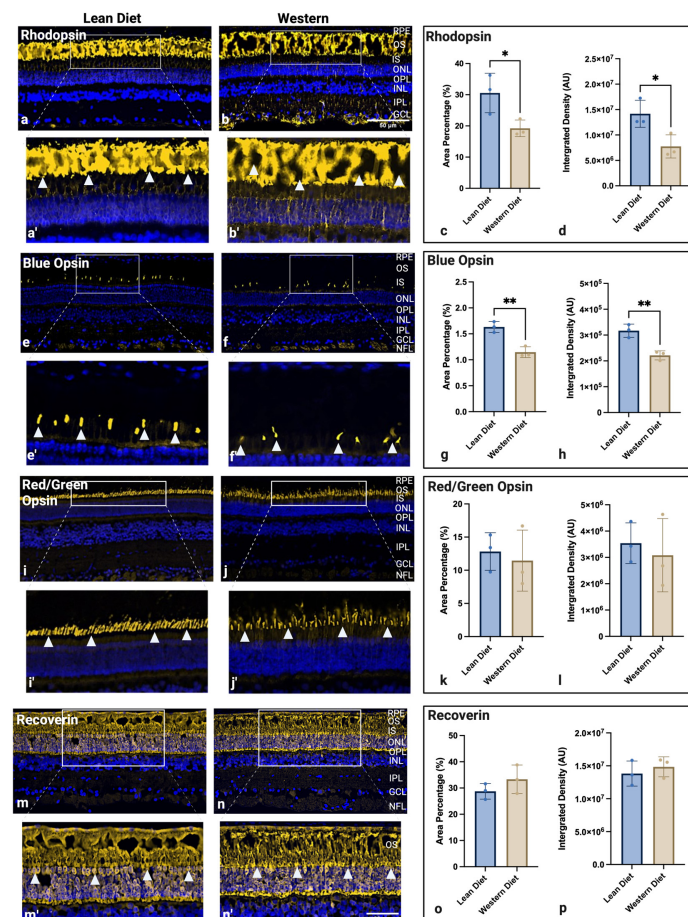


Figure 2. Retinas of Ossabaw minipigs fed on Lean or Western diet were immunostained for photoreceptors using rhodopsin, blue opsin, red/green opsin, and recoverin markers. (a,a'): Rhodopsin strongly stained rod outer segments (OS) and the outline of the cell bodies. (white arrowhead). (b,b'): Gaps in staining (arrowhead) suggest rod degeneration and impaired visual function in MetS Ossabaw minipigs. (c,d): Area percentage and integrated density confirmed significant differences between the Lean and the Western diet. (e,e'): Blue cone opsin is sparsely distributed in the pig retina (white arrows). (f,f'): Partial absence and thinning were seen in the MetS minipig group. (g,h): Significant reduction in blue opsin stain between the Lean and the Western diet. (i,i'): Red/green cone opsins are densely distributed in Lean diet minipigs. (j,j'): Similarly, partial absence and thinning of

cones were seen in the Western diet pigs (arrows). (j,k,l): Recoverin showed no significant differences between staining in both groups. (m,n,m',n'): Total cones and rods visualized using anti-recoverin antibody revealed whole photoreceptors from the OS through the outer plexiform layer (OPL). No overt changes in staining distribution were observed. (o,p): Slight increased expression in the MetS model was not statistically significant. Data represented as Mean $\pm$ SD (n = 3). *, $p < 0.05$; **, $p < 0.01$.

3.3. Retinal Neurons: Amacrine, Horizontal, Bipolar, and Ganglion Cells

Since we previously found increased degenerating neurons in the Western diet-fed pigs using Fluoro-Jade C staining [28], we wanted to comprehensively examine neuronal changes across retinal layers. Immunostaining was conducted with specific antibodies for parvalbumin [41], calbindin [42], protein kinase C alpha (PKC α) [43], and neuron-specific nuclear protein (NeuN) [44], which are markers for amacrine, horizontal, rod bipolar, and ganglion cells, respectively. Parvalbumin staining (Figure 3a,a',b,b') revealed phenotypic disruption of amacrine cells along with an increase in expression in the ganglion cell layer (GCL) of the retinas of Western diet-fed Ossabaw minipigs compared to those on a Lean diet.

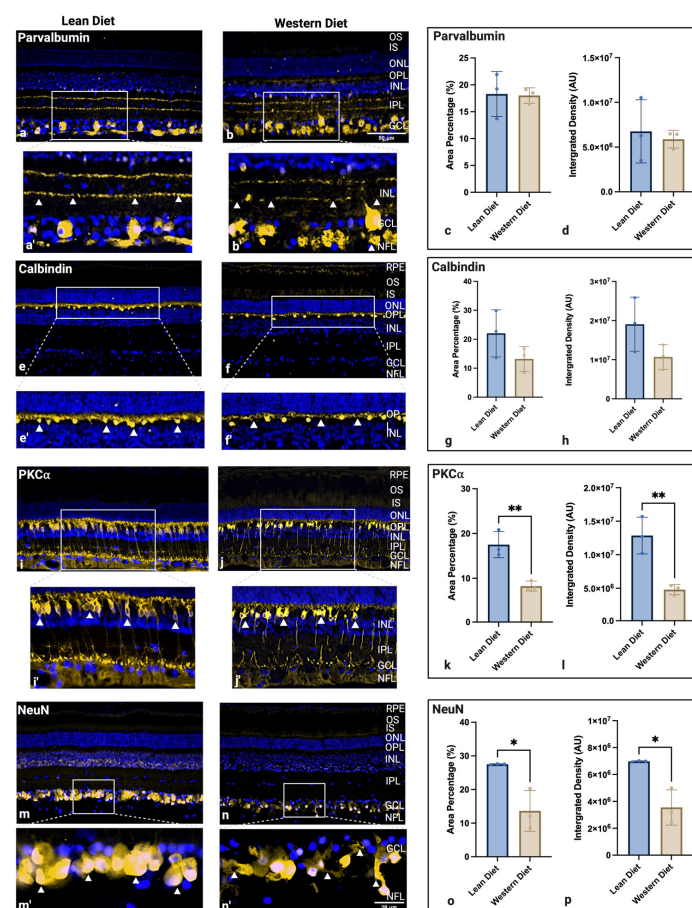


Figure 3. Retinas of Ossabaw minipigs fed a Lean or Western diet were stained for amacrine, horizontal, bipolar, and ganglion cells. (a,b,a',b'): Parvalbumin immunostaining shows disruption of amacrine cell organization within the inner nuclear layer (INL) and increased parvalbumin expression in the ganglion cell layer (GCL) (arrowhead) in the Western diet-fed pigs. (c,d): No significant differences in area percentage and integrated density between the two diet groups. (e,f,e',f'): Calbindin immunostaining of horizontal cells in the OPL (thick arrows) demonstrates reduced calbindin expression in the Western diet-fed pigs. (g,h): Area percentage and integrated density measurements remained comparable between groups. (i,j,i',j'): Bipolar cells were visualized with monoclonal PKC α

antibody. The MetS Ossabaw minipig model exhibits degeneration of bipolar cells in the INL. (k,l): Decreases in area percentage and integrated density in the Western diet pigs confirm overall bipolar cell loss compared to the healthy Lean diet. (m,n,m',n'): NeuN staining of ganglion cells in the GCL (white boxed regions) highlights histological alterations in the Western diet-fed pigs. (o,p): NeuN area percentage and integrated density were reduced in the MetS pigs. Data represented as Mean $\pm$ SD (n = 3). *, $p < 0.05$; **, $p < 0.01$.

However, there are no quantitative differences in the area percentage and integrated density of parvalbumin in response to the Western diet (Figure 3c,d). Calbindin-positive horizontal cell bodies assembled along the OPL and were seen to have reduced neuronal density in the Western diet-fed pigs (Figure 3e,e',f,f'). However, it did not reach statistical significance (Figure 3g,h). Protein kinase C alpha (PKC α ; Figure 3i,i',j,j'), a specific marker for rod bipolar cells, showed fewer bipolar cells in the inner plexiform layer (IPL) in Western diet mini pigs, which was significantly decreased compared to their Lean counterparts ($p = 0.0030$; Figure 3k) and integrated density ($p = 0.0061$; Figure 3l). Additionally, we observed significant histological changes in NeuN, a marker for ganglion cells (Figure 3m,m',n,n'), consistent with a reduction in the area percentage ($p = 0.0355$; Figure 3o) and integrated density ($p = 0.0355$; Figure 3p) of MetS diet Ossabaw minipigs.

3.4. Retinal Presynaptic and Postsynaptic Connections

Synaptophysin, a presynaptic vesicle marker [45], is found in the terminals of both photoreceptors within the OPL and second-order neurons situated in the IPL, thus serving as a crucial indicator of synaptic activity and vesicle release within the retina (Figure 4a,a',b,b'). Synaptophysin staining showed reduced integrated intensity ($p = 0.0277$; Figure 4d), reflecting Western diet-induced changes in presynaptic vesicles. Postsynaptic density protein (PSD-95), a molecular scaffold responsible for synaptic function and plasticity [46], was used to visualize postsynaptic densities of excitatory synapses in both experimental groups (Figure 4e,e',f,f'). PSD-95 depicts significantly less intense immunohistochemical staining in the retinal tissues of the Western diet-fed Ossabaw minipigs compared to the Lean diet. Statistical analyses show both a decrease in PSD-95 area percentage ($p = 0.023$; Figure 4g) and integrated density ($p = 0.0121$; Figure 4h).

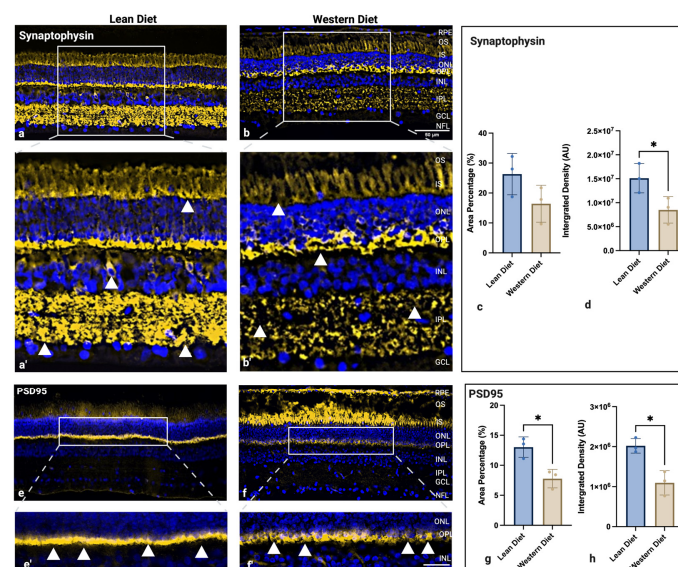


Figure 4. Retinas of Ossabaw minipigs fed a Lean or Western diet were stained for synaptic sites (presynapses and postsynapses) using synaptophysin and PSD95. (a,a'): Synaptophysin exhibited collective alignment throughout the Lean diet group. (b,b'): Limited staining and disrupted thin synaptic sites were exhibited in the Western diet pigs (white arrowhead). (c,d): While area percentage

was not significantly reduced, integrated density was significantly decreased in the Western diet groups. (e,e'): Visible signaling displayed in PSD95 staining in Lean diet retina (white arrowhead). (f,f'): Degeneration in post-synaptic processes seen as diffused staining in the MetS minipig group (white arrows). (g,h): PSD95 was reduced in the Western diet MetS minipigs compared to the Lean counterparts. Data represented as Mean $\pm$ SD ($n = 3$), *, $p < 0.05$.

3.5. Retinal Macroglia: Müller Glial Cells and Astrocytes

Müller cells and astrocytes were assessed using glutamine synthetase (GS) and glial fibrillary acidic protein (GFAP) antibodies. Gliosis in the MetS Ossabaw minipig model is a critical factor in DR pathogenesis. The GS antibody was used to stain Müller cells [47].

While the cellular processes were well distributed in Müller cells of Lean diet pigs, they were disrupted in the Western diet-fed pigs (Figure 5a,a',b,b'). However, the GS expression showed no discernible differences in staining intensity between the two groups (Figure 5c,d). GFAP is a key marker of astrocytes and is also found in activated Müller cells [48] (Figure 5e,e',f,f'). GFAP expression was intense in the GCL, showing a significant increase in area percentage ($p = 0.0158$; Figure 5g) and integrated density ($p = 0.027$; Figure 5h), indicating gliosis in the Ossabaw pigs after the Western diet.

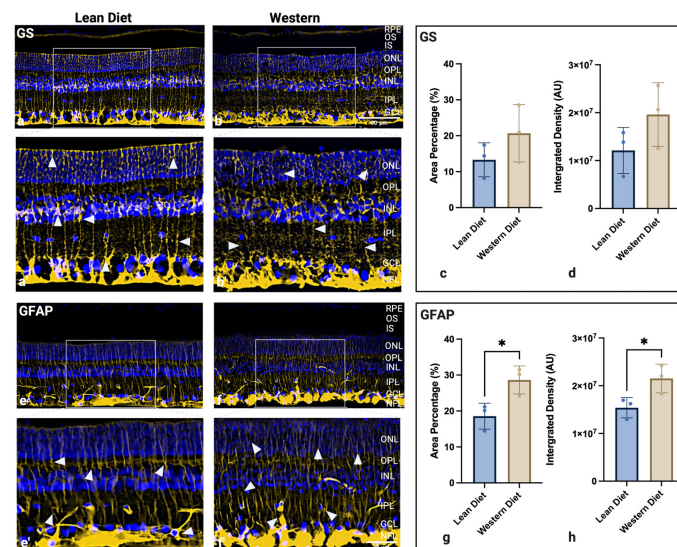


Figure 5. Retinas of Ossabaw minipigs fed a Lean or Western diet were stained with GS for Müller cells and GFAP stain for retinal astrocytes and activated Müller cells. (a,a'): Equally distributed Müller cells can be seen displayed throughout the outer nuclear layer (ONL) and nerve fiber layer (NFL). (b,b'): Müller processes were disrupted in MetS condition minipigs. (c,d): No significant differences in expression were seen between groups. (e,f,e',f'): MetS pigs showed intense GFAP staining in the ganglion cell layer (GCL), indicative of increased glial activity (white arrowhead). (g,h): Increased area percentage and integrated density confirmed glial activation in Ossabaw minipigs fed a Western diet. Data represented as Mean $\pm$ SD ($n = 3$); *, $p < 0.05$.

3.6. Retinal Microglia

Retinal microglial cells are the primary responders to retinal stress or injury [49]. Ramified retinal microglia are predominantly localized in the GCL and IPL of the Lean pig retina (Figure 6a,a'). However, microglia in the Western diet-fed mini pigs were morphologically amoeboid and rounded, suggestive of activation. Moreover, elongated microglia were translocating from the INL towards the outer retina (Figure 6b,b'). These changes are not quantitative, as no significant changes are observed in the area percentage and integrated intensity (Figure 6c,d). These MetS-induced changes may indicate cellular stress and inflammation, which play a critical role in the progression of retinal diseases.

While area percentage and integrated density did not show significant differences, the total number of activated amoeboid microglia was significantly increased in the Western diet-fed Ossabaw mini pigs.

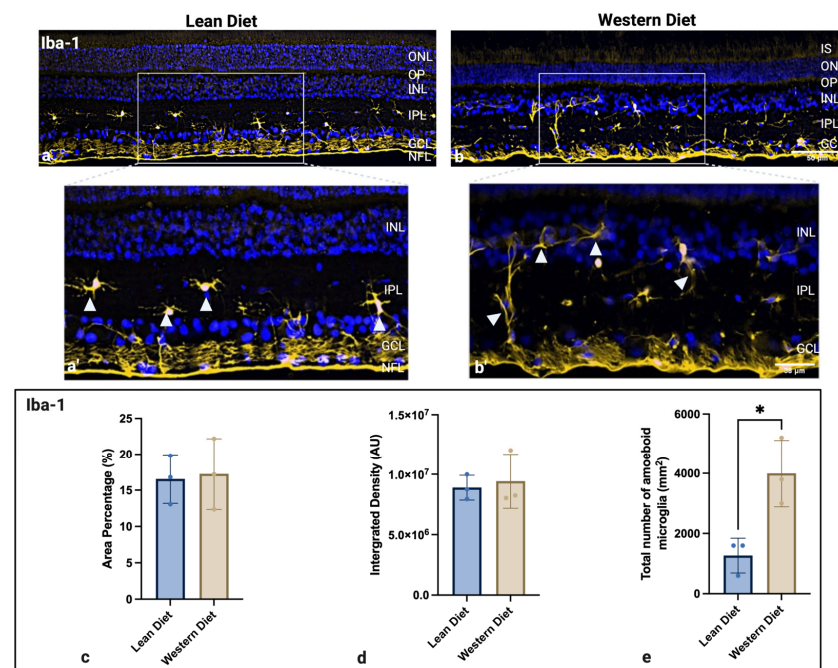


Figure 6. Retinas of Ossabaw minipigs fed a Lean or Western diet were stained for microglia using Iba-1 (a,b). (a,a'): Healthy homeostatic ramified microglia (white arrowhead) were seen in the inner plexiform layer (IPL) of Lean pigs. (b,b'): Microglia were seen to be amoeboid and rounded in the Western diet pigs, and migrating towards the outer retina, indicating activated microglia. (c,d): Area percentage and integrated density for microglia staining in Ossabaw minipigs fed a Western diet. (e): Quantification of total amoeboid microglia representing activated microglia in the Lean vs. Western diet-fed Ossabaw mini pigs. Data represented as Mean $\pm$ SD (n = 3); *, $p < 0.05$.

4. Discussion

Vision loss is one of the most debilitating conditions, exerting a profound adverse effect on the quality of life. It has been associated with depression among adults [48], increases the dependency of affected elderly individuals on their caregivers [49–51], and constitutes a substantial economic burden in the United States. According to a consensus from the National Eye Institute and Prevent Blindness America, the leading causes of age-related eye diseases are cataracts, glaucoma, age-related macular degeneration, and DR. Several rodent models are available; however, none accurately recapitulate human retinal disease, as they lack a central cone-rich region and are predominantly chemically induced or possess mutations [50,51]. In contrast, pigs have a cone-rich visual streak extending both nasally and temporally [52], and Ossabaw minipigs in particular have a high propensity for obesity, attributed to their “thrifty genotype,” rendering them appropriate models for MetS research [53]. However, their retinal structure has not yet been extensively studied in MetS. Retinal tissue is considered the most metabolically active tissue in the body and is highly susceptible to metabolic alterations and reprogramming [54]. In this study, we examined retinal pathomorphological alterations in Ossabaw minipigs subjected to a high-fat, high-fructose corn syrup, and high-calorie diet (Western diet, also referred to as MetS diet) for ten consecutive weeks. Our study showed evident retinal damage, along with morphological, histopathological, and neuronal changes across multiple retinal layers, suggesting early signs of MetS-induced retinal damage and providing early pathological indicators of developing DR.

The RPE is crucial for light adaptation in the visual function [55]. The RPE constitutes the outer blood–retina barrier and facilitates epithelial transport, light absorption, visual cycle, phagocytosis, spatial ion buffering, immune modulation, and secretion [56]. CRALBP plays a critical role in the visual cycle by facilitating the transport of retinaldehyde, which is vital for photoreceptor function and the regeneration of visual pigments. Ezrin serves as a marker of RPE polarity and stains the apical surface and microvilli of the RPE [36], indicating cellular integrity. Both CRALBP and Ezrin markers showed no significant quantitative differences among dietary groups, emphasizing the resilience of RPE cells and their related cellular functions despite early diet-induced morphological changes in the inner retina.

Recoverin, a calcium-binding protein with a crucial role in the phototransduction cascade by regulating rhodopsin kinase activity, stained both rod and cone photoreceptors with no difference between the diet groups. Next, the outer segments of rods and cones were analyzed separately. The rod photoreceptors were stained with the Rhodopsin marker, a photosensitive G-protein-coupled receptor (GPCR) found in the rods that mediates scotopic vision [57]. As rhodopsin is essential for phototransduction [58], a significant decrease in the Western diet pigs suggests that MetS may adversely affect rhodopsin synthesis or stability, potentially impairing rod cell function and overall visual acuity. Blue opsin and red/green opsins are critical for color discrimination and high-resolution vision, contributing to color vision and visual acuity [38,58]. Blue opsin was quantitatively reduced in the Western diet pigs, while red/green opsin had a sparse distribution in the Western diet pigs. Together, these suggest potentially compromised color vision. These findings highlighted early changes to the photoreceptor OS, which are metabolically demanding and require continuous daily renewal supported by the RPE. Since RPE apical processes were thickened in the Western diet-fed pigs, this may indicate early RPE dysfunction and photoreceptor degeneration resulting from MetS.

Photoreceptor-derived neural signals propagate along a vertical pathway through bipolar and ganglion cells, while horizontal and amacrine cells establish a horizontal pathway that refines information by lateral signal integration [59]. The processed data are subsequently transmitted from the retina to the brain via the optic nerve, utilizing ganglion cell axons. MetS Ossabaw minipigs predominantly displayed pathomorphological changes throughout the amacrine, horizontal, bipolar, and retinal ganglion cell layers. Neurotransmitter retinal neurons, such as parvalbumin-positive amacrine cells, play roles in dopamine and γ -aminobutyric acid (GABA) secretion and synaptic release, and modulate retinal neuronal activity during dark- and light-adaptation [60]. Parvalbumin is a key Ca^{2+} binding protein found in AII amacrine cells, knotty type 2 amacrine cells, starburst amacrine cells, and displaced amacrine cells with cell bodies located in the ganglion cell layer. Paralbumin-immunopositive cells showed irregularities in the INL and an increase in displaced amacrine cell bodies in the GCL in retinas collected from MetS Ossabaw minipigs. Horizontal cells participate in glucose transport, regulate light-dependent phosphoinositide metabolism, and require a stable metabolic input to support the aspartate-malate shuttle, which recycles GABA and provides inhibitory feedback within the outer plexiform layer of the retina. Calbindin stains both the A-type and B-type horizontal cells in the retina. The integrated intensity observed in the retinal tissues of the Western diet-fed Ossabaw mini pigs is reduced compared to those on a Lean diet. There are also fewer horizontal cell nuclei in the MetS pigs than in the Lean group along the OPL.

Bipolar cells are the principal interneurons that function as the primary conduits from photoreceptors to ganglion cells [61]. They rely on glucose to meet their high energy demands and regulate diverse cellular activities, including cell growth, differentiation, and apoptosis, thereby rendering them susceptible to metabolic stress [62]. Retinas from MetS

Ossabaw minipigs stained for PKC α showed several pathohistological defects, including loss of dendrites and synaptic signals from bipolar cells and degenerative rod bipolar cell axons throughout the INL and IPL, indicating that rod bipolar cells are the early target of MetS.

RGC dendrites receive synaptic inputs from bipolar cells and extend within the inner plexiform layer (IPL) [63]. Despite the distinctive metabolic characteristics of the IPL, RGC dendrites predominantly rely on oxidative phosphorylation (OXPHOS) for ATP production and are notably vulnerable to metabolic stress or injury [64]. We tested NeuN to assess the structural integrity of retinal ganglion cells in the Lean and Western diet-induced MetS pigs. NeuN-positive RGCs exhibited distortion and mislocalization within the designated GCL, extending into the NFL, with a significant reduction in area percentage in the Western diet group. There were also noticeably fewer ganglion cells in the MetS Ossabaw minipigs than in the Lean pigs, suggesting early signs of neuronal degeneration and increased ganglion cell death [65], seen in patients with diabetes and other related ocular diseases [66–68]. These findings provide further insights into the role and structure of retinal neurons, leading to impaired function and increased susceptibility to retinal diseases, including DR and DME.

Next, we investigated presynaptic and postsynaptic morphology in the MetS minipig model. Synaptophysin is crucial for synaptic vesicle trafficking and neurotransmitter release [45], and PSD-95 plays a pivotal role in postsynaptic signaling and plasticity by anchoring synaptic proteins and receptors at the postsynaptic membrane [46]. Both markers were significantly reduced in the Western diet-fed Ossabaw retina. This corroborates the structural changes in bipolar and ganglion cells, suggesting reduced presynaptic vesicle presence or activity, and depletion of synaptic density or alterations in synaptic structure and function in response to MetS stress.

CRALBP in Müller cells is essential for M-cone function [69]. Relatively lower CRALBP was seen in the inner retina of the Western diet-fed pigs, suggestive of gradual Müller dysfunction. In Western diet-fed pigs, glutamine synthetase was unchanged, suggesting that glial function related to glutamate metabolism and detoxification remains unaffected by short-term dietary differences. Conversely, GFAP, a biomarker of activated astrogliosis, was associated with astrocyte pathology and migration in the MetS pigs. The upregulation of GFAP has been associated with DR pathology [70], further corroborating our findings that Ossabaw mini pigs fed a Western diet for 10 weeks exhibit an early indication of retinal degeneration. These observations also align with prior research on streptozotocin (STZ)-induced mice, where retinal astrocytes are prone to early degeneration in diabetes [71,72].

Retinal microglial cells are the primary responders to retinal stress or injury [73]. Although we did not observe differences in Iba-1 staining intensity between the diet groups, we observed noticeable changes in microglial morphology and migration toward the outer retina. Our recent study on the diabetic pig retina indicates that microglia under MetS release damage-associated molecular pattern (DAMP) proteins, such as S100A9, which are detrimental to retinal cells and play a critically important role in prolonging the sterile inflammatory stages of DR [74].

Recent clinical data indicate that the age at which diabetes onset occurs may worsen the progression of diabetic retinopathy (DR) in youths [75]. Type 2 diabetes mellitus (T2DM) is rarely seen in prepubertal adolescents; however, post-pubertal adolescents with T2DM have a 4.8-fold increased risk of developing DR, which also tends to progress more rapidly. In our previous study, using transmission electron microscopy (TEM), these MetS pigs exhibited early disruption of cellular architecture across neural layers [28]. Specific discrepancies across retinal cell types are quantified in this systematic examination; however, the small sample size limited statistical power. Studies have shown that pigs share genomic similarities with the human immune system [76]. Although pig models were

limited in previous studies [77], Ossabaw minipigs, in particular, have emerged as a unique preclinical model for MetS [26]. In summary, this study found subtle changes in the RPE, photoreceptor OS, bipolar and ganglion synaptic connections, and morphological activation of microglial cells post ten weeks on a Western diet in Ossabaw pigs. Our findings suggest a link between diet-induced metabolic changes and neuroretinal disorders and underscore the importance of Ossabaw mini pigs, which are prone to diabetes, exhibit phenotypic traits evident in retinal histology, and are suitable not only for T2DM models but also for studying the long-term pathological progression of DR and DME.

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Abbreviations

The following abbreviations are used in this manuscript:

MetS	Metabolic Syndrome
DR	Diabetic Retinopathy
DME	Diabetic Macular Edema
HDL	High-Density Lipoprotein
T2DM	Type 2 Diabetes Mellitus
CRAO	Central Retinal Artery Occlusion
AMD	Age-related Macular Degeneration
FDA	U.S. Food and Drug Administration
mm	millimeter
PBS	Phosphate-Buffered Saline
PFA	Paraformaldehyde
OCT	Optical Cutting Temperature
SD	Standard Deviation
NIH	National Institutes of Health
ROI	Region of Interest
MC	Monoclonal
PC	Polyclonal
RPE	Retinal Pigment Epithelium
CRALBP	Cellular Retinaldehyde-binding Protein
OPL	Outer Plexiform Layer
IPL	Inner Plexiform Layer
OS	Outer Segments
IS	Inner Segments
ONL	Outer Nuclear Layer

NFL	Nerve Fiber Layer
PKC α	Protein Kinase C alpha
NeuN	Neuron-Specific Nuclear Protein
GCL	Ganglion Cell Layer
PSD-95	Postsynaptic Density Protein
RGC	Retinal Ganglion Cells
GS	Glutamine Synthetase
GFAP	Glial Fibrillary Acidic Protein
Iba1	Ionized Calcium-binding Adaptor Molecule 1
GPCR	G-protein Coupled Receptor
GABA	γ -aminobutyric acid
OXPHOS	Oxidative Phosphorylation
ATP	Adenosine Triphosphate
STZ	Streptozotocin
DAMP	Damage-associated Molecular Pattern
TEM	Transmission Electron Microscopy
MetS Pigs	Pigs exhibiting Metabolic Syndrome
DAPI	4',6-diamidino-2-phenylindole

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