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# Maternal undernutrition model of two generations of rats: Changes in the aged retina

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**Summary.** The impact of maternal undernutrition on morphological changes of the retina was assessed in two generations of aged offspring. Wistar 18 rats (9 of each generation of 20-month-old female offspring; in total -27 eyes) were analyzed. The first generation offspring were born to mothers who: (a) were restricted to food only before pregnancy (pre-pregnancy); (b) whose food was restricted before and during pregnancy. The control group and all the offspring were fed normally. After enucleating the eyes, paraffin sections were stained with hematoxylin and eosin. The thickness of retina layers was measured. Cryosections were immunostained using glial fibrillary acidic protein, ionized calcium-binding adaptor molecule1, RNA-binding protein with multiple splicing for evaluation of macroglia, microglia and retinal ganglion cells by digital image analysis tools. Our data have shown atrophy of photoreceptor layer and degeneration of outer nuclear layer in all investigated groups, but less damage was found in the control group. Higher Müller cell activity and greater number of microglial cells was observed in the second generation offspring born from both restricted diet groups. Higher numbers of microglial and retinal ganglion cells were observed in the second generation in comparison to the first generation offspring. Malnutrition of the mother may be one of the possible causes of degeneration of the outer layers of the retina and activation of Müller cells in the second generation offspring. The effect of maternal nutritional restriction on the number of microglial and retinal ganglion cells is unclear.

**Key words:** Macroglia, Maternal malnutrition, Microglia, Photoreceptors, Rat's retina

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## Introduction

Many different stress factors exist that can affect the development and growth of a human being at an early stage of their life. One of these factors is unbalanced, low calorie diet. Starvation is still a huge problem all over the world, more than 690 million people go hungry (Sustainable Development Goal 2, Zero Hunger). Another problem in well-developed countries is negative body image and different cultural perceptions of a perfect body shape, which often pressure young women to go on a strict diet and lose weight. As a result, pregnant women and their fetuses may experience nutritional deprivation (Linna et al., 2014).

Malnutrition in early growth period can impair postnatal health of the offspring in many different ways. Many researchers have explored environmental factors that affect the metabolism of a naturally growing organism during pregnancy, lactation and early childhood and revealed their possible harmful effects on long-term health (Barker, 1995, 1998, 2007; Gluckman et al., 2008; Barker et al., 2009; Padmanabhan et al., 2016; Zheng et al., 2016; Mandy and Nyirenda, 2018; Koletzko et al., 2019). This widely recognized phenomenon was also assigned a term of "developmental or metabolic programming" (Barker, 1995). Obesity, metabolic syndrome, cardiovascular problems in the later stages of adult life of the offspring

**Abbreviations.** DAPI, 4', 6-diamidino-2-phenylindole dihydrochloride; DIA, digital image analysis; FR, food restricted; GCL, ganglion cell layer; Generation1, first generation; Generation2, second generation; GFAP, glial fibrillary acidic protein; Group1, first group; Group2, second group; HE, Hematoxylin and eosin; Iba1, ionized calcium-binding adaptor molecule 1; ILM-OLM, inner limiting membrane to outer limiting membrane; ILM-PL, inner limiting membrane to photoreceptors layer; INL, inner nuclear layer; INL-ONL, inner to outer nuclear layer; INL-ONL/ILM-OLM, INL-ONL and ILM-OLM ratio; IPL, inner plexiform layer; IPL/ ILM-OLM, IPL and ILM-OLM ratio; ONL, outer nuclear layer; RBPMS, RNA, binding protein with multiple splicing.

are nowadays well known and are considered a consequence of maternal stress experienced during the critical periods of offspring growth (Barker, 1995, 1998, 2007; Painter et al., 2005; Roseboom et al., 2006; Barker et al., 2009; Padmanabhan et al., 2016; Mandy and Nyirenda, 2018; Kinra et al., 2020).

Nutritional deprivation as a significant early stressor can also affect sensory systems in different ways. Vision is one of the most critical sensory functions for living a fulfilling life. There are data on the importance of various nutrients to miscellaneous eye structures (Jayaratne et al., 2017). Early stressors such as prenatal hypoxia may affect the developing retina and due to fetal programming predetermine age-related retinal pathologies (Bourque et al., 2013). However, many diseases of the retina remain unclear and untreatable, causing blindness all over the world.

It is evident that the environment in utero is very important for later quality of life. Fetal hypoxia, malnutrition and various other stressors program disorders in adults (Gluckman et al., 2008; Kinra et al., 2020). Experimental studies also support the concept of Developmental Origins of Health and Disease (Araminaite et al., 2014; Gonzalez-Bulnes et al., 2016; Roepke et al., 2020).

The pre-pregnancy period has previously been little studied. However, as the number of metabolic diseases increases among children and adults, the peculiarities of the preconception period is of growing interest, revealing the importance of women's nutrition and lifestyle for the long-term health of their offspring (Dunneram and Jeewon, 2015; Fleming et al., 2018; Moholdt and Hawley, 2020; Stephenson et al., 2018).

It is already known that the period around conception is critical for the health of the next generation. The influence of a mother's lifestyle is observed even in several later generations (Waterland et al., 2008; Giraudo et al., 2010; Araminaite et al., 2014), however, the analysis of multiple generations is very challenging.

To the best of our knowledge, there is a lack of studies examining retinal changes in the later life of aged rats caused by mother malnutrition. For better understanding of the possible pathological changes, we investigated the effect of nutritional deprivation in early growth of aging rat retinas.

The purpose of the presented study was to evaluate the impact of maternal nutritional restriction before pregnancy and in utero on morphological changes of retina in the first and second generation of aged offspring.

## Materials and methods

### *Animals*

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the EC Directive 2010/63/EU for animal experiments, using protocols approved and monitored by the Animal Welfare Ethical Board of Lithuania (Permission no. 0211, 2009 an no. G2-20, 2015).

Experiments were carried out on Wistar albino rats. Female rats (10-12 months of age, weighing 220±20 grams) were randomly divided into three groups subjected to nutritional restriction and were supplied with different nutrition 1 month before and throughout the pregnancy period. Further investigations of the first (*Generation1*) and second generation (*Generation2*) offspring were made: first group (Group1) - mothers were 50% food restricted (FR) (10 grams feed per day) 1 month before pregnancy only, second group (Group2) - 1 month before and throughout the pregnancy period, and control group - mothers as well as offspring were fed normally (fixed calorie diet - recommended daily allowance of nutrients; mothers got 20 grams per day *Kiss Py* (*Terra animalis*, Kaunas, Lithuania) feed whose composition was well balanced (chips (corn, wheat, hay), wheat, oats, locust beans, maize, barley, pea flakes, corn flakes, two-colored sorghum, safflower seeds, sunflower seeds, wheat popcorn, dried carrots, peas,

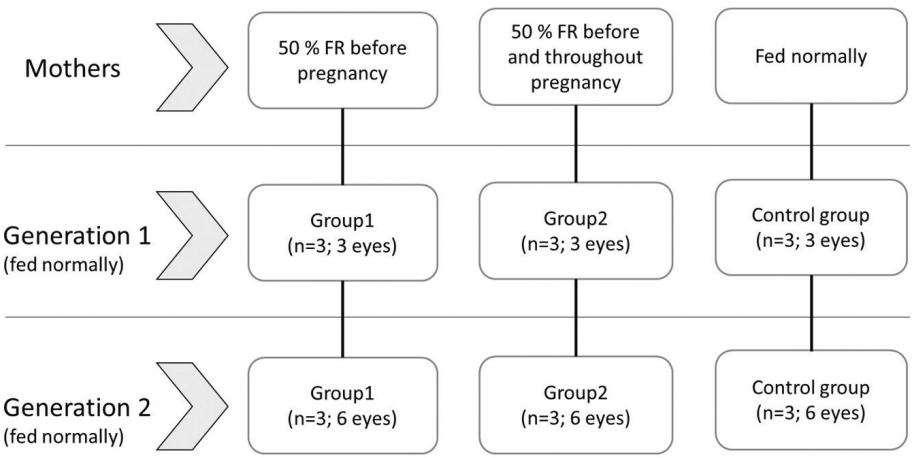


**Fig. 1.** Flow chart of the experiment.

peanuts) and met the needs of rodents for optimal energy value.

Other living conditions (singly-housed rats in vivarium) and illumination (all rats exposed to the same light) were equal for all groups. At the age of 20 months (old age), rats of both generations were euthanized and their eyeballs were removed. 9 rats (9 eyes) from Generation1 and 9 rats (18 eyes) from *Generation2* were enrolled into the final analysis (Fig. 1).

### *Animal sacrifice and tissue collection*

At the end of the study, rats were deeply anesthetized with a mixture of xylazine (Sedaxylan, Eurovet animal health) and ketamine (Ketamidor, Richter Pharma). Thereafter animals were sacrificed by transcardial perfusion first 3 min with 0.9 % NaCl solution, then 15 min with 4% paraformaldehyde in 0.1M phosphate buffer solution, pH 7.4. Eyes were collected and stored in 4% paraformaldehyde solution overnight and then transferred to cryoprotectant (tissue collecting solution consisting of etilenglycol, glycerol and phosphate buffer) for further analysis.

### *Tissue processing and staining*

Paraffin-embedded retinal sections

Paraffin-embedded eye bulb sections of 9 eyes from *Generation2* rats were prepared for examination. One paraffin block from each of 9 rat's eyes was made. Serial 3-µm-thick sections through the optic disc were obtained by a microtome (Leica SM2010R) and mounted on glass slides.

Hematoxylin and eosin (HE) staining.

All sections on glass slides were deparaffinized in xylene, rehydrated in a series of alcohols, rinsed in distilled water and stained with HE. After staining, sections were dehydrated in a series of ascending alcohols, cleared in xylene, and mounted with cover glass using Depex mounting media.

Cryo-embedded retinal sections

We embedded 18 eyes (9 of each generation) in optimal cutting-temperature (O.C.T.) compound (Tissue-Tek; Sakura Finetek, Torrance, CA, USA) and frozen in liquid nitrogen. Cryosections (10-µm-thick) of eyes were performed at the optic nerve level using a cryostat (Leica CM1860) and collected on SuperFrost® (Thermo Fisher Scientific) slides for fluorescent immunohistochemistry analysis.

Immunohistofluorescence

Frozen sections were processed for immunostaining using antibodies against glial fibrillary acidic protein (GFAP), ionized calcium-binding adaptor molecule 1(Iba1), RNA-binding protein with multiple splicing (RBPMS) and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) staining to ascertain layers and nuclei rows. Antibodies and dilutions are provided in Table 1.

### *Imaging*

Immunofluorescence slides were examined and scanned by Fluorescence Microscope Leica DM6B Upright Microscope including Leica DMC62000 Camera and LAS X Software at 20x magnification. Paraffin-embedded and HE stained retinal sections were captured by using the Aperio Scan-Scope XT Slide Scanner (Aperio Technologies, Vista, CA, USA) under 20x objective magnification (0.5 µm resolution).

### *Digital image analysis*

Digital image analysis (DIA) was performed using the HALO Multiplex IHC v1.2 and HALO Area Quantification FL v2.1.2 and HALO algorithms (IndicaLabs, NM, USA) with a manually selected region of interest (ROI) enclosing the tissue sections. The software enables automated recognition of selected tissue areas and cell segmentation in scanned images. The nuclear analysis was calibrated to enumerate cell profiles in retina layers, while tissue areas in immunofluorescence images were quantified by calibrated Area Quantification algorithm.

### *Morphometry and cell counts*

Thickness of retina layers

Thickness of inner plexiform layer (IPL), inner to outer nuclear layer (INL-ONL; instead of evaluating

**Table 1.** List of antibodies used in the study.

| Target | Molecular marker | Antibody | Company | Dilution |
|---|---|---|---|---|
| Macroglial cells | GFAP[1] | Mouse monoclonal | Sigma (G3893) | 1:1000 |
| Microglial cells | Iba1[2] | Rabbit polyclonal anti-ionized calcium binding adaptor molecule 1 | WAKO (019-19741) | 1:500 |
| Retinal ganglion cells (RGCs) | RBPMS[3] | Rabbit polyclonal | Phosphosolutions (1830-RBPMS) | 1:200 |
| DNA | DAPI[4] | 4′,6-diamidino-2-phenylindole dihydrochloride | Sigma - Aldrich (D9542) | 1:10000 |

[1]: GFAP, glial fibrillary acidic protein; [2]: Iba1, ionized calcium-binding adaptor molecule 1; RBPMS, RNA - binding protein with multiple splicing; [4]: DAPI, 4',6-diamino-2-phenylindole dihydrochloride.

INL and ONL separately as usual, we decided to measure these layers continuously to avoid inaccuracies because of existing obvious ONL degenerative changes), inner limiting membrane to outer limiting membrane (ILM-OLM), inner limiting membrane to photoreceptors layer (ILM-PL) were manually measured (in steps of every 300 µm) using Halo software in digitalized HE slides (Fig. 2C). INL-ONL and ILM-OLM ratio (INL-ONL/ILM-OLM), IPL and ILM-OLM ratio (IPL/ ILM-OLM) were calculated to evaluate the proportion of INL-ONL and IPL layers to the whole retina. ILM-OLM thickness was equated to the thickness of the entire retina according to observed atrophy of the photoreceptor layer. The length of the remaining segments of photoreceptors (µm) was measured in whole retina scans.

Nuclear layers

INL-ONL total layer area ($mm^2$) was marked manually in HE scans, total INL and ONL cells (units) and Average nuclei area ($\mu m^2$) were calculated by calibrated HALO software (Fig. 2). INL-ONL nuclear density was calculated by dividing total amount of cells (units) by total layer area ($mm^2$) provided by software.

Immunoreactivity in Müller glial cells

To evaluate the degree of glial activation, a scoring system based on the extent of GFAP staining was used (Anderson et al., 2008). A minimum of three sections per animal was examined with a magnification of 40x under the fluorescent microscope and scores were assigned for central retinal regions (at 300 µm either side of the optic disc) according to the five-point scoring system (see in Table 2). The best quality scanned immunofluorescence images were assessed to different intensity of green colour fluorescence of Müller glial cells analysis by Halo software: GFAP Green Area Analyzed ($\mu m^2$), Green Area ($\mu m^2$), Green Weak Area ($\mu m^2$), Green Moderate Area ($\mu m^2$), Green Strong Area ($\mu m^2$), % Green Area ($\mu m^2$), % Green Weak Area ($\mu m^2$), % Green Moderate Area ($\mu m^2$), % Green Strong Area ($\mu m^2$), % Negative Area, Green Average Positive Intensity (Fig. 3). The mean length (µm) of Müller cells processes was measured and compared between groups and generations.

Retinal ganglion cells

RGCs in retina were counted manually in fluorescent microscopic field of view as fluorescent red round cells across the ganglion cell layer (GCL). Moreover, digitalized images were analyzed by DIA tools detecting RBPMS red stained area and evaluating: RBPMS Area Analyzed ($\mu m^2$), Red Area ($\mu m^2$), Red Weak Area ($\mu m^2$), Red Moderate Area ($\mu m^2$), Red Strong Area ($\mu m^2$), % Red Area ($\mu m^2$), % Red Weak Area ($\mu m^2$), % Red Moderate Area ($\mu m^2$), % Red Strong Area ($\mu m^2$), % Negative Area, Red Average Positive Intensity.

Microglia

Microglia cells (active and silent), identified by the shape of their fluorescent nuclei stained with Iba1, were

**Table 2.** GFAP five-score system (modified according Anderson et al., 2008).

| Score | Immunostaining |
|---|---|
| 0 | Insignificant staining |
| 1 | Müller cell endfeet region in ganglion cell layer (GCL) only |
| 2 | Müller cell endfeet region in GCL plus a few proximal processes |
| 3 | Müller cell endfeet plus many processes up to ONL[5] |
| 4 | Müller cell endfeet plus processes with some in ONL |
| 5 | Müller cell endfeet plus lots of dark processes to outer margin of ONL |

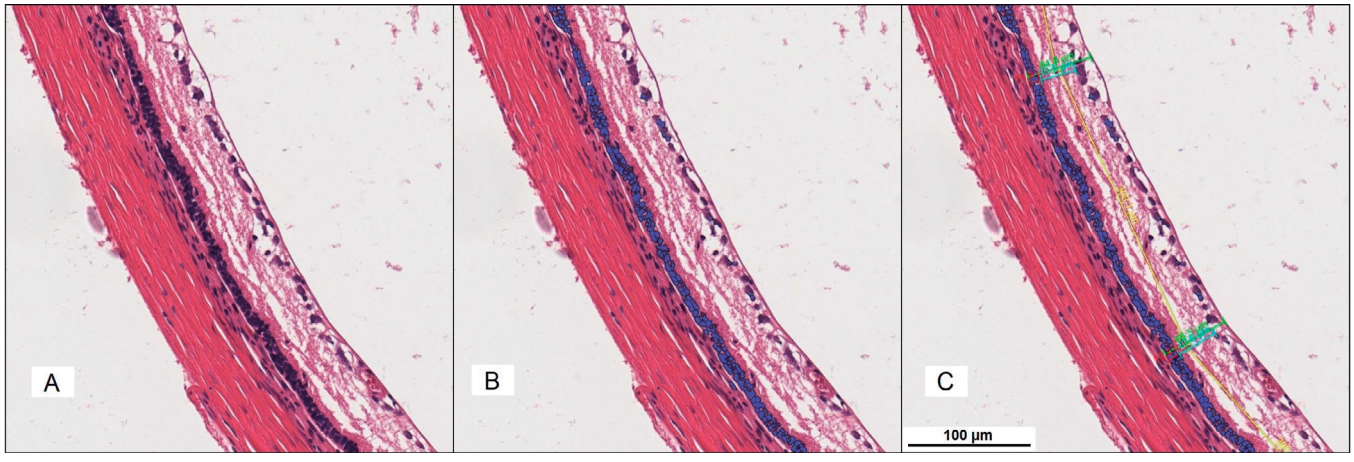


**Fig. 2.** Retina scans stained with HE and their analysis by Halo program: scan of the retina **(A)**, automated segmentation of nuclei in INL-ONL **(B)**, measurements of retina layers **(C)** (yellow line - measurement of retina every 300 *µ*m, green line - thickness of ILM to OLM, blue line - IPL). HE, Hematoxylin and eosin; INL-ONL, inner to outer nuclear layer; ILM, inner limiting membrane; OLM, outer limiting membrane; IPL, inner plexiform layer.

quantified on sections per µm length of retina. Automatic analysis was also performed by Halo program to determine Iba1 red stained area: Iba1 Area Analyzed (µm$^2$), Red Area (µm$^2$), Red Weak Area (µm$^2$), Red Moderate Area (µm$^2$), Red Strong Area (µm$^2$), % Red Area (µm$^2$), % Red Weak Area (µm$^2$), % Red Moderate Area (µm$^2$), % Red Strong Area (µm$^2$), % Negative Area, Red Average Positive Intensity.

### *Statistics*

Data were analysed using statistical analysis software package SPSS for Windows version 23.0 to assess whether differences between groups were statistically significant. Normality was checked using the Shapiro-Wilk test. The groups and generations were compared using Mann-Whitney U, Kruskal Wallis test. Results were presented as mean values with standard deviation (SD). The results were considered significant when p value was less than 0.05.

## Results

### *Retina layers*

*Inner plexiform layer (IPL).* No statistically significant difference between groups of Generation2 offspring was observed ($p>0.05$).

*Inner to outer nuclear layer (INL-ONL).* INL-ONL was thicker in control group than in nutritionally deprived groups of *Generation2* offspring (20.7 ±7.32 µm; 14.9±6.24 µm; $p<0.01$). Comparing the three investigated groups separately, the thickest INL-ONL was identified in control group (Group1, 14.42±6.89 µm; Group2, 15.3±7.87 µm; control group, 20.7±7.32; $p<0.01$) (Fig. 4).

*Inner limiting membrane to outer limiting membrane (ILM-OLM).* ILM-OLM was the thickest in Group2 (59.87±18.99) compared with Group1 (52.29±16.76) and with control group (59.10±18.12) ($p=0.01$) of *Generation2*.

*Inner to outer nuclear layer (INL-ONL) and inner limiting membrane to outer limiting membrane (ILM-OLM) ratio.* The ratio was calculated to evaluate the thickness of INL-ONL relative to ILM-OLM. INL-ONL formed a larger proportion of the retina in control group (0.35) than in Group1 (0.3) and Group2 (0.27) ($p<0.05$).

*Inner plexiform layer (IPL) and inner limiting membrane to outer limiting membrane (ILM-OLM) ratio.* The ratio was calculated to evaluate the thickness of IPL relative to ILM-OLM. IPL formed a smaller proportion of the retina in control group (0.43) than in Group1 (0.49) and Group2 (0.49) ($p<0.05$).

*Inner limiting membrane to photoreceptors layer (ILM-PL).* 56% of eyes had degeneration of PL. The thickness of ILM-PL was significantly lower in Group1 ($p<0.05$).

*Photoreceptors* (length of remaining segments - µm). We have not observed any remaining segments of

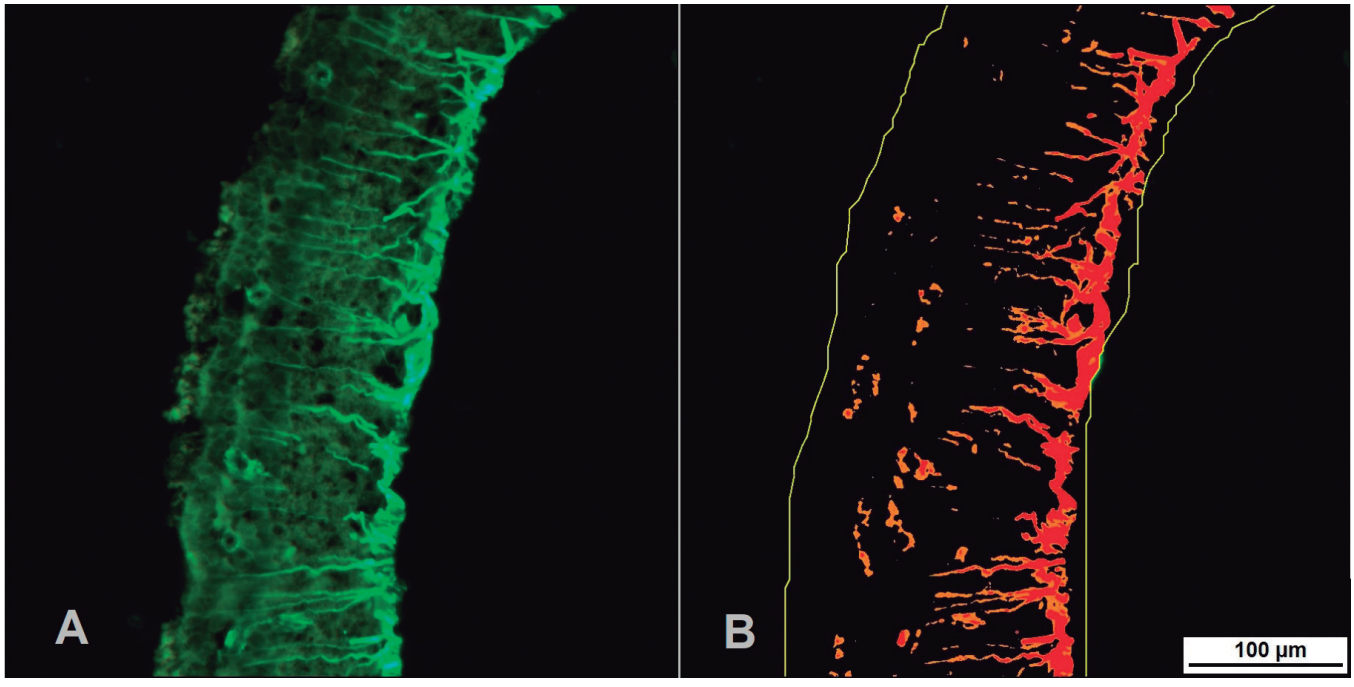


**Fig. 3.** GFAP immunofluorescence detection: green immunostained Müller cell endfeet plus processes **(A)**, analysis of Müller cells (red marked area) by DIA **(B)**. GFAP, glial fibrillary acidic protein; DIA, digital image analysis.

photoreceptor layer in Group1. Photoreceptor segment was only extant in one eye in Group2: 1482.55 µm, while it was present in all eyes within the control group: 1433.71±563.08 (1040.56 µm; 1181.81 µm; 2078.77 µm). Loss of photoreceptor layer was present in all groups, but larger remaining segments were observed in the control group.

*Nuclear layers*

The quantification of nuclear layer (INL and ONL) was performed by automated image analysis. INL-ONL total layer area ($mm^2$) was marked manually in all scanned retina tissue sections. There were no differences between the marked areas among investigated groups (Group1, 0.36±0.16 $mm^2$; Group2, 0.33±0.19 $mm^2$; control group, 0.37±0.17; $p>0.05$). Total amount of cells and average nuclei area ($\mu m^2$) were counted automatically. We found significantly more cells in control group than in nutritionally deprived groups (5154±516.14 cells; 3500±308.26 cells; $p=0.024$) in *Generation2*. Average nuclei area was unequal - significantly smaller in control group than in nutritionally deprived groups (11.49±0.68 µm2 vs 13.28±1.01; $p<0.05$) (Fig. 5). INL-ONL density was calculated by dividing the total amount of cells by area of the total layer. The mean nuclear density in Group1 was 11446±6640 cells/$mm^2$, in Group2 it was 14629±10286 cells/$mm^2$ and in control group 15595±6086 cells/$mm^2$. However, no statistically significant difference in nuclear density between all of the groups was found ($p>0.05$).

*Macroglia - GFAP*

We present data, which can demonstrate higher

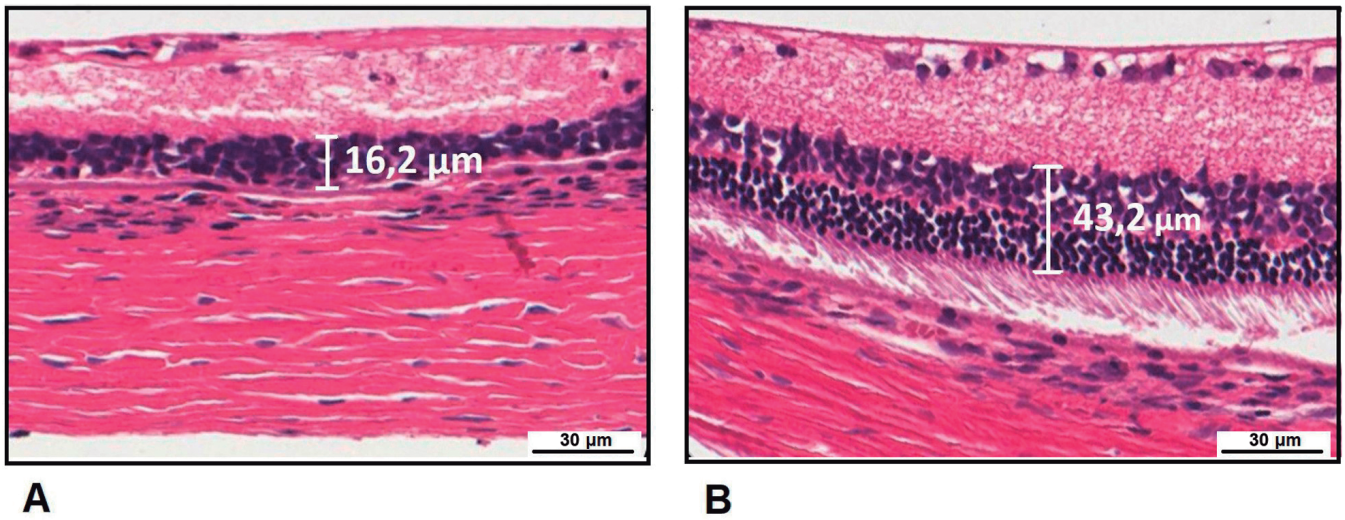


**Fig. 4.** INL-ONL thickness (16.2 µm) in nutritionally deprived group **(A)**, INL-ONL thickness (43.2 µm) in control group **(B)**. INL-ONL, inner to outer nuclear layer.

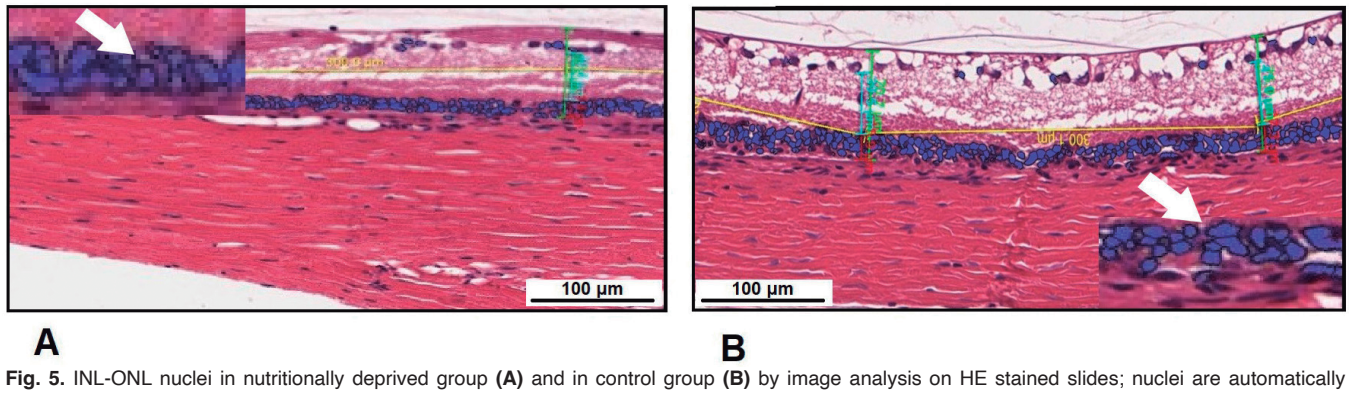


**Fig. 5.** INL-ONL nuclei in nutritionally deprived group **(A)** and in control group **(B)** by image analysis on HE stained slides; nuclei are automatically segmented by DIA and marked in blue colour. White arrows show magnified segment of nuclear layer. Yellow line - measurement of the retina at every 300 $\mu$m, green line - thickness of ILM to OLM, blue line - thickness of IPL. INL-ONL, inner to outer nuclear layer; HE, Hematoxylin and eosin; DIA, digital image analysis; ILM, inner limiting membrane; OLM, outer limiting membrane; IPL, inner plexiform layer.

activity of Müller cells in *Generation2* of the offspring, whose grandmothers were nutritionally constrained. Mean GFAP score in Group1 was equal to Group2 (2.3±0.5 vs 2.3±0.8; p>0.05), whereas control group had statistically significant lower score of Müller cells in the retina (1.1±0.4; p<0.01). GFAP staining can implicate moderate activation of macroglia in the retina of aged (20 months) rats (Fig. 6). However, no difference in any intensity of green colour area (GFAP-positive area indicating Müller cells) between the generations and groups was observed. The average length of Müller cell processes was 56.64±44.38 µm. We have not found significant difference in average length of Müller cells processes between the two generations and investigated groups (p>0.05).

### *Retinal ganglion cells*

We carried out manual analysis under fluorescent microscopy and observed higher RGCs counts in *Generation2* compared to *Generation1* (p<0.05) (Fig. 7). We detected controversial results in analysis of red colour area (RBPMS-positive area) between the two generations. Red Area (µm²), Red Weak Area (µm²), Red Average Positive Intensity and Weak + Moderate + Strong Red Area (µm²) indicators were significantly higher in Generation1 (p<0.05), possibly driven by higher RGC counts or larger cells (Fig. 8).

*Generation1 results:* no significant difference in RGCs was found (p>0.05) between the groups of *Generation1*. The result was consistent under both methods. *Generation2 results:* manual analysis under microscopy (p<0.05) suggested that Group1 had reduced number of RGCs compared to Group2 and control group (Fig. 7).

### *Microglia*

We observed lower microglial cell numbers in *Generation1* vs *Generation2* (p<0.05) (Fig. 9).

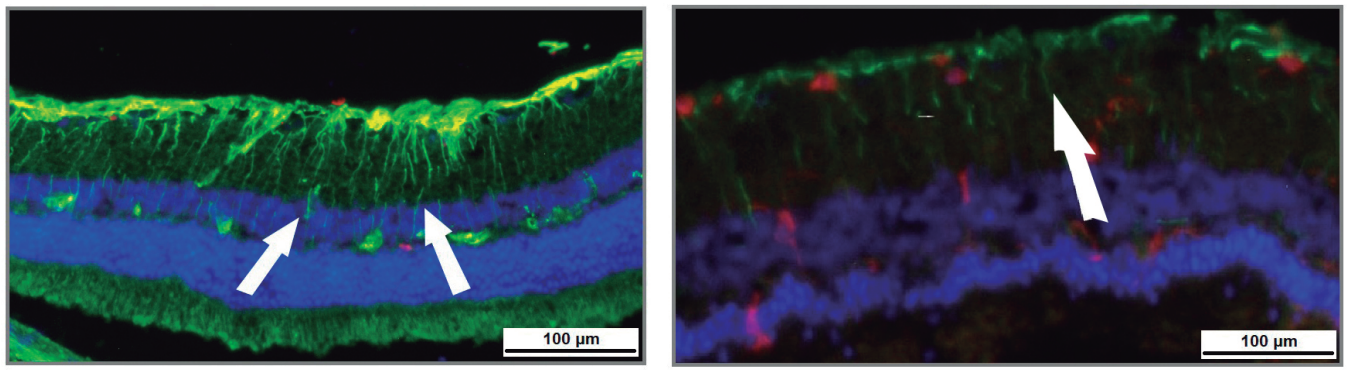


**Fig. 6.** Glial cell activity. GFAP immunofluorescence in nutritionally deprived group **(A)**, control group **(B)**. White arrows: Müller cell processes. GFAP, glial fibrillary acidic protein.

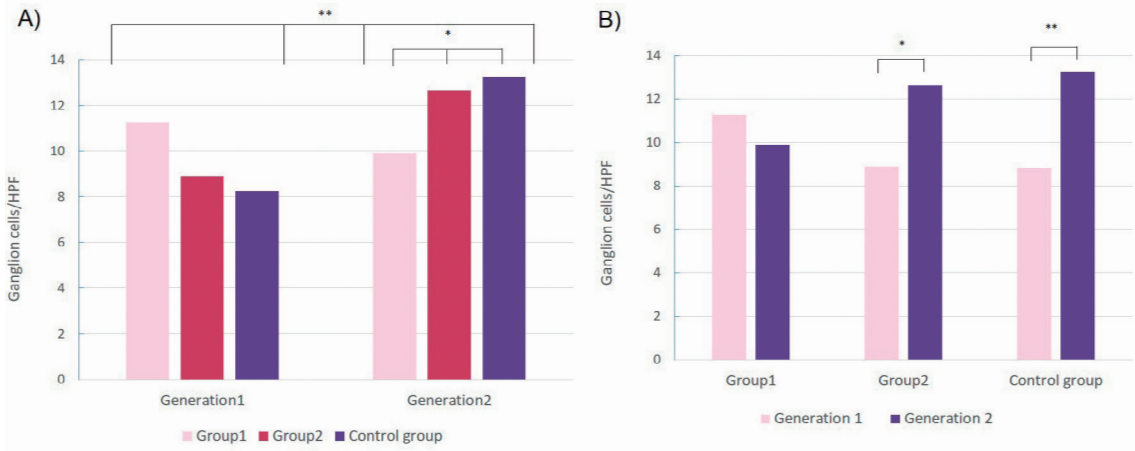


**Fig. 7.** RGCs between groups and generations. **A.** *: lower RGC counts in Group1 compared to Group2 and control group (p<0.05) in *Generation2*, **: higher RGC counts in *Generation2* vs *Generation1* (p<0.05). **B.** *: higher RGC counts in Group2 of *Generation2* vs *Generation1* (p<0.01), **: higher RGC counts in control group in *Generation2* vs *Generation1* (p<0.01). RGCs, retinal ganglion cells.

Generation1: lower microglial cell number was found in Group2 compared to the other groups ($p<0.05$). *Generation2*: offspring rats from Group1 and Group2 had no significant difference in the number of microglial cells ($p>0.05$), whereas both of these groups had significantly more cells compared to the control group ($p<0.01$) (Fig. 9). No results of Halo Iba1 red area analysis were significant ($p>0.05$) (Fig. 10).

## Discussion

In this study, we disclose histological changes in the retina of aged albino rats due to different maternal nutrition during pre-pregnancy and pregnancy period in

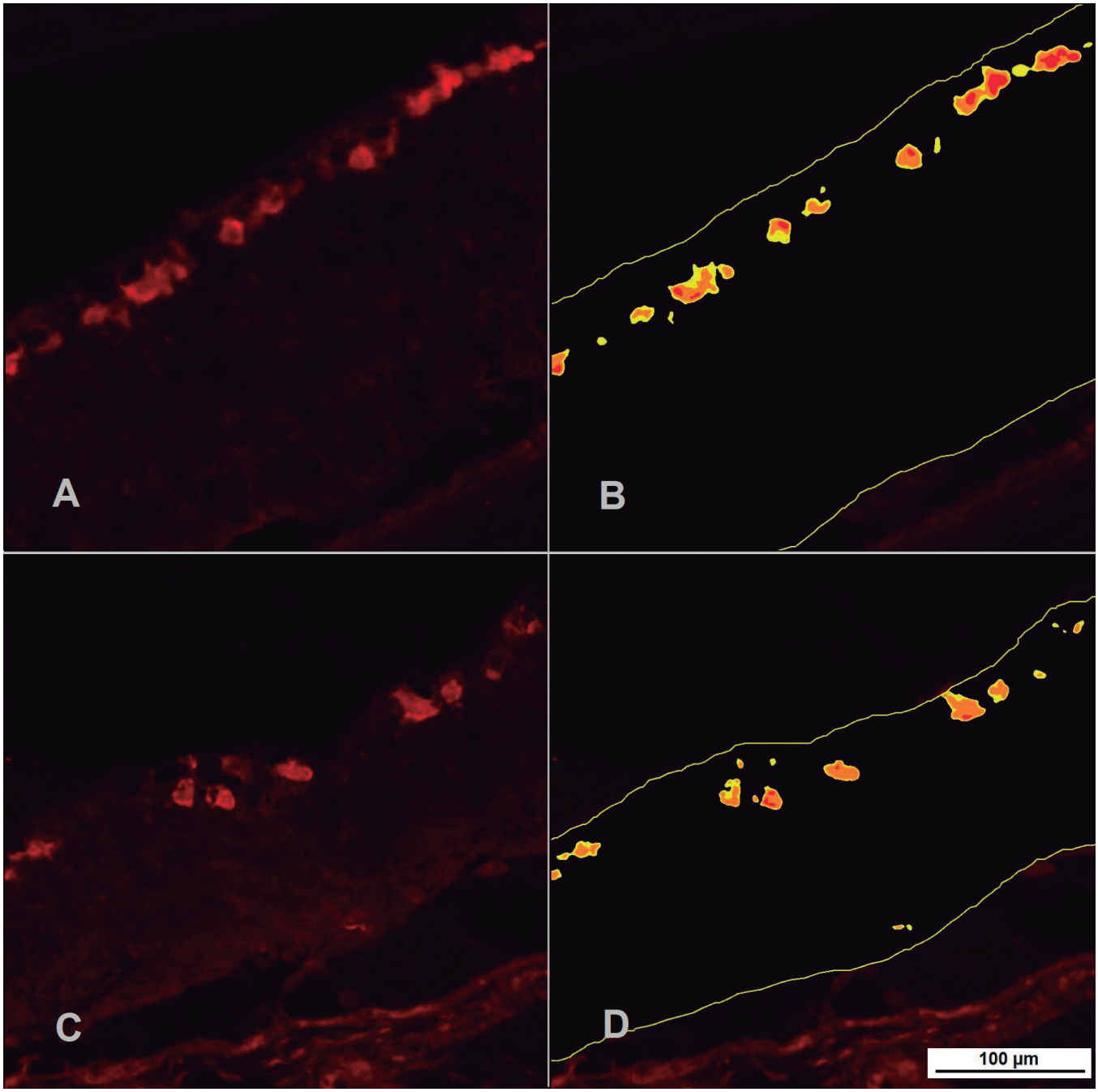


**Fig. 8.** RGCs in *Generation1* (red immunostained RGCs **(A)**, analysis of RGCs **(B)** (orange marked area) by Halo software) vs *Generation2* (red immunostained RGCs **(C)**, analysis of RGCs **(D)** (orange marked area) by Halo software) detected by DIA. RGCs, retinal ganglion cells; DIA, digital image analysis.

*Generation1* and *Generation2* of offspring. Various studies have investigated morphological changes in the retina of healthy aged rats and those affected by known factors. To the best of our knowledge, there are no other studies examining maternal nutritional restriction effects on the retina of aged offspring in two successive generations.

The use of automated and manual methods in the evaluation of different retina cells allowed us to have a wider view of possible tendencies in how mothers' nutrition could impact normal aging of the retina. Neither of these methods is impartial, but gives us more valuable information. Selection of methodology varies across studies. Automatic quantification is used in some of the studies (Nadal-Nicolás et al., 2018), but visualization of the retina using a microscope remains irreplaceable (El-Sayyad et al., 2014; Fernandez-Bueno et al., 2017; Raghu et al., 2017).

### *Retina layers. Nuclear layers*

Our data revealed obvious atrophy of photoreceptor layer and degeneration of ONL in all investigated groups. A logical explanation for these findings is changes of rat retina due to aging and exposure to light during the lifetime. As an example, El-Sayyad et al. analysed different age Wistar albino rat retinas and showed data of ONL and INL cell reduction and deterioration of photoreceptors induced by aging (El-Sayyad et al., 2014). There are more data supporting the occurrence of photoreceptors loss in aged rat retina. Bourque et al. found age-related photoreceptor dysfunction, however, without any anatomical changes in a group with prenatal hypoxia (Bourque et al., 2013). In comparison to other studies that have been done in this field, our study brings a new perspective by analysing the differences between groups with different maternal nutrition: offspring whose mothers were fed normally had larger segments of photoreceptor layer remaining and had more cells in INL-ONL than those whose mothers were nutritionally deprived.

Previous studies of the aging process demonstrated extended lifespan and protection of the central nervous system by caloric restriction in mice and rats (Obin et al., 2000; Velingkaar et al., 2020).

Obin et al. propose that caloric restriction decreases the loss of photoreceptors in aging pigmented rats (Obin et al., 2000). However, these studies analysed individual malnutrition effects for the aging process, which is different from our study model where offspring were fed normally. With the view of developmental programming, the negative effect of mothers' malnutrition in early fetal growth period on photoreceptors can be a result of metabolic stress in the organism (Barker, 1995, 1998, 2007; Painter et al., 2005; Roseboom et al., 2006; Barker et al., 2009; Padmanabhan et al., 2016; Zheng et al., 2016; Mandy and Nyirenda, 2018; Koletzko et al., 2019; Kinra et al., 2020).

ONL cell density is also associated with age, diet and light (Obin et al., 2000). Obin et al. demonstrated greater loss of ONL cell density in central and peripheral retina in caloric restricted group of 12-18 month old rats. ONL cell loss was associated with caloric restriction only in central retina for 18-24-month-old rats (Obin et al., 2000).

Findings of lower numbers of nuclei in INL-ONL and greater loss of photoreceptor layer for *Generation2* of offspring whose mothers were nutritionally restricted before and throughout pregnancy, confirms the effect of diet on the aforementioned retina structures. According to other studies, different light exposure or rat age is critically important for the status of photoreceptors (Obin et al., 2000). In our study, the only variable factor was exposure to different maternal diet. All rats were similarly aged (in parallel with humans, 20-month-old rat offspring were considered to be aged (Sengupta, 2013) and kept in equal environmental conditions, including light exposure. In other studies, taking into

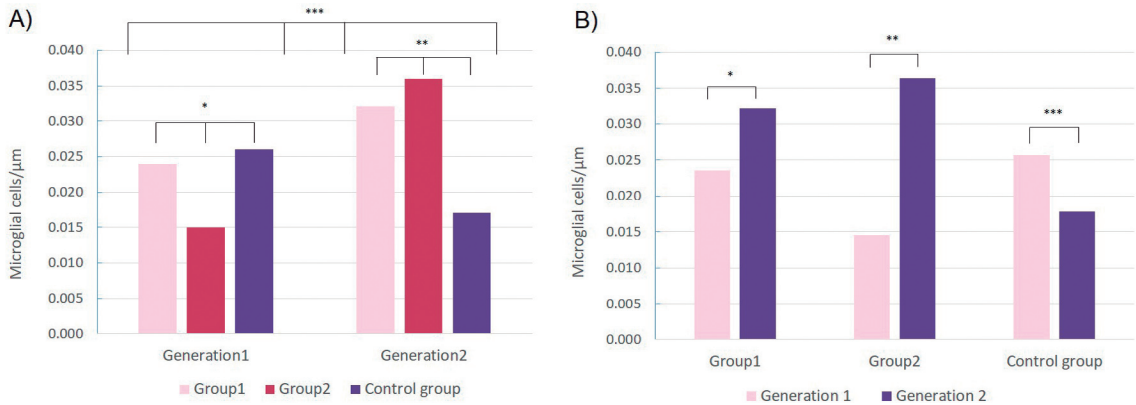


**Fig. 9.** Microglial cells between groups and generations. **A.** *: lower microglial cell number in Group2 compared to Group1 and control group (p<0.05) in *Generation1*, **: higher microglial cell number in Group1 and Group2 compared to the control group (p<0.01) in *Generation2*, ***: lower microglial cell number in *Generation1* vs *Generation2* (p<0.05). **B.** *: lower microglial cell number in Group1 of *Generation1* vs *Generation2* (p<0.01), **: lower microglial cell number in Group2 of *Generation1* (p<0.01), ***: higher microglial cell number in control group of Generation1 (p<0.05).

account caloric restriction (O'Steen and Landfield, 1991; Obin et al., 2000) during the period of early growth (before and throughout pregnancy) was not analysed. To our knowledge, this is the first study demonstrating the possible relationship between maternal nutrition and photoreceptor loss of aged *Generation2* offspring.

*Macroglia*

Human retinal macroglia consist of Müller cells and astrocytes. Macroglia maintain normal homeostasis of photoreceptors and neurons of the retina (Hoz et al., 2016; Reichenbach and Bringmann, 2020). Damage of

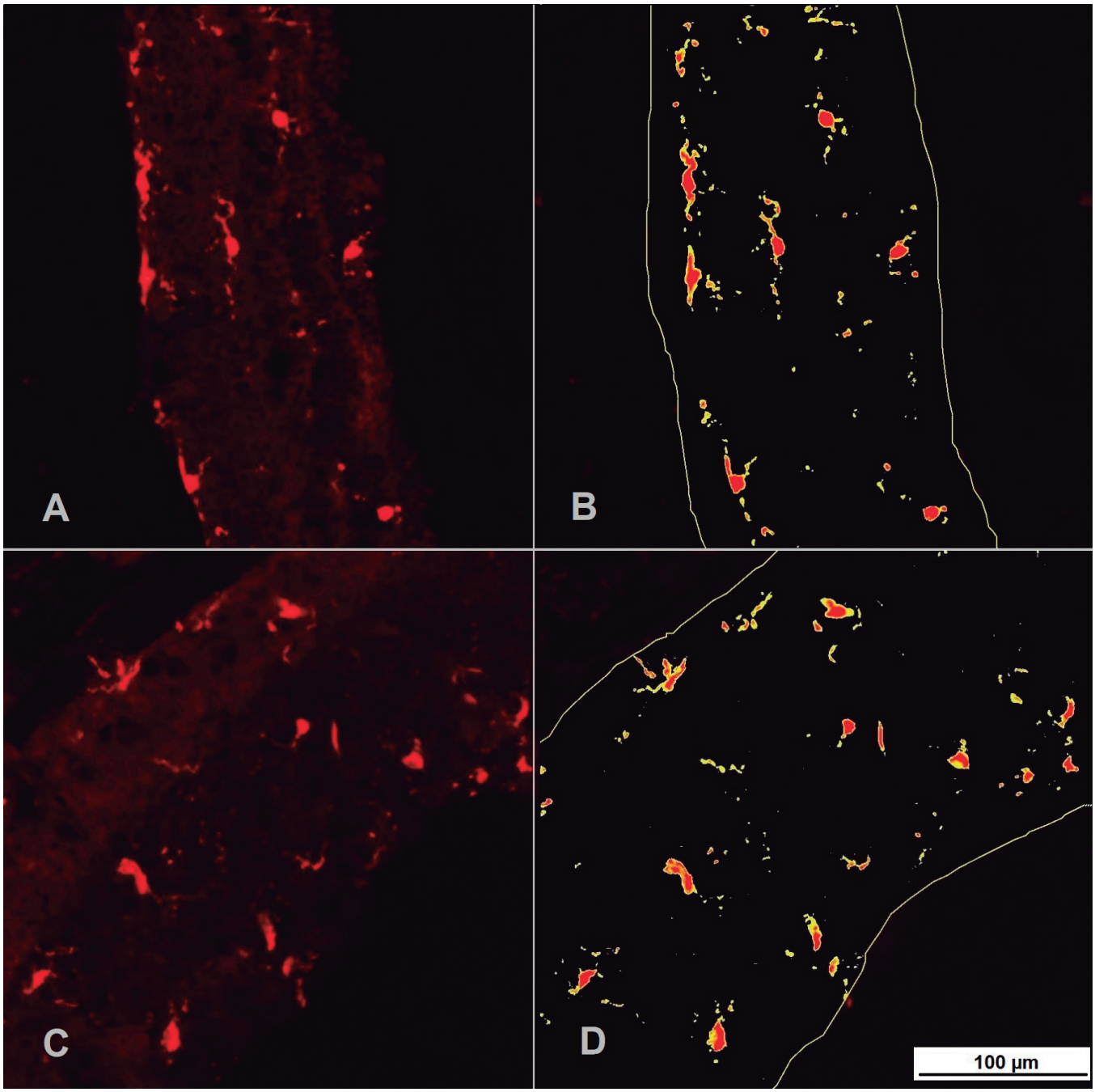


**Fig. 10.** Microglial cells in first generation (red immunostained microglial cells **(A)**, analysis of microglial cells **(B)** (red marked area) by Halo software) and second generation (red immunostained microglial cells **(C)**, analysis of microglial cells **(D)** (red marked area) by Halo software) analysed and detected by DIA. DIA, digital image analysis.

the retina results in active gliosis (Hoz et al, 2016). Gliosis of astrocytes protects RGCs from damage. Any pathogenic stimuli activate Müller cells to protect neurons, although this process can lead to degeneration of neurons as well (Reichenbach and Bringmann, 2015). Müller cells' epigenetic plasticity enables retina regeneration in some species. Epigenetically glia are similar to late-born retinal neurons (Dvoriantchikova et al., 2019). Such a characteristic augments the importance of glia in the retina.

GFAP is a major component of astrocyte intermediate filaments and is expressed only in Müller cells and astrocytes. Antibodies against GFAP mark normal and pathologically activated Müller cells.

Our data demonstrated higher macroglia activity in the *Generation2* offspring, whose mothers were nutritionally deprived before and throughout pregnancy. These findings suggest the idea that malnutrition in early growth period may provoke greater changes of aging macroglia even for *Generation2*. A subtle increase in GFAP staining was concluded as a response to prenatal stress (hypoxia) in Bourque et al. study (Bourque et al., 2013). Moreover, active gliosis has been indicated as an expression of neurodegeneration in the aging process (Mohamed et al., 2019; Reichenbach and Bringmann, 2020). Our data also support the idea that aging induces gliosis in the retina. We observed GFAP positive staining in all of the investigated groups, showing a moderate activation of macroglia in the retina of aged rats.

Further analysis is needed to understand what provokes macroglia activation in the offspring of malnourished mothers. Nutritional deprivation in early growth period may be one significant stressor. According to the well-known Barker's theory, due to developmental plasticity, malnutrition during fetal life can induce structural and functional body changes (Barker, 1995, 1998, 2007; Barker et al., 2009).

### *Ganglion cells*

Our data indicate that maternal nutritional deprivation had no clear effect on the amount of RGCs in albino rats' retina. The results obtained under two different methodologies comparing both generations also did not show any evident tendency on differences in the number of RGCs. More intense indexes of red area may suggest a higher number or larger area of cells in the *Generation1* vs the *Generation2*. Neufeld and Gachie demonstrated natural RGC loss in rats' retina during the lifetime and the loss of RGCs was lower for rats under caloric restriction, as it slowed the growth of the retina (Neufeld and Gachie, 2013). Such data support the idea that malnutrition does not have any negative impact on the malnourished person, but raises a question about the possible effect on future generations. If we consider the theory of Barker about developmental programming, we may assume that malnutrition can be a component which worsens the natural aging of the retina (Barker, 1995, 1998, 2007; Barker et al., 2009).

Other authors declare that retinal outer segment degeneration is followed by remodelling of the inner segment of retina. Garcia-Ayuso et al. concluded that following an unknown period after photoreceptor loss, RGCs wither too (Garcia-Ayuso et al., 2018). In the light of these findings we can argue that RGCs might be affected more in areas where photoreceptors are lost. To verify this theory, more detailed investigations would be needed.

The main variable factor in our study was maternal nutrition, which restricted our ability to estimate the impact of aging on the cell count the way it was done in several other studies. There are studies which report RGC loss in aged rats' retina (Calkins, 2013; El-Sayyad et al., 2014), while others show that RGCs do not change with age (Nadal-Nicolás et al., 2018). Nadal-Nicolás et al. showed that even the volume of retinal nerve fiber layer stayed unaffected in senescence (Nadal-Nicolás et al., 2018).

Further studies of greater extent are needed to specify possible changes of RGCs. Findings on RGCs can be an indicator of central nervous system neurons.

### *Microglia*

Microglia are primary immune cells of the retina located in the RGC layer, plexiform layers and around the vessels in healthy retina (Hoz et al., 2016; Reichenbach and Bringmann, 2020). Microglia main functions are protecting against microorganisms which may cause inflammation and repairing tissue (Reichenbach and Bringmann, 2020). It is known that retinal microglia migrate and become activated by stress (Couturier et al., 2014). Microglia cells protect neurons from existing pathological stimuli, although long term continuous stressors can trigger cells to damage the surrounding tissue (Rashid et al., 2019).

In an experiment, we used Iba1 molecular marker to label all microglia cells in the retina. Grotegut et al. co-localized ED1 and Iba1 markers to label the active form of microglia. This enables us to compare counts of active form cells between the groups (Grotegut et al., 2019). While the active form in our study was not distinguished separately, we still managed to find the trend in changes of microglia population between the different groups. We found a higher amount of microglia cells in nutritionally deprived groups compared to normally fed group in *Generation2*. Offspring from *Generation2* had a higher number of microglia cells compared to *Generation1*. As we have already established, microglia cells protect the tissue of the retina. At first sight, the higher number of cells could lead to better protection of the retina, however, if we consider that microglia cell migration and activation are caused by negative factors, higher counts of cells might mean an inflammatory process of the tissue (Rashid et al., 2019; Rathnasamy et al., 2019). In addition, our data suggest that undernutrition can act as a stressor causing activation of microglia by increasing the number of cells. Even aging

of microglia may lead to age-related retinal diseases, and activated microglia becomes the main objective of therapies that aim to treat retinal diseases (Rathnasamy et al., 2019). The trends we have seen as part of this study motivates us to continue our research with more focus on subtypes of microglia (Stratoulias et al., 2019).

There is evidence that developmental origins of health and disease driven by fetal programming under adverse environment may be visible even in subsequent generations.

Skinner showed that environmental factors during the gestational period directly exposed first and second generations (Skinner, 2008). Exposure of pregnant mice to a low-protein diet affected pancreas of even three generations of offspring (Frantz et al., 2011). In our study, retinal changes observed were more obvious in the second generation than in the first generation of rats. This could be just an incidental finding given a small sample size, but could also be related to compound intergenerational inheritance effects. Retina, eyes, brain and other organs of the nervous system are imperative to survival and could be more resistant to adverse environmental factors in the first generation, while compound effects become more obvious in the second generation.

### *Limitations of the present study*

The main limitation of this study is a small sample size of animals investigated. Additionally, we have assumed that sampling does not take into account the total retinal layer, which is another limiting factor of the study. We believe the study still provides a good basis for evaluating the trends of possible changes. Findings of significant differences between the investigated groups motivate us to continue our research with examination of retinal wholemounts.

Possible bias due to manually selecting the most representative areas (best quality) for image analysis in immunofluorescence images.

### *Conclusions*

In conclusion, our results support the possibility that maternal undernutrition is linked to aggravation of age-related changes in the retina of offspring. Maternal nutritional restriction at pre-pregnancy and pregnancy may entail degeneration of outer layers of the retina and activate Müller cells in *Generation2*. A malnourished preconception period might result in reduced retinal ganglion cell counts in *Generation2* but there was no statistically significant evidence to support the impact of nutrition on retinal ganglion cells counts in *Generation1*. Differences in retinal ganglion cell counts between generations are still unclear due to controversial results obtained by different evaluation methodologies. Higher counts of microglial cells in nutritionally restricted groups of *Generation2* can be linked to possible microglial activation in aging retina. To the best of our knowledge, there are no other studies examining maternal nutritional restriction effects on the retina of aged offspring in two generations, and this study is the first one to analyze the possible relationship between maternal nutrition and photoreceptor loss of aged *Generation2* offspring. Our findings suggest that the damage caused by nutritional deprivation may be distributed across all organ systems, including those that are non-imperative to survival. The data provide the baseline to understand the possible effects of maternal nutritional deficit on aging retinas, although further investigation is needed.

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