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Purpose: The exact cellular types that form the human fovea remain a subject of debate, and few studies have been conducted on human macula to solve this question. The purpose of this study was to perform immunohistochemistry on fresh human samples to characterize the glial cells that form the human fovea.
Methods: Immunohistochemistry was performed using antibodies against proteins expressed in astrocytes or in retinal Müller glial cells or both types of cells on six human macula obtained from eyes enucleated for peripheral intraocular tumors and on two postmortem eyes from healthy donors. The posterior poles of the enucleated eyes were cryosectioned and stained with antibodies against the glial proteins GFAP, vimentin, CRALBP, glutamine synthetase, and connexin 43.
Results: A population of cells positive for GFAP and negative for glutamine synthetase and CRALBP that express connexin 43 were identified at the roof of the foveal pit. These cells are distinct from the Müller cone cells described by Yamada and Gass, suggesting that another type of foveal glial cells, most likely astrocytes, are present in the human fovea.
Conclusions: This study showed that in humans, astrocytic glial cells cover the foveal pit. Their roles in macula homeostasis and mechanisms of macular disease remain to be determined.

The human macula is a highly specialized retinal area, located at the center of the visual axis that comprises less than 5% of the total retinal surface. The macula ensures visual acuity and photopic and color vision. With the development of imaging technologies, such as spectral domain optical tomography technology (SD-OCT), the macula morphology is being extensively explored and described in healthy and pathological conditions. In contrast, literature on the histology of the human macula is scarce due to the limited access to fresh human eyes. To improve the interpretation of OCT imaging, a better understanding of the cells that form the macula and the fovea is needed.

The macula is an area 5.5–6.0 mm in diameter, where the ganglion cell layer (GCL) is the thickest of the whole retina [1]. It is divided in concentric regions defined by the number of nuclei in the different cellular layers and by the orientation of the fibers in the outer plexiform layer. In the fovea, which is 1.5 mm in diameter, there are only cones (around 0.3% of the total number of cones). Their density is highest at the foveola where it reaches around 200,000/mm^2 with high interindividual variability [2]. In the foveola, there are only

cones and retinal Müller glial (RMG) cells and one or two rows of inner nuclei but no nerve fiber layer, no GCL and no inner plexiform layer, as these layers are displaced laterally. In the monkey fovea, equal numbers of Müller cell trunks and cone terminals were described with each Müller cell partially coating two to three cone terminals [3]. Thus, the density of RMG cells is higher in the fovea, where there are only cones. The parafovea is the region (500 µm in diameter around the fovea) with the largest fiber layer and a thick Henle's layer where RMG cells have a Z shape and are bound with cone axons by junction proteins [4].

During development of the retina, the macula forms progressively and continues maturing during childhood until the age of 10–12 years [5]. Astrocytes appear first near the optic disc, and by migrating subsequently further peripherally, they guide the vessels' development, avoiding the fovea that remains avascular during retinal angiogenesis [6,7]. The glial composition of the macula is thought to be exclusively composed of RMG cells. However, the structure of the macroglial cells in the fovea differs from that of the RMG cell structure in other parts of the retina. In 1969, E. Yamada reported the electron microscopy observation of a human retina where he described that the inner half of the foveola was composed of an inverted cone-shaped zone of RMG cells, which was called the "Müller cells cone" (MCC) Interestingly, in this description, a few nuclei of some "atypical glial

cells" were observed within the MCC, while the exact location of the foveal MCC cell nuclei was not identified. Yamada also reported that the inner limiting membrane (ILM) at the inner surface of the MCC was much thinner (10 nm to 20 nm) compared to the ILM in the perifoveal region [8], although the density of the RMG cells in this region is high. In 1999, D. Gass supported the existence of the MCC and hypothesized that it could serve as a reservoir for macular pigments, a plug for the maintenance of foveal cones, and that it could explain the pathogenesis of congenital foveoschisis and of age-related macular hole [9]. More recently, Govetto et al. showed that RMG cells in the MCC were stained differently by toluidine blue than other RMG cells on semithin sections of two human maculas and one monkey macula. They explained the foveal cone traction occurring in epiretinal membrane retraction, by the vertical traction of the cone RMG cells [10]. In this observation, the exact location of the vertical RMG cells nuclei was also not clearly identified. Finally, in the monkey retina, a recent immunohistochemistry study raised the possibility that another type of glial cells, probably astrocytes, might exist in addition to RMG cells in the MCC of non-human primates [11].

However, the structure and cellular glial composition of the human fovea remains a subject of debate. To provide additional information on glial cells of the human macula, we performed immunohistochemical analysis of human maculas using RMG and astrocyte markers.

METHODS

Human ocular tissues: The use of human samples adhered to the tenets of the Declaration of Helsinki and was approved by the local Ethics Committee of the Swiss Department of Health on research involving human subjects (CER-VD N°340/15) that the study adhered to the ARVO statement on human subjects. The subjects signed informed consent. Postmortem whole globes were received from the Lausanne Eye Bank, when the cornea had been considered unsuitable for transplantation. Table 1 summarizes information on the ocular samples. Five retinas were obtained from eyes enucleated between 2013 and 2018 for anterior uveal tumors or for conjunctival epidermoid carcinoma but an intact posterior pole. The enucleation procedure allowed to collect fresh tissues for analysis. Two retinas were taken from donor eyes, obtained within 10 h post mortem. The eyes were sectioned, and the anterior part (including the retina up to the equator) removed; the posterior poles were used for immunohistochemistry on cryosections.

Immunohistochemistry: Human samples were fixed in 4% paraformaldehyde for 24 h at 4 °C and then processed for immunohistochemistry. The samples were rinsed in saline and included in Tissue-Tek® (Sakura, Finetek, Netherlands). For fluorescence immunohistochemistry, 10-µm-thick neuroretina sections were incubated in blocking buffer (PBS 1X-137 mM, NaCl, 2.7 mM, KCl, 8 mM, Na_2HPO_4, and 2 mM KH_2PO_4, PH 7.4 with 10% fetal calf serum and 0.5% Triton X-100) for 2 h at room temperature, then rinsed in PBS and incubated at 4 °C in primary antibodies listed in Table 2, and diluted in PBS supplemented with 10% fetal calf serum and 0.1% Triton X-100. After washing in the same

ID	Sex	Age (year)	enucleation	Post mortem (h)	Comments
			TABLE 1. DATA RELATED TO THE OCULAR SAMPLES.		
P1	F	54	+		Peripheral temporal melanoma
					Normal macula
P2	F	53	+		Peripheral nasal melanoma
					Normal macula
P3	F	65	+		Ciliary body melanoma
					Normal macula
P4	F	75		7 h	Normal macula on post mortem observation
P5	M	69		9 h	Kidney failure
					No ocular history
P6	M	71	+		Ciliary body nasal melanoma
					Normal macula
P7	M	78	+		Epidermoid carcinoma of the conjunctiva - Normal macula
P8	F	85		34 h	Hypertension. No ocular history - Normal macula

TABLE 2. LIST OF ANTIBODIES USED.				
Antibodies	**Species**	**References**	**Lab provider**	**Dilution**
Anti-GFAP polyclonal central domain of the protein	Rabbit	ZO334	Dako cytomation	1/200
Anti-glutamine synthetase clone GS-6	Mouse	MAB 302	Merck Millipore	1/300
Anti-CRALBP monoclonal	Mouse	MA1-813	Thermo Fisher Scientific	1/300
Anti-vimentin polyclonal	Chicken	Ab24525	Abcam	1/500
Anti-CX43 C-ter domain	Goat	Sc-6560	Santa Cruz	1/200
Anti-ZO1 polyclonal	Rabbit	Sc-10804	Santa Cruz	1/200
Alexa-fluor 488 and 594			Invitrogen	1/300
4',6'-diamino 2-phenylindo DAPI				1/5000

buffer, the sections were incubated with their corresponding secondary antibodies AlexaFluo®488 or AlexaFluo®594 (Invitrogen/Thermo Fisher scientific Carlsbad, CA) for 3 h (Table 2). Sections were counterstained with 4′, 6-diamino-2-phenylindole (DAPI, Sigma Aldrich, Saint Quentin Fallavier, France), washed in saline, and mounted in Fluoromount (ThermoFisher Scientific, Herblain, France). Imaging was done with a laser scanning confocal microscope (LSM 710, Carl Zeiss, Munich, Germany) equipped with Zeiss 63x Plan-Apochromat oil immersion objective and the Zeiss ZEN software.

To differentiate the different types of microglial cells in the macula, we used several glial markers, expressed either in astrocytes or in RMG cells. Glial fibrillary acidic protein (GFAP) is expressed in astrocytes and is a marker of astrocytes in the brain and in the retina [12]. GFAP is also faintly expressed in RMG cells, located at the inner part of the RMG cell extension at the ILM. However, GFAP can be overexpressed all along RMG cells in the case of astrogliosis [13,14]. Vimentin is expressed in RMG cells, and not in mature astrocytes [12,15], although it can be expressed in some transformed astrocytes [16]. Glutamine synthetase (GS) is specifically expressed in RMG cells and not in astrocytes [17]. Cellular retinaldehyde-binding protein (CRALBP) is also a marker of RMG cells and is not expressed in astrocytes [18,19]. Connexin 43 is mostly expressed in GFAP-positive astrocytes in the retinal GCL to a lesser extent in the processes of RMG cells [20,21]. To control for nonspecific binding of the secondary antibody, staining was performed with secondary antibodies while omitting the primary antibodies (Appendix 1)

RESULTS

GFAP-positive cells are present at the surface of the foveal pit: Sections of the foveola pit from three patients (P1, P2, and P4) showed the presence of cell nuclei located at the innermost part of the pit, where only photoreceptor nuclei are present in the outer retina. All other retinal nuclei layers are absent, defining the foveola. The GCL, inner nuclear layer (INL), and outer nuclear layer (ONL) were clearly distinguished at the edges of the foveola. The cells present in the inner layer of the foveola were positively stained by the GFAP antibody, demonstrating their glial origin (Figure 1). These cells had lateral extensions over the retinal surface (Figure 1 insets) and were clearly different from radial central Müller glial cells. Importantly, although present in all the observed specimens, this retinal layer showed inter-individual variability with variable thickness and density.

Glial cells at the roof of the foveal pit express astrocytes but not Müller glial cells markers : To characterize the foveal glial cells, we performed coimmunolocalization of different markers of astrocytes and RMG cells on serial sections. Most of the glial proteins were expressed in the astrocytes and the Müller glia, except GS and CRALBP, which are considered specific RMG cell markers. In the P1 macula, GFAP stained the astrocytes in the nerve fiber layer (NFL) and around the retinal vessels up to the inner plexiform layer (Figure 2A and inset and B). However, GFAP also stained the Z-shaped Müller glial cells (Figure 2B, inset). GS labeled the Müller glial cells as transversal longitudinal cells spanning the entire retina from the INL to the ONL and harboring a Z shape in Henle's fiber layer at the macula (Figure 2C inset and F, and 2D inset). At the foveola, the GFAP-positive cells are not stained by GS (Figure 2E,B,D insets).

In the macula from patient 2 (Figure 3), GFAP stained the foveal inner glial cells and astrocytes around the vessels (Figure 3A,B). Vimentin nicely stained the foveal glial cells at the roof of the pit (called the glial plug) and the RMG cells of the fovea and all along the retina (Figure 3C,D inset). Interestingly, GS also stained the Müller glial cells at the fovea and all along the retina, but did not stain the foveal glial plug (Figure 3E,F inset). Colabeling of glial cells with GFAP and

GS showed that the two markers did not colocalize in the foveal glial plug (Figure 3G, arrow).

The macula of patient 3 (Figure 4) showed two sections of the fovea stained with GFAP (Figure 4A–D and insets) showing GFAP-positive cells around the retinal vessels and GFAP-positive cells in the foveal glial plug (Figure 4A,B inset), which send extensions up to the inner plexiform layer (Figure 4C,D inset). These cells did not extend up to the ONL like the foveal Müller glia (Figure 4I), indicating that two types of glial cells are present at the foveola. Vimentin stained the Müller glial cells and faintly stained the foveal glial plug (Figure 4G,H inset), CRALBP, which is a specific marker for Müller cells, did not stain the foveal glial plug (Figure 4E,F inset), demonstrating that those cells were not common RMG cells.

The macula of patient 4 (Figure 5) showed GFAP-positive cells at the roof of the foveola pit (Figure 5A,B inset), which sent some extensions toward Henle's fiber layer. CRALBP, which is a specific marker of Müller glial cells, labeled the Z-shaped Müller glia (Figure 5C,D insets) and the foveal Müller glia, but did not stain the foveal glial plug (Figure 5E, arrow).

The macula of patient 5 was folded during the section procedure. Nevertheless, the GFAP and GS colabeling showed again GFAP-positive cells in the foveal pit (Appendix
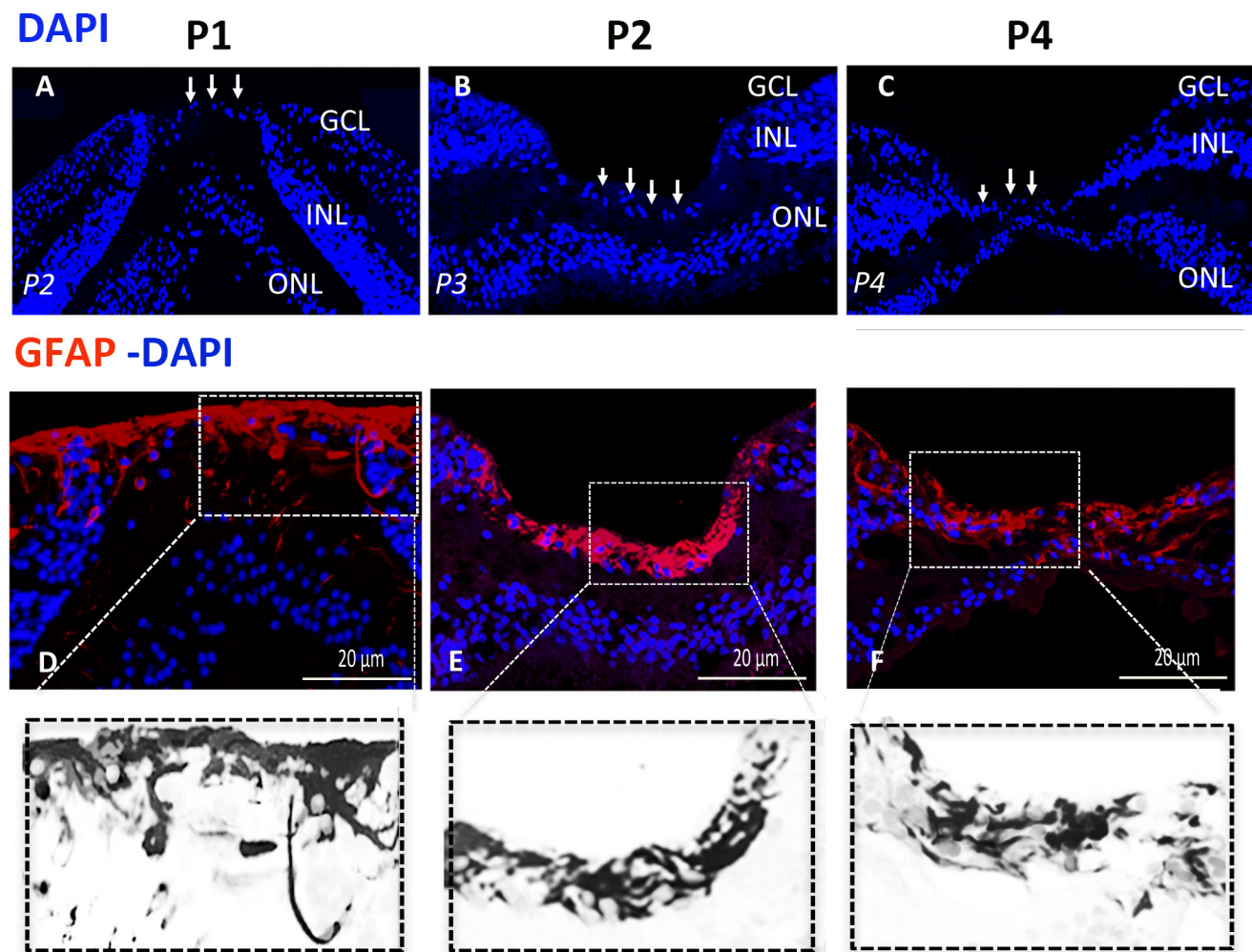

Figure 1. Cryosections and immunolabeling of cells at the roof of the foveal pit. Cryosections (10 μm thick) of the foveola from P1 (**A**), P2 (**B**), and P4 (**C**) showing the presence of cell nuclei (4′, 6-diamino-2-phenylindole [DAPI] staining) at the roof of the foveal pit (arrows). Costaining with the glial marker glial fibrillar acidic protein (GFAP) of the foveola from P1 (**D**), P2 (**E**), and P4 (**F**) shows that GFAP-positive cells with lateral extension lie on the roof of the fovea (black and white higher magnification insets). The nuclei of those cells are in the innermost layer and do not send radial extensions toward the outer nuclear layers, demonstrating that these cells are distinct from Müller cone cells. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.
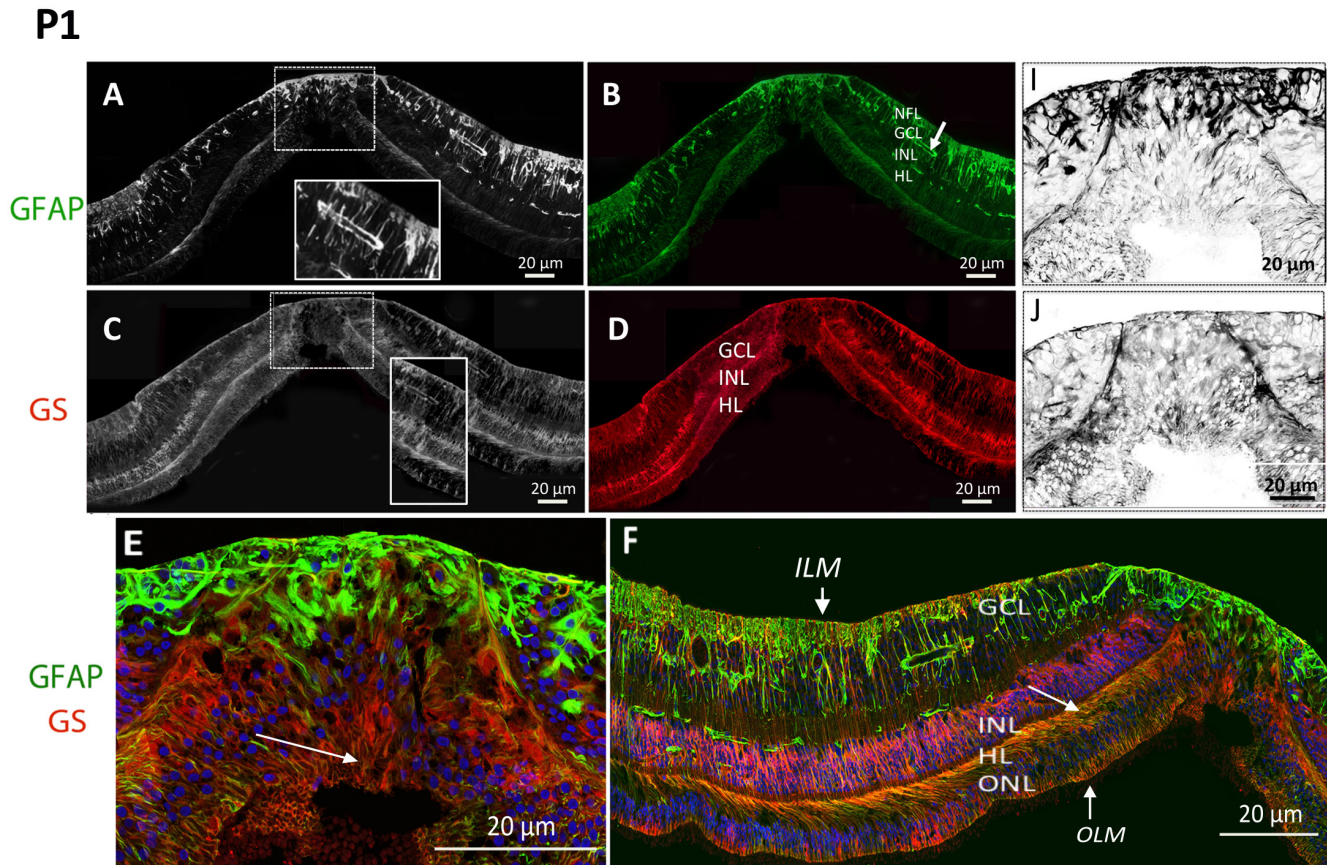
Figure 2. Coimmunostaining of P1 foveal glial cells with the Müller cell marker, GS, and GFAP. Coimmunostaining of foveal cryosections from P1 with glutamine synthetase (GS), which is a specific marker of Müller cells in humans, and with glial fibrillar acidic protein (GFAP) which stains astrocytes and activated retinal Müller glial cells, shows that GFAP-positive cells constitute the roof of the foveal pit **A**, **B**, and inset) and are not stained with GS (**C**, **D**, and inset). GFAP stains perivascular astrocytes (**B**, arrow), and GS stains the Müller cells' radial extensions at the fovea (**E**, arrow) and Z-shaped Müller cells in Henle's fiber layer (**F**, arrow). Note that the Z-shaped retinal Müller glial cells also express GFAP (**F**, yellow labeling). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; HL, Henle's fiber layer; OLM, outer limiting membrane.

2 and A inset), and Müller glial cells stained by GS in Henle fiber's layer and in the fovea (Appendix 2 inset). There was no costaining of the foveola glial plug by the two markers (Appendix 2). In the macula of patient 7, similar GFAP and GS costaining was observed (Appendix 3).

Glial cells at the roof of the foveal pit express Connexin 43: Connexin 43 (Cx43) is the main astrocytic connexin allowing close communication between astrocytes in the brain and in the retina. Cx43 is not specifically expressed in astrocytes, as retinal Müller glial cells have also been shown to express Cx43 in rodents [22] and in humans [20]. In the macula from three patients (P2, P4, and P7), Cx43 was highly expressed in the GFAP-positive foveal cells that formed the roof of the foveola. Cx43 was also expressed faintly in the retinal Müller

glial cells that form Henle's fiber layer, but was not expressed along the foveal cone Müller cells (Figure 6).

DISCUSSION

Using different glial markers, we identified a population of glial cells, resembling astrocytes, that forms the roof of the foveal pit. These cells have a star shape with longitudinal lateral extensions, and their nuclei are located at the innermost part of the foveola. They express astrocyte markers (GFAP, vimentin, and Cx43). We previously showed that they also express aquaporin 4 [23], but they do not express specific markers of Müller glial cells, such as GS and CRALBP, demonstrating that those cells are different from the MCC described by Yamada [8] followed by Gass [9]. Interestingly, in human macula specimens, the nuclei have repeatedly been
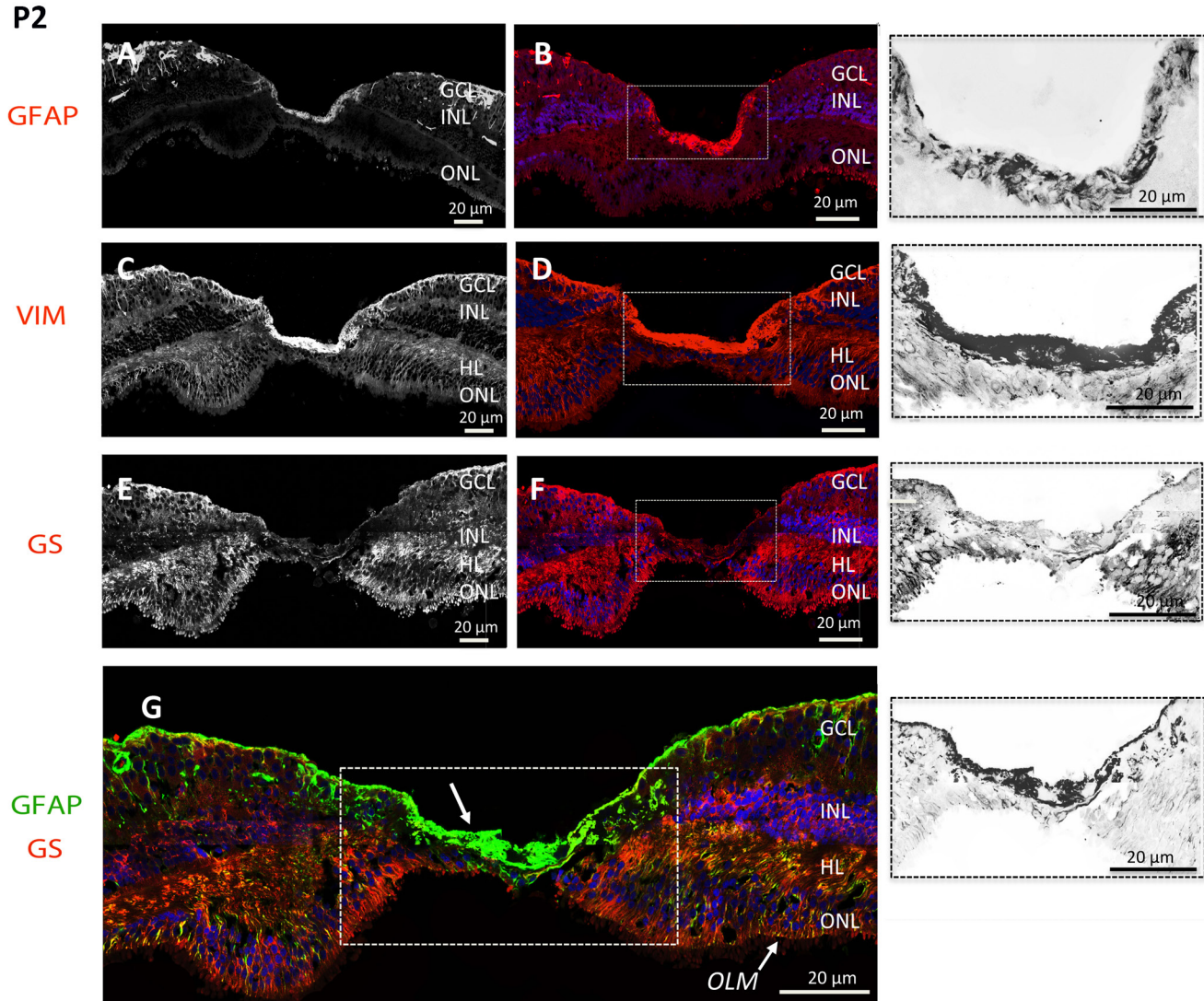
Figure 3. Coimmunostaining of P2 foveal glial cells with the Müller cell marker, GS, GFAP, and vimentin. Coimmunostaining of foveal cryosections from P2 with glutamine synthetase (GS), which is a specific marker of Müller cells in humans, glial fibrillar acidic protein (GFAP), and vimentin (VIM), which stains all glial origin cells, shows that the GFAP-positive cells at the roof of the foveal pit (**A**, **B**, and inset) are stained with vimentin (**C**, **D**, and inset) but do not express GS (**E**, **F**, and inset). Note that vimentin and GS stain the retinal Müller glial cells of the fovea (**C**, white arrow and inset black arrow) and the Z-shaped Müller cells of Henle's fiber layer (**D**). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; HL, Henle's fiber layer; OLM, outer limiting membrane.

found at the roof of the foveola, but the type of cells they belong to has never been clarified. Gass hypothesized that these nuclei could be those of the MCC [9], which was not supported by our observations. The presence of astrocytes in the foveola is intriguing. Controversy persists regarding the presence of astrocytes in the central retina before the fovea is formed. Two hypotheses have emerged from studies performed during the development of non-human primate retinas. One hypothesis is that the area where the macula will further develop remains devoid of astrocytes and vessels at all times [24]. The other is that astrocytes first invade the central retina but then decline prenatally in the perifoveal region [25]. The mechanisms by which the number of astrocytes declines have not been elucidated, as no cell death markers that support the death of astrocytes could be identified during macula development [25]. One of the hypotheses was that foveal astrocytes could lose the expression of GFAP and other glial markers, rather than die, simulating depletion. Thus, foveal astrocytes could be still present in the foveola but be undetectable with classical glial immunolocalization. Interestingly, in

Distler et al.'s study showing a decrease in perifoveal astrocytes, although a clear ring devoid of astrocytes was shown, a subset of stellate astrocytes was still present in the center of the foveola in one adult macaque flatmounted retina, which expressed GFAP [25]. There is still controversy regarding the presence of astrocytes in the fovea. In the present study, we collected adult human maculas from eyes enucleated because of peripheral malignant tumors and from two human donors with a short postmortem delay (less than 10 h). The fact that we could perform immunohistochemistry on fresh tissues is important as the expression of several glial markers, such as glutamine synthetase can decrease after death. All of the observed samples showed GFAP-positive astrocytes at the roof of the foveal pit, although with interindividual
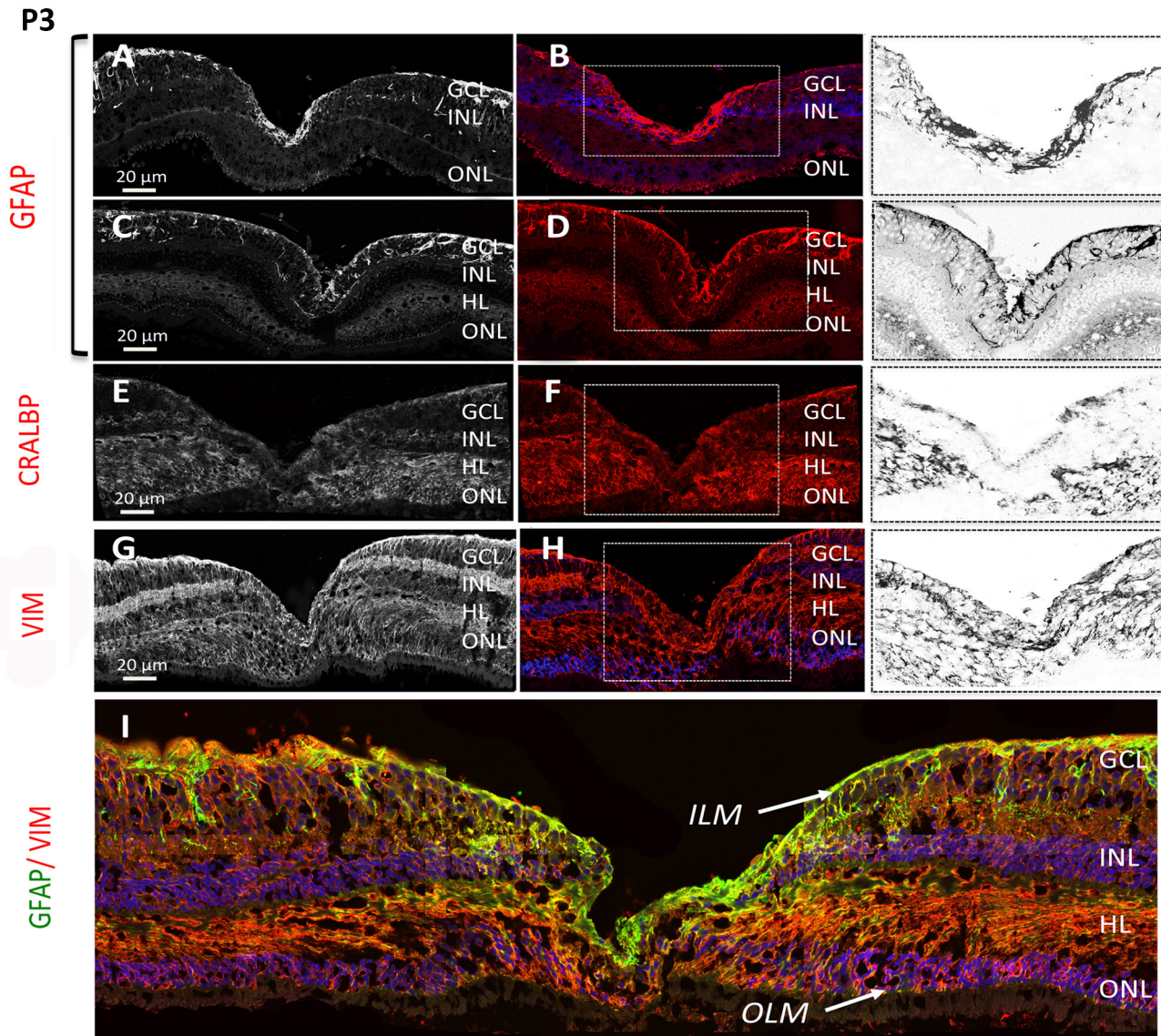

Figure 4. Coimmunostaining of P3 foveal glial cells with the Müller cell marker CRALBP, GFAP, and vimentin. Coimmunostaining of foveal cryosections from P3 with cellular retinaldehyde-binding protein (CRALBP), which is a specific marker of Müller cells in humans, glial fibrillar acidic protein (GFAP), and vimentin (VIM), which stains all glial origin cells, shows that the GFAP-positive cells at the roof of the foveal pit (**A**, **B**, **C**, **D**, and inset) are stained with vimentin (**G**, **H**, and inset) but do not express CRALBP (**E**, **F**, and inset). **I**: Costaining with GFAP and vimentin shows that the GFAP-positive cells at the roof of the pit (arrow) are faintly stained with vimentin and are distinct from the vimentin-positive Müller glial cell extensions (red arrow). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; HL, Henle's fiber layer; OLM, outer limiting membrane.
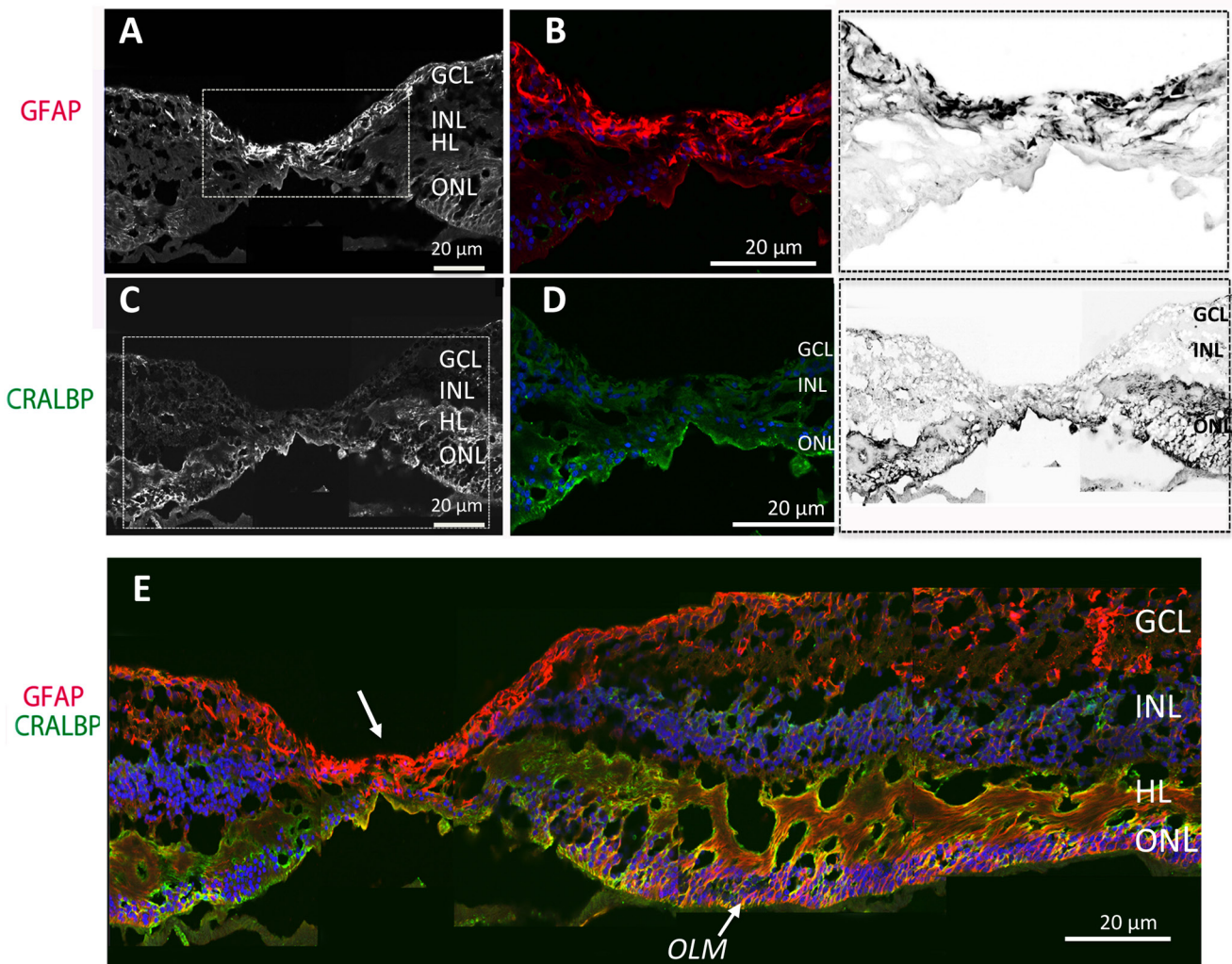
P4

Figure 5. Coimmunostaining of P4 foveal glial cells with the Müller cell marker CRALBP and GFAP. Coimmunostaining of foveal cryosections from P4 with cellular retinaldehyde-binding protein (CRALBP), which is a specific marker of Müller cells in humans, and glial fibrillar acidic protein (GFAP) shows that the GFAP-positive cells at the roof of the foveal pit (**A**, **B**, and inset) do not express CRALBP (**C**, **D**, and inset). **E**: Costaining with GFAP and CRALBP shows that the GFAP-positive cells at the roof of the pit (arrow) do not express CRALBP and are distinct from the CRALBP-positive Müller glial cells.

density, indicating that in adult humans, not only MCC but also astrocytes are present in the avascular fovea. To detect this cell population, it is important to perform immunohistochemistry on sections of the posterior segment of the eye, without removing the vitreous, whose traction can induce their detachment, explaining that they might not be detected on flatmounted maculas. As shown in a previous macaque study [24], in the human macula, the glial Z-shaped Müller cells of the perifoveal region that form Henle's fiber layer express vimentin but also GFAP.

The exact roles of foveal astrocytes have not been determined, but we hypothesize that they could (i) intervene in the foveal pigment import from the vessels to the fovea through the inter-glial communication network, (ii) protect foveal cones from excitotoxicity, (iii) and act as a belt that holds foveal cones and the MCC, in a region where the

inner limiting membrane is the thinnest. This hypothesis is supported by the clinical observation that vitreous traction on the foveal pit can induce the detachment of a dense material during the formation of the lamellar hole, without causing significant functional damage [26] (Appendix 4). If the detached foveal glial cells correspond to the MCC, which is closely linked and attached to cone pedicles, it is unlikely that cones could continue to survive and function normally.

This study has several limitations, such as the limited number of fresh maculas and that some originated from eyes that had a peripheral tumor, which could have induced the activation of glial cells. However, interestingly, we did not observe in these samples overexpression of GFAP in the retinal Müller glial cells. In addition, the same observation was made on two human donor eyes that were devoid of any known ocular pathology.

In conclusion, this study showed that in the adult human macula, a specific astrocytic cell population covers the roof of the foveal pit. This finding could help understand mechanisms of macular interface pathologies. Further studies are required to determine the origin and roles of this cell population.
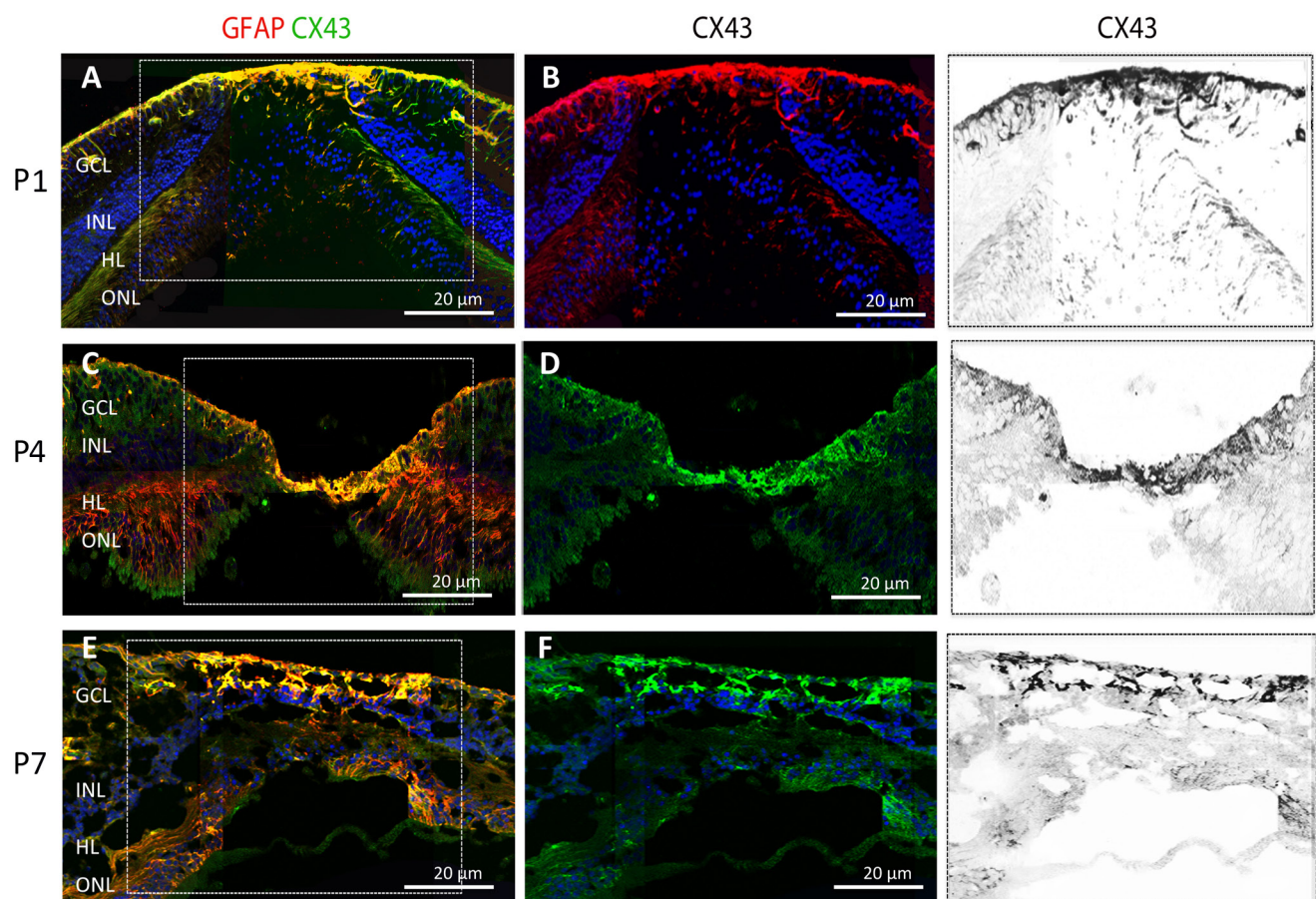

Figure 6. Cx43 and GFAP immunostaining. Glial fibrillar acidic protein (GFAP)-positive astrocytes at the roof of the foveal pit from P1 (**A**, **B**, and inset), P4 (**C**, **D**, and inset), and P7 (**E**, **F**, and inset) express connexin 43 (Cx43). Cx43 is also faintly expressed in retinal Müller glial (RMG) cells in Henle's fiber layer.

APPENDIX 1. TO CONTROL FOR NONSPECIFIC BINDING OF THE SECONDARY ANTIBODY, STAINING WAS PERFORMED WITH SECONDARY ANTIBODIES WHILE OMITTING THE PRIMARY ANTIBODIES.

To access the data, click or select the words "Appendix 1." Except from non-specific auto fluorescence of the outer segments, there was no staining with none of the secondary antibodies used in our experiments.

APPENDIX 2. CO-IMMUNOSTAINING OF P5 FOVEAL GLIAL CELLS WITH THE MÜLLER CELL MARKER GLUTAMINE SYNTHETASE (GS) AND GFAP.

To access the data, click or select the words "Appendix 2." Co-immunostaining of foveal cryosections from P5 with GS, which is a specific marker of Müller cells in humans and GFAP shows that the GFAP positive cells at the roof of the foveal pit that is folded (A, B and inset) do not express GS (C and D and inset). Co staining with GFAP and GS (E) shows that the GFAP positive cells at the roof of the pit do not express GS and are distinct from the GS positive glial Müller cells, in the fovea and in the Henle layer (E).

APPENDIX 3. CO-IMMUNOSTAINING OF P7 FOVEAL GLIAL CELLS WITH BOTH MÜLLER CELL MARKER GLUTAMINE SYNTHETASE (GS) AND RETINALDEHYDE- BINDING PROTEIN (CRALBP), WITH GFAP.

To access the data, click or select the words "Appendix 3." Co-immunostaining of foveal cryosections from P7 with CRALPB and GS, which is are specific marker of Müller cells in humans and GFAP shows that the GFAP positive cells at the roof of the foveal pit that is folded (C, E, and F) do not express GS (D and inset) not CRALBP (B and inset). Co staining of GFAP with CRALBP (E) or GS (F) shows that the GFAP positive cells at the roof of the pit do not express the Muller cells markers that are expressed by cells at the Henle layer.

APPENDIX 4. SD-OCT IMAGES OF MACULAE A.SD-OCT B SCAN OF A WOMAN WHO WAS 75 YEARS OLD, PRESENTING WITH A LAMELLAR HOLE IN FORMATION.

To access the data, click or select the words "Appendix 4." The posterior hyaloid remains attached to the dense hyper-reflective roof of the foveal pit, while separation of inner layer occurs at the OPL. B. Five years later, the Henle layer is stretched and the hyperreflective dense part of the foveola remains on the detached posterior vitreous in front of the foveola (arrow). Note that no epiretinal membrane is detected and that the external limiting membrane and ellipsoid zone are intact. Vision has remained 20/20 during the whole process and the patient has remained asymptomatic.

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