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# Expression patterns of aquaporins 1 and 4 in stroke

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## Abstract

Ischemic stroke occurs through embolic or thrombotic obliteration of an artery from cerebral circulation and represents over 80% of all stroke cases. One of the fiercest complications after stroke is edema, which results from imbalanced water diffusion around the blood vessels walls. Water diffusion around blood vessel walls occurs physiologically mainly through two protein-formed pores, namely aquaporins (AQPs) 1 and 4. Here, we compare for the first time the expression patterns and colocalization degrees of the two AQPs in control brain tissue and in peri-ischemic regions, on tissue obtained from eight patients with confirmed ischemic pathology and from five control cases. Our analysis showed that AQP4 is more abundant that AQP1, especially in the cortex and in the organized scar areas. The colocalization of the two markers was high, both located on the astrocytes membranes, but the colocalization degree decreased in the scar peri-ischemic regions. Colocalization with basement membranes was also lower for AQP1 compared to AQP4, in all regions analyzed.

***Keywords***: aquaporin 1, aquaporin 4, ischemic stroke, human brain tissue.

## Introduction

In the world, stroke represents the leading cause of death in developed countries and the prevalence of this condition is increasing slightly [1]. Ischemic stroke is the most common type, represents 80% of all stroke patients and occurs from a sharp decrease of blood flow to a particular region of the brain; neurological dysfunctions are the main consequence of this disease [2].

Cerebral edema is the most notable acute complication of stroke, for which there is no pathogenic therapy at present, but parenteral administration of hypertonic solutions like mannitol is accepted because it causes the passage of water from the cerebral parenchyma into the bloodstream and reduces intracranial pressure [3]. Cerebral edema in ischemic stroke usually occurs in the first week, with a peak between the first and third day [4]. Cerebral edema has a cytotoxic form that appears in the first minutes of ischemia and a vasogenic form that appears due to alterations of the blood–brain barrier (BBB) [5, 6]. Cytotoxic edema is characterized by intracellular accumulation of water without alteration of the BBB, while in vasogenic edema the blood proteins followed by water migrate into the tissue after disruption of the BBB [5].

Aquaporins (AQPs) are a family of proteins, made up of more than 10 subtypes, with different locations in the human body, whose main role is in facilitating the transport of water in different cells, first identified in the early 1990s [7, 8]. Recent studies have shown the presence of eight AQPs in the brain: AQP9, AQP1, AQP5, AQP4, AQP8, AQP3, AQP11, AQP7; of all the best represented are AQP1 and AQP4. These water channels are in the form of tetramers and are located in the cell membranes. In the brain, AQP4 is the most abundant water channel, is located in the astrocyte extensions and acts according to the local osmotic forces as a bidirectional channel [9].

Besides their importance in reducing cerebral edema, AQP are also involved in other diseases, such as traumatic brain injury, brain tumors, epilepsy, *neuromyelitis optica* (NMO), Parkinson's disease, Alzheimer's disease (AD), hydrocephalus, major depressive disorder schizophrenia, and autism [10–14].

In this article, we focused on describing the location of AQP1 and AQP4 in stroke and on observing their degree of colocalization in different areas like the peri-lesional scar, perilesional white matter (WM), control cortices and WM areas.

## Materials and Methods

We have utilized here brain tissue from eight patients with confirmed ischemic pathology and from five patients that died of non-central nervous system (CNS) related causes (Table 1).

These patients had been admitted and diagnosed in the Department of Neurology, Clinical Hospital of Neuro-psychiatry, University of Medicine and Pharmacy of Craiova, Romania, and their biological material had been included in the brain bank project that is undergoing in the Department of Histology from University of Medicine and Pharmacy of Craiova. The average ages were of 67.13± 5.03 years for stroke patients, and of 66.4±7.02 years for control cases.

**Table 1 –** ***Description of the patients***

| Age [years] | Gender | Pathology |
|---|---|---|
| 56 | M | Bilateral acute temporal lesion |
| 67 | M | Cortical ischemic event in both occipital lobes, right hippocampus and brainstem |
| 68 | F | Acute cortical ischemic event in the left fronto-temporal lobes |
| 66 | M | Large old right temporo-parietal ischemic lesion |
| 68 | M | Old cortical and subcortical ischemic lesion with cavitation and gliotic scar |
| 68 | F | Old cystic cavitation in the white matter beneath the left temporal lobe |
| 71 | M | Old cortical and subcortical ischemic event of the right temporal lobe |
| 73 | M | Old cortical and subcortical ischemic event of the left parietal lobe |
| 56 | F | Digestive tract tumor |
| 64 | F | Myocardial infarction |
| 68 | M | Lung tumor |
| 69 | F | Digestive tumor |
| 75 | M | Digestive tumor |

M: Male; F; Female.

Selected paraffin coupes were included in this study after re-confirmation of acute and organized hypoxic changes and liquefactive necrosis with variable surrounding areas of gliosis. No particular changes were noted for control tissue, except accumulation of *corpora amylacea* under the *pia mater* and around blood vessels. For immunohistochemistry (IHC), seriate sections were cut, deparaffinized, re-hydrated, and first processed for antigen retrieval in citrate buffer (0.1 M, pH 6) by microwaving for 20 minutes, at 650 W. After cooling down to room temperature, endogenous peroxidase was inhibited with a 1% solution of water peroxide for 30 minutes, then the unspecific binding sites were blocked in 3% skimmed milk (Biorad, California, USA) for another 30 minutes. For enzymatic detection, the slides were next incubated for 18 hours, at 4°C, with the primary antibodies (Table 2).

Next day, the signal was amplified with a species-specific peroxidase labeled polymer (Nichirei Biosciences, Tokyo, Japan) and visualized with 3,3'-diaminobenzidine (DAB) (Nichirei Biosciences). After a Hematoxylin counterstaining, the slides were coverslipped with xylene-based mounting medium (Sigma-Aldrich, St. Louis, MO, USA).

**Table 2 –** ***The antibodies utilized in this study***

| Name | Clonality | Staining pattern | Immunization target | Dilution | Vendor |
|---|---|---|---|---|---|
| *anti-AQP1* | Mouse, IgG1, clone 1/A5F6 | AQP1 on astrocyte membrane | An epitope within the cytoplasmic domain of the water-specific channel AQP1 | 1:200 | NB600-749 Novus Biologicals, UK |
| *anti-AQP4* | Rabbit, polyclonal | AQP4 on astrocyte membrane | Amino acids 244–323, C-terminal, of human AQP4 | 1:1000, 1:3000 (fluorescence) | sc-20812, Santa Cruz, USA |
| *anti-Col. IV* | Mouse, IgG1k, clone CIV22 | Basement membranes | Purified pepsin fragments of Col. IV isolated from human kidney | 1:30, with tyramide | M0785, Dako, Denmark |
| *anti-Col. IV* | Rabbit, polyclonal | Basement membranes | Col. IV from human and bovine placenta | 1:50 | NB120-6586, Novus Biologicals, UK |
| *anti-GFAP* | Mouse, IgG1k, clone GA5 | Astrocyte cytoskeleton | GFAP from porcine spinal cord | 1:500 | ABIN125137, Antibodies-online, Germany |
| *anti-GFAP* | Rabbit, polyclonal | Astrocyte cytoskeleton | GFAP isolated from cow spinal cord | 1:20 000 | Z0334, Dako, Denmark |

*Antigen retrieval utilized: microwaving in 0.1 M citrate, pH 6. AQP: Aquaporin; Col. IV: Collagen type IV; GFAP: Glial fibrillary acidic protein; IgG: Immunoglobulin G.

For fluorescence multiple stainings, the slides were processed as described above before adding the primary antibodies. All washings steps were done in a 0.1 M neutral phosphate-buffered solution (PBS).

For triple staining for AQP4/AQP1/glial fibrillary acidic protein (GFAP), the slides were first incubated with highly diluted anti-rabbit AQP4 (1:3000) together with the anti-mouse AQP1. Next day, the slides were incubated with an anti-rabbit peroxidase labeled polymer (Nichirei) together with anti-mouse Alexa Fluor 596 (1:300, Invitrogen) for one hour, washed in PBS, and then the peroxidase signal was detected with tyramide Alexa 488 (1:200, Invitrogen, Life Technologies GmbH, Germany) for 10 minutes. After thorough washing, the slides were incubated again with the third primary antibody, the anti-rabbit GFAP, for 18 hours at 4°C. The signal was detected with an anti-rabbit Alexa Fluor 555 for 30 minutes, this secondary being enough to visualize the highly abundant GFAP target, but too weak to visualize the anti-rabbit AQP4 from the first round of staining.

For double stainings for AQP4/collagen IV and AQP1/collagen IV, slides were incubated simultaneously overnight, at 4°C, with the pairs of primary antibodies of different species (Table 2), and the next day with a pair of species-specific Alexa Fluor 596- and 488-labeled antibodies, for 30 minutes. All slides were washed again in PBS and finally coversliped with a 4',6-diamidino-2-phenylindole (DAPI)-containing coverslipping medium (Invitrogen).

Images were collected randomly from the regions of interest (closest WM and perilesional cortices, as well as random areas from control tissue), capturing five microscopic fields for each region. Imaging was performed on our Nikon 90i (Nikon, Elta 90, Romania), fluorescence microscope equipped with a 16 Mp DS-Ri1 complementary metal oxide semiconductor (CMOS) camera, custom made narrow band filters able to differentiate DAPI, Alexa 488, Alexa 555 and Alexa 594 spectra without cross-bleed (Chroma Technology Corp., Bellows Falls, USA), and the Nikon NIS-Elements Advanced Research package including automation and deconvolution algorithms. Deconvoluted fluorescence images were analyzed in what it regards signal areas of individual stainings, and overlapping coefficients for paired fluorescent channels. Data were averaged for all the images from each patient, and then averaged again for the control group, respectively the stroke group. All the

data were represented graphically as average ± the standard error of the means in Excel 2016, and a Student's *t*-test was utilized to search for differences between pairs. In all cases, $p<0.05$ indicated statistical significance.

## Results

We have first evaluated the immunoexpression of AQP1 *versus* AQP4 on seriate sections from perilesional tissue, in both the cortex and WM (Figure 1). In the perilesional cortex, as well as in control tissue, AQP1 had a much fainter expression compared to AQP4, although the signal was distributed in the same compartments: around blood vessels and in the intraparenchymal astrocytes (Figure 1, A, A1, B and B1). In the immediate peri-liquefaction core, in the glial scar, the signal was much more diffuse and denser for both markers, again with apparent less staining for AQP1 (Figure 1, A, A2, B and B2). The stainings were even more diffuse, and the differences even more subtle for the perilesional WM (Figure 1, A, A3, B and B3).

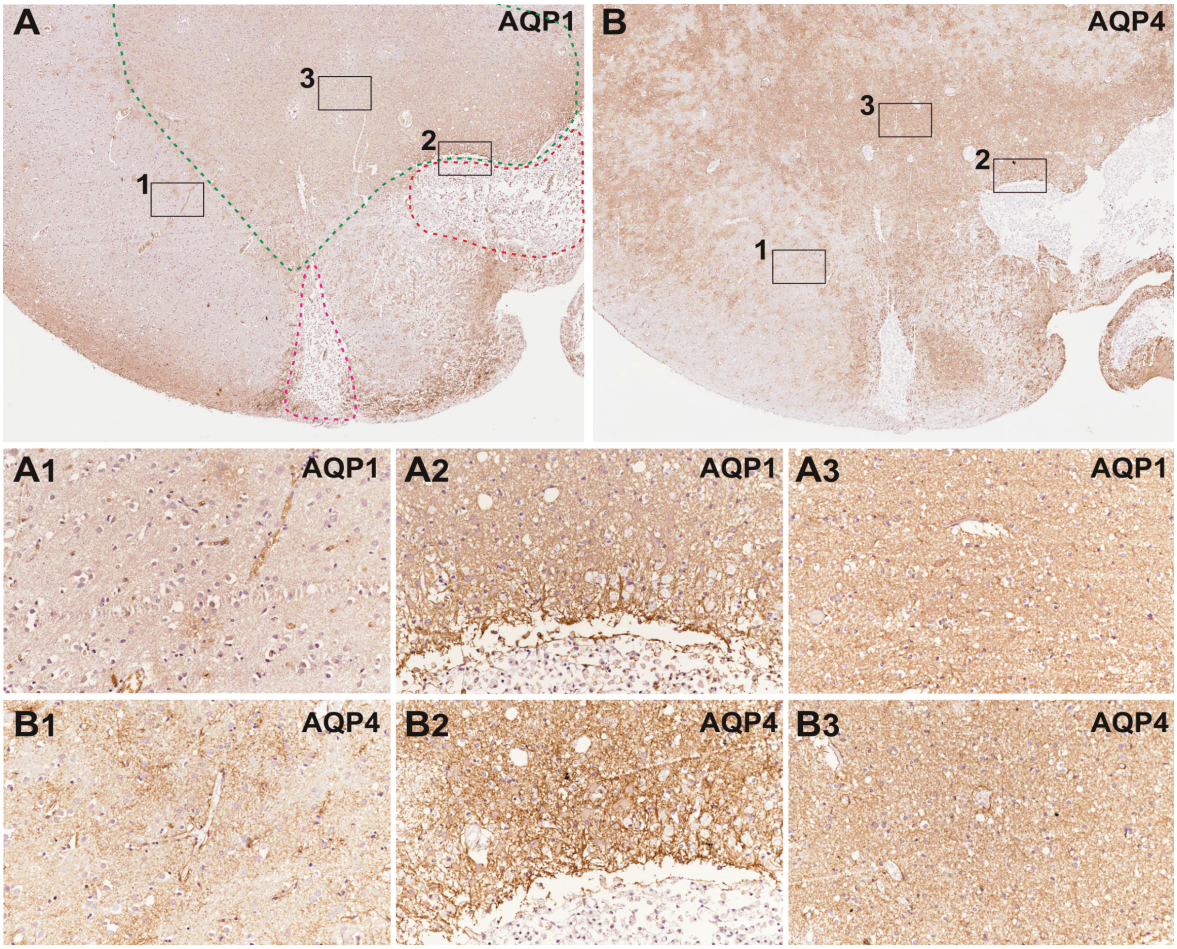


**Figure 1** – ***Immunoexpression patterns of AQP1 and AQP4 in a peri-liquefactive organized scar region. Perilesional cortex shows more AQP4 signal both around blood vessels and in the parenchymal astrocytes (A, B, A1 and B1); perilesional scar tissue reveals a dense-diffuse staining for both markers (A, B, A2 and B2); and white matter shows a patchy and more intense staining for AQP4 (A, B, A3 and B3). (A and B) 5× scan; (A1–B3) 20×. AQP: Aquaporin.***

Next, we assessed comparatively the expression of the two AQPs, in fluorescently stained sections from perilesional cortices (Figure 2). While the diffuse expression patter showed a high degree of colocalization for the WM, the cortex itself showed distinctive patterns for the two proteins (Figure 2, A–C). Thus, while AQP4 was intensely expressed by the *pia mater* and by focal astrocytes in the cortex itself, AQP1 was almost non-existent at the level of the *pia*, and with only sparse astrocytes being positively co-labeled by this marker (Figure 2, c1–c4). An interesting finding was that while most AQP1 signal co-localized with GFAP, not all AQP4 signal came from GFAP-labeled astrocytes. However, this apparent overexpression of AQP4 over AQP1 was not generalized, but it varied with the histological area. For example, there were perilesional cortices where AQP4 signal was restricted to rare astrocytes, while AQP1 showed a much more spread area of representation (Figure 3, A–C). It was in these regions that AQP4 was either co-colocalized with AQP1 or present alone. The most interesting phenomenon, however, was in the glial scar surrounding the necrotic core, where AQP1 expression seemed to be restricted to the immediate vicinity of the gemistocytes' membrane, with the perinuclear areas being devoided of signal (Figure 3, D–F). This feature was not observed for AQP4 in the scar regions. Around blood vessels, both AQPs were expressed around the basement membranes on the abluminal surface of the vessel, with AQP1 being mostly restricted to this area, and with AQP4 being intensely expressed in the surrounding astrocytes (Figure 3, G–J). However, on astrocytic endfeets, there was clear-cut colocalization of AQP4, AQP1 with GFAP cytoskeleton (detail in Figure 3, G–J).

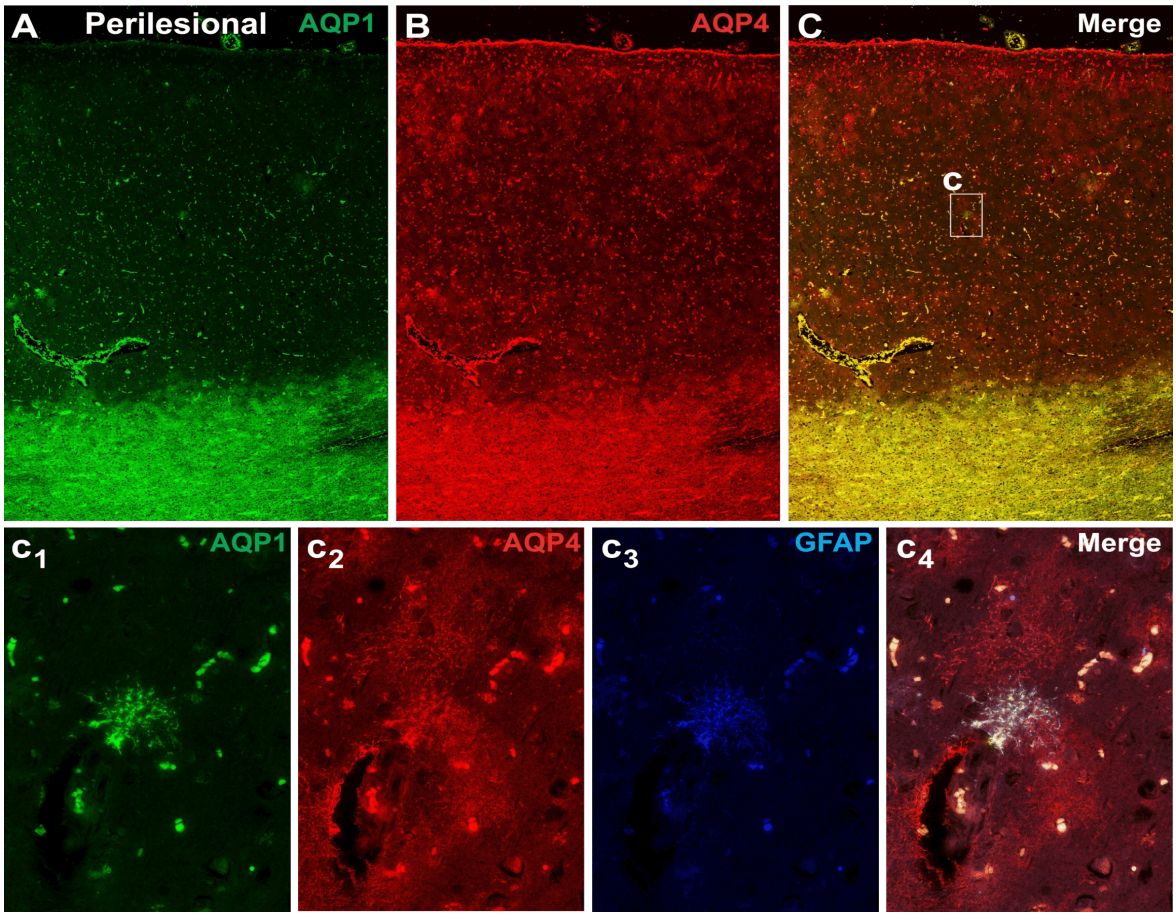


**Figure 2** – *Co-expression of AQP1, AQP4 and GFAP in perilesional cortices. AQP1 is expressed sparsely in the cortex and more intensely in the white matter, where it also colocalize more with AQP4 (A–C). Almost all AQP1 astrocytes are also AQP4 and GFAP positive, but not all AQP4 astrocytes express GFAP (c1–c4). (A–C) 5× scan; (c1–c4) 20×. AQP: Aquaporin; GFAP: Glial fibrillary acidic protein.*

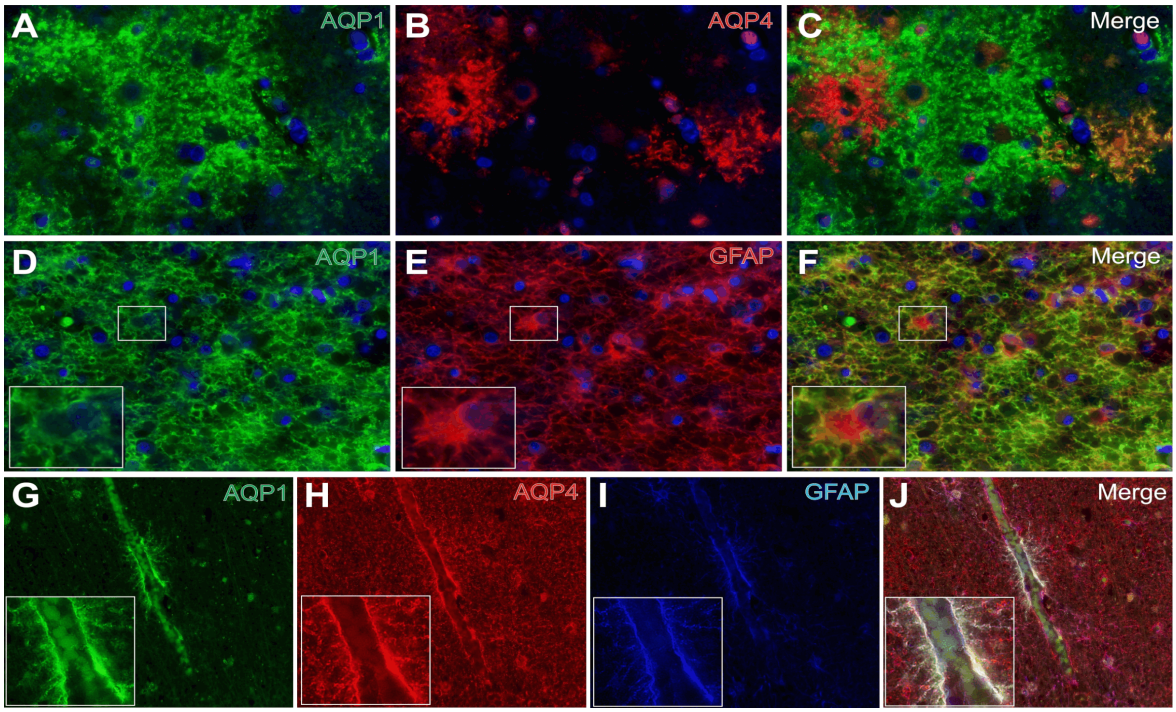


**Figure 3** – *A variety of AQP1–AQP4 co-expression patterns is present. AQP4 signal exceeds that of AQP1, with both AQP1 and AQP4 single stained cells being evidentiated (A–C). In a perilesional scar region, gemistocytes (GFAP-positive) retain only a peripheral staining of AQP1 (D–F and enlarged insets). Around blood vessels, both markers are colocalized in the perivascular astrocyte end-feets (GFAP-positive) (G–J and enlarged insets). (A–F) 40×; (G–J) 20×. AQP: Aquaporin; GFAP: Glial fibrillary acidic protein.*

We next assessed the expression of AQP1, AQP4 and GFAP, in recent and organized perilesional scar, WM and control tissue (Figure 4A). As expected, GFAP showed higher values in organized scar compared to recent scar, but due to the nature of much more diffuse staining in the WM, no statistical significance could be recorded between control and perilesional WM stainings. Both AQP4 and AQP1 had lower expressions than that of GFAP, with AQP1 showing a constant tendency toward lower values compared to AQP4. Although AQP1 was significantly lower than AQP4 only in organized scar and control WM ($p$<0.05), and in almost all instances AQP1 was significantly lower than GFAP (at least $p$<0.05), while AQP4 showed a significant difference only for old scar tissue ($p$<0.05). We next analyzed the colocalization degrees of AQP4/AQP1, as the overlap coefficients, and basically in the scar areas pooled together (recent and organized) the degree of overlapping was significantly low than for WM lesional regions pooled together (recent and organized), compared to control WM values [$F$(2,36) = 3.4, $p$<0.05] (Figure 4B).

Lastly, we wanted to evaluate the colocalization degree of AQP4 and AQP1 with collagen IV, given the demonstrated close relationship of the astrocyte end-feets with the vascular basement membranes (Figure 4, C–H). If we quantified the colocalization degrees as Pearson's correlation coefficients between AQPs and collagen IV, the average values were higher for AQP4 compared to AQP1 in all instances (Figure 4I), but with significant differences only for the WM in both control and perilesional areas. It seemed in fact that in control cases the difference was more accentuated, and corroborated with the fact that water diffusion occurs mostly at the level of WM around axons, it is very likely that although AQP1 is also present in the perivascular compartment, AQP4 contributes in fact more to water diffusion.

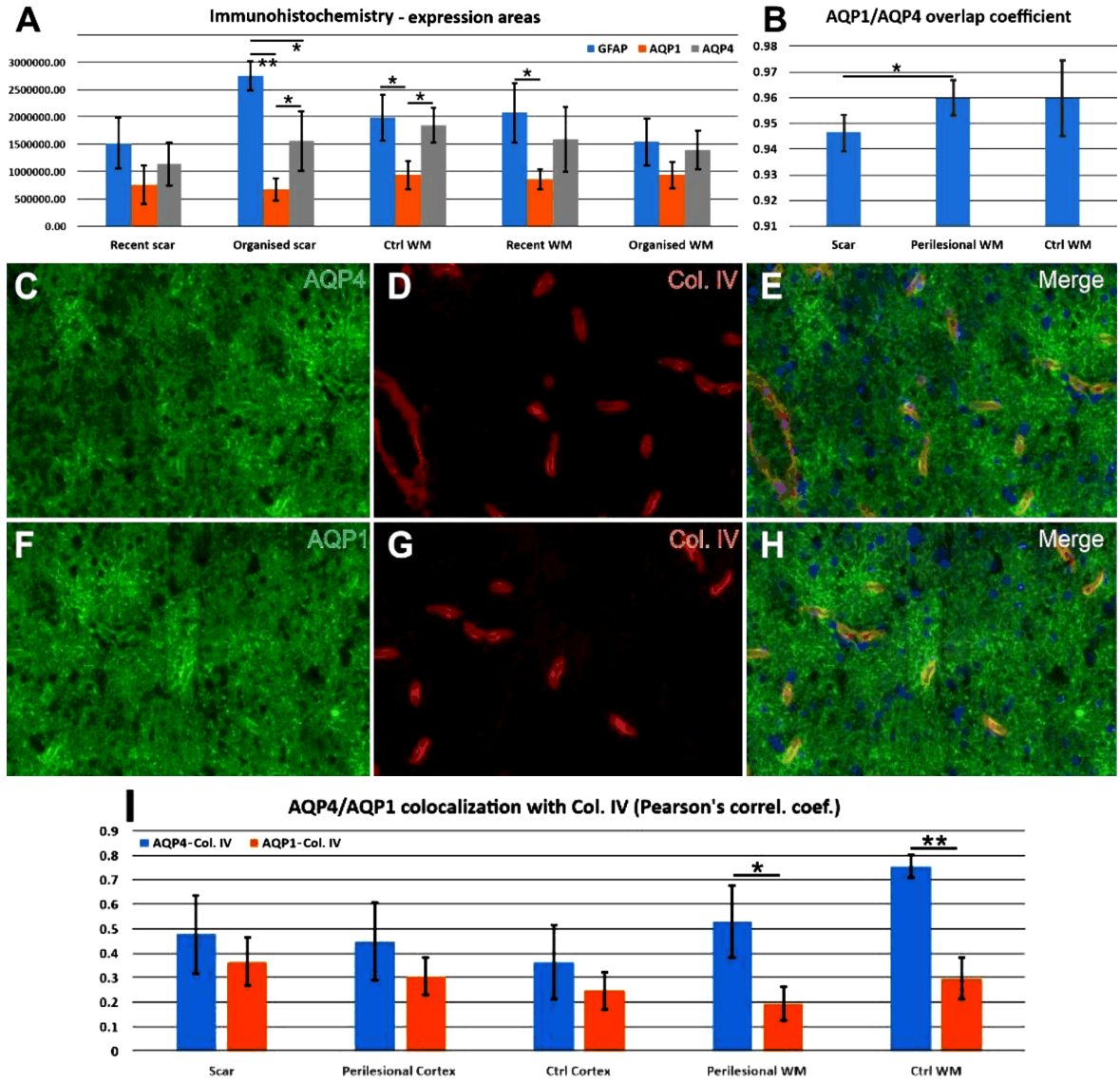


**Figure 4** – ***Comparative expression areas for AQP1, AQP4 and GFAP reveal constantly lower levels for AQP1, although significance is not attained for all instances (A), while colocalization of the AQP1 and AQP4 signals reveal a decreasing overlap in the scar region (B). Both AQP4 and AQP1 show a strong expression in the perivascular end-feets, and colocalize with Col. IV in the basement membranes (C–I). *p<0.05, **p<0.001, ANOVA testing; error bars represent standard errors of the means. (C–H) 20×. AQP: Aquaporin; GFAP: Glial fibrillary acidic protein; Col. IV: Collagen type IV; ANOVA: Analysis of variance; Ctrl: Control; WM: White matter.***

## Discussions

AQPs are a family of proteins, made up of more than 10 subtypes, with different locations in the human body, whose main role is in facilitating the transport of water in different cells, first identified in the early 1990s [7, 8]. Recent studies have shown the presence of eight AQPs in the brain: AQP9, AQP1, AQP5, AQP4, AQP8, AQP3, AQP11, AQP7; of all the best represented are AQP1 and AQP4. These water channels are in the form of tetramers and are located in the cell membranes. In the brain, AQP4 and AQP1 are the most abundant water channels, located mostly in the astrocyte extensions and act according to the local osmotic forces as bidirectional channels [9].

AQP1 is a protein that plays a role in water transport and was the first identified member of the AQP family. Studies that analyzed the phenotype of transgenic mice deficient in AQP1 showed its importance in normal functions of placenta, kidney, gland secretion, lung, heart, peritoneum, cells, eye, nervous system [15, 16]. At the level of the CNS, AQP1 was identified in epithelial cells of the choroid cells, and trigeminal ganglia, in small diameter sensory neurons in the spinal cord [17–19]. Besides these areas, AQP1 is expressed also in the *glia limitans* and astrocytes in the WM [20]. AQP1 expression has been shown to correlate positively with the degree of malignancy of astrocytomas, is associated with tumor invasion and angiogenesis [21–23]. Recent studies have found AQP1-positive astrocytes in the temporal neocortex of Parkinson's patients [24]. It has been shown that AQP1 plays an important role in angiogenesis, neurodegenerative disease, tumor growth and neoplastic invasiveness [1, 25–28]. In our study, we showed that AQP1 was present in both perivascular astrocytes and cerebral parenchyma. AQP1 was also present in the brain of stroke patients in the glial scar and in the immediate peri-liquefaction scar, but with lower values than for AQP4. There were also areas, such as the *pia mater*, where its expression was almost non-existent but there were some positive astrocytes for this marker, as opposed to AQP4, which was intensely positive at this level.

Recent studies have described AQP1 in the nonhuman primate in a subpopulation of astrocytes, suggesting species differences. Its role in water homeostasis has also been demonstrated [20]. Interestingly, AQP1 although highly positive in peripheral endothelial cells is not found in normal brain capillary endothelium [29, 30]. It has been observed that the osmotic permeability of water in choroid plexuses is reduced fivefold in AQP1-null mice compared with wild-type mice; this means a 50% reduction in intracranial pressure and may have important implications in ischemia [31]. Inhibition of AQP1 could be a therapeutic strategy in counteracting excessive cerebrospinal fluid (CSF) accumulation in hydrocephalus, ischemic stroke or intracranial hypertension [32, 33].

After various brain lesions, AQP4 is overexpressed in reactive astrocytes [34]. Mice with AQP4 deficiency have showed hemispheric enlargement, improved neurological deficit and reduction of cerebral edema at 24 hours in a focal ischemic stroke model [9]. Because this AQP has a bidirectional role in water transport, it allows it to participate in both the edema of the initial phase and the elimination of the water from the parenchyma in the blood vessels in the late phases [35]. AQP4 is a major contributor to brain disorders accompanied by cytotoxic edema when impaired BBB is not present. In ischemia, pneumococcal meningitis, glioblastoma or trauma, AQP4 upregulation was associated with worse outcome but edema was reduced with AQP4 deletion or downregulation [36–38]. In this study, we tried to analyze the expression of AQP1 and AQP4 in different areas of the infarcted brain but also in the normal brain. In stroke patients, we analyzed their expression in WM and recent and organized peri-lesional scar; we found a positive signal for the two AQPs in all the analyzed areas but as in other studies [39], the AQP4 values were increased in stroke patients. In the treatment of brain injuries, an important goal is to reduce and control cerebral edema. Because the critical role of AQP4 in the progression of cerebral edema has been demonstrated, this could be a promising therapeutic target for this intervention. Recent studies have found 2-(nicotinamide)-1,3,4-thiadiazole (TGN-020) to be a potential inhibitor of AQP4 [40]; the ability of TGN-020 to inhibit AQP4 has been demonstrated in animal models of cerebral ischemia, in which single-dose pretreatment reduced cerebral edema [41]. Moreover, even after the onset of ischemia, TGN-020 showed to reduce the edema and block water entrance into the parenchyma at the level of the vascular basement membranes [42]. AQP4 expression was rapidly up-regulated in perivascular extensions of astrocyte in a mouse model of transient cerebral ischemia, with a peak one hour after ischemia and the second was observed in the penumbra after 48 hours; the first peak coincided with early cerebral swelling in the border and core of the ischemia and the second peak coincided with the degree of brain swelling [43]. These findings suggest that AQP4 is the main water channel involved in water movements after stroke [6].

We have analyzed here for the first time the immuno-expression of AQP4 *versus* AQP1 on seriate sections through the brain from patients suffering from stroke but also through the normal brain. Although the immuno-expression of both AQPs was positive in the same areas, in the astrocytes and around the blood vessels. In all the areas analyzed by us, peri-liquefaction core, in the glial scar, perilesional WM and cortex the expression of AQP4 was higher than that of AQP1. It would be of interest to show in the future, the three-dimensional relationship between the blood vessels and the AQP1- respectively AQP4-positive astrocytes on scanned seriate sections [44].

Our study, as well as other studies that have analyzed the immunohistochemical expression of AQP4 in the normal brain, have shown that it is present in glial cells with astrocyte structure and in ependymal cells. They also showed that the glial cells were stained in mesencephalon, spinal cord, thalamus and cerebellum. Just as in our study, the neurons in the cerebellum, spinal cord and brain were not positive for these markers. The positivity of the astrocytes for AQP4 was different, so in the perivascular processes it had the highest intensity, in the *pia* and in the ependymal layer, data similar to those obtained by us [45]. Other studies have shown that AQP4 is present in the osmosensory regions of the brain, as well glial lamellae surrounding vasopressin-secretory neurons in

supraoptic nucleus [46]. It has also been identified in the skeletal muscle sarcolemma [47].

The expression of the two AQPs was evaluated further by fluorescently stained sections in perilesional areas. The degree of colocalization of the two AQPs was increased in the white substance, while in the *pia mater* AQP1 it was not present at all and AQP4 was intensely positive. The expression of the two AQPs was different depending on the histological area analyzed by us, so AQP1 had a higher expression in some areas of perilesional cortex.

Although in the literature AQP1 is described in the epithelial cells of the choroid plexus [30], we found a clear colocalization between the two AQPs at the level of the perivascular astrocytic end-feets, but with a much higher expression for AQP4. We were also interested to see the expression of AQP1, AQP4 and GFAP in WM, recent and organized perilesional scar and control tissue. In all the areas analyzed by us, we had a positive signal for all the marked structures; AQP1 had the lowest expression in all regions, while GFAP had the highest expression everywhere; the expression for AQP4 was higher than for AQP1. The expression for GFAP was best represented in the organized scar.

## Conclusions

Corroborating all the data obtained by us both through the microscopic images and through the statistical analysis, we showed that the expression of the two AQPs increases in stroke, colocalizing with both collagen IV and GFAP, but the AQP4 values are constantly higher.

**Conflict of interests**

None to declare.

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Gabriela-Camelia Roşu and Ion Cristian Gîlceavă have contributed equally to the manuscript.

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